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COMMISSION ON GENETIC RESOURCES FOR FOOD AND AGRICULTURE

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INTERGOVERNMENTAL TECHNICAL WORKING GROUP ON ANIMAL GENETIC RESOURCES FOR FOOD AND AGRICULTURE

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INNOVATIONS IN CRYOCONSERVATION OF ANIMAL GENETIC RESOURCES – DRAFT TECHNICAL GUIDELINES

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BACKGROUND

The Commission on Genetic Resources for Food and Agriculture (Commission), at its Thirteen Regular Session,[1] endorsed the *FAO guidelines – Cryoconservation of Animal Genetic Resources*[2] which were published in 2012. They cover the fundamental issues involved in developing and operating gene banks as elements in comprehensive national strategies for the management of animal genetic resources for food and agriculture, and generally emphasize the goal of conservation to reconstitute a population that has gone extinct *in vivo*.

At its Seventeenth Regular Session, the Commission requested FAO to continue developing and updating guidelines to facilitate the application of new scientific discoveries related to the identification, characterization and conservation of animal genetic resources.[3] It further requested FAO to strengthen partnerships with stakeholders and donors to continue technical and policy support for country implementation of the Global Plan of Action for Animal Genetic Resources (Global Plan of Action).[4]

Recent years have seen substantial advancements in biotechnologies for the sustainable use and conservation of genetic resources for food and agriculture.[5] Impacts of these advancements have been particularly important for cryoconservation (*in vitro* conservation), for which new developments in genomics, reproductive biotechnologies and bioinformatics have opened new opportunities for management of animal genetic resources for food and agriculture.

In addition to advances in biotechnologies, evolution has occurred in the prospects for utilization of material collected in gene banks. Although opportunities for other objectives were known, in the past gene banks were often regarded primarily as "insurance" against breed extinction. More and more frequently, gene banks are being viewed as tools for improved management of *in vivo* populations, as well as a resource for research.

The number of countries operating gene banks or planning to develop them is increasing. According to the survey undertaken to prepare the document *Synthesis progress report on the implementation of the Global Plan of Action for Animal Genetic Resources – 2020*,[6] nearly 60 percent of the countries reported that at least a portion of their breeds are subject to cryoconservation measures. In an additional 20 percent of the countries, such actions are planned and funding has either been identified or is being sought.

Despite these suggestive results, the data in the Domestic Animal Diversity System (DAD-IS) indicate that only about 10 percent of local breeds have material stored in gene banks.[7] The large proportion of countries with existing or planned gene banks indicates potentially high demand for technical knowledge on cryoconservation of animal genetic resources. On the other hand, the comparatively small number of breeds with material reported in gene banks implies a need for support in application of this knowledge, including support for management of the information associated with cryoconservation.

To support countries in the cryoconservation of their breeds and to facilitate the adoption of recent technological innovations, FAO has developed new guidelines on cryoconservation, which are given in the annex of this document. The draft guidelines will complement and update the *FAO guidelines – Cryoconservation of Animal Genetic Resources*.

[1] CGRFA-13/11/Report, paragraph 79.
[2] http://www.fao.org/3/i3017e/i3017e00.pdf
[3] CGRFA-17/19/Report, paragraph 84.
[4] CGRFA-17/19/Report, paragraph 86.
[5] CGRFA/WG-AnGR-11/21/8; CGRFA/WG-AnGR-11/21/Inf.11.
[6] CGRFA/WG-AnGR-11/21/Inf.3.
[7] CGRFA/WG-AnGR-11/21/Inf.7.

The draft guidelines have been prepared through extensive cooperation with collaborators from around the world. From 2016 to 2020, FAO was a partner in the project "Innovative Management of Animal Genetic Resources" (IMAGE),[8] which was supported by the European Union's Horizon 2020 Research and Innovation Programme. The IMAGE project was coordinated by INRAE, the National Research Institute for Agriculture, Food and the Environment of France. One of the deliverables of IMAGE was a review of the previous FAO guidelines and recommendations for content of a potential new edition.[9] These recommendations were considered in the preparation of the new draft guidelines. The draft guidelines were coordinated and edited by staff members of the Nordic Genetic Resource Center (NordGen),[10] a partner of IMAGE. Each section of the draft guidelines was prepared under the supervision of two or more lead authors, one of which was from an IMAGE partner institution. To ensure a wider perspective, other lead author(s) were drawn from stakeholder organizations across the world. Text boxes presenting examples were also provided by invited authors from across all regions. Individual sections of the document have been made available for voluntary review by subscribers of the Domestic Animal Diversity Network (DAD-Net)[11] and have been subject to review by invited scientists.

Relative to the previous guidelines, increased emphasis is placed on the active utilization of gene-bank material, especially in the management of *in situ* populations. The sections of the draft guidelines are the following: (i) Building a gene banking strategy; (ii) Quality management for improved organization and implementation; (iii) Choice of biological material to be preserved; (iv) The economics of gene banking; (v) Developing and using gene bank collections; (vi) Collection and cryopreservation of germplasm and tissues; (vii) Sanitary issues and recommendations; (viii) Databases and documentation; (ix) Legal issues: Acquisition, storage and transfer of gene bank material; and (x) Capacity building and training.

[8] http://www.imageh2020.eu
[9] https://www.imageh2020.eu/deliverable/D7.13.pdf
[10] https://www.nordgen.org/en/
[11] https://dgroups.org/fao/dad-net

ANNEX

INNOVATIONS IN CRYOCONSERVATION OF ANIMAL GENETIC RESOURCES

DRAFT TECHNICAL GUIDELINES

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Foreword

Acknowledgements

The goal and structure of the guidelines

Abbreviations and acronyms

ABS	Access and benefit-sharing
ABS-CH	ABS Clearing House
AI	Artificial insemination
AnGR	Animal genetic resources (for food and agriculture)
AnGRC	Animal Genetic Resources of Canada
ART	Assisted reproductive technologies
BC*n*	Backcross (*n* indicates the generation number)
CBD	Convention on Biological Diversity
CBA	Cost-benefit analysis
CEA	Cost-effectiveness analysis
CNA	Competent National Authority
CPA	Cryoprotectant (or cryo-protective agent)
C:P	Cholesterol to phospholipid ratio
DAD-IS	Domestic Animal Diversity Information System
DBMS	Database management systems
DMF	Dimethyl formamide
DMP	Data management plan
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
ERFP	European Regional Focal Point (for animal genetic resources)
ET	Embryo transfer
EU	European Union
EUGENA	European Genebank Network for Animal Genetic Resources
FAO	Food and Agriculture Organization of the United Nations
FSH	follicle stimulating hormone
GDP	Gross domestic product
GIS	Geographic information systems
GRIN	Genetic Resources Information Network
IC	Intercross
ICSI	Intracytoplasmic sperm injection
ID	Identification
IETS	International Embryo Technology Society
IMAGE	Innovative management of animal genetic resources project
iPSC	Induced pluripotent stem cells
IVC	*In vitro* culture
IVF	*In vitro* fertilization
IVM	*In vitro* maturation
IVP	*In vitro* production
ISO	International Organization for Standardization
LN	Liquid nitrogen
MAA	Material acquisition agreement
MSC	Mesenchymal stem cells
MTA	Material transfer agreement
OIE	World Organisation for Animal Health
OPU	Ovum pick up

PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGC	Primordial germ cells
QMS	Quality management system
SSC	Spermatogonial stem cells
SCNT	Somatic cell nuclear transfer
SDG	Sustainable Development Goals
SWOT	Strengths, weaknesses, opportunities and threats
SNP	Single nucleotide polymorphism
TUMASG	Transrectal ultrasonic-guided massage of the accessory sex glands
TUNEL	Terminal deoxynucleotidyl transferase nick end labeling
USA	United States of America
WGS	Whole genome sequencing

SECTION 1

Building a gene banking strategy

Building a gene banking strategy

1.1 INTRODUCTION

Gene banking is a powerful tool for the management of animal genetic resources for food and agriculture (AnGR). Although gene banking is technically demanding and can require substantial (initial) investments, once genetic materials are cryopreserved, properly stored and documented, they can remain viable for a practically endless amount of time. Many countries have invested in national gene banks as part of their strategy for AnGR management. The primary goals of gene banks are to have "insurance" to protect against breed extinction or catastrophic loss and to support the *in situ* populations across species and breeds. Additional goals are being identified because of a growing interest in active management of genetic diversity.

The *Global Plan of Action for Animal Genetic Resources* (Global Plan of Action; FAO, 2007) has a single Strategic Priority (Number 9) devoted specifically to *ex situ* conservation, but gene banking can contribute to other strategic priorities and actions for the sustainable management of AnGR. For example, Strategic Priority 4 is to "establish species and breed development strategies and programmes". An *ex situ* collection can complement the *in situ* population and increase options for genetic improvement or adaptation to changing environmental or economic conditions. Strategic Priority 11 is to "develop approaches and technical standards for conservation". Operation of a gene bank allows for the development, adaptation, and refinement of approaches to build collections and cryopreserve genetic material. To reflect the growing role of gene banks and to maximize returns on investment in them, countries should consider a wide range of approaches to utilize their *ex situ* collections.

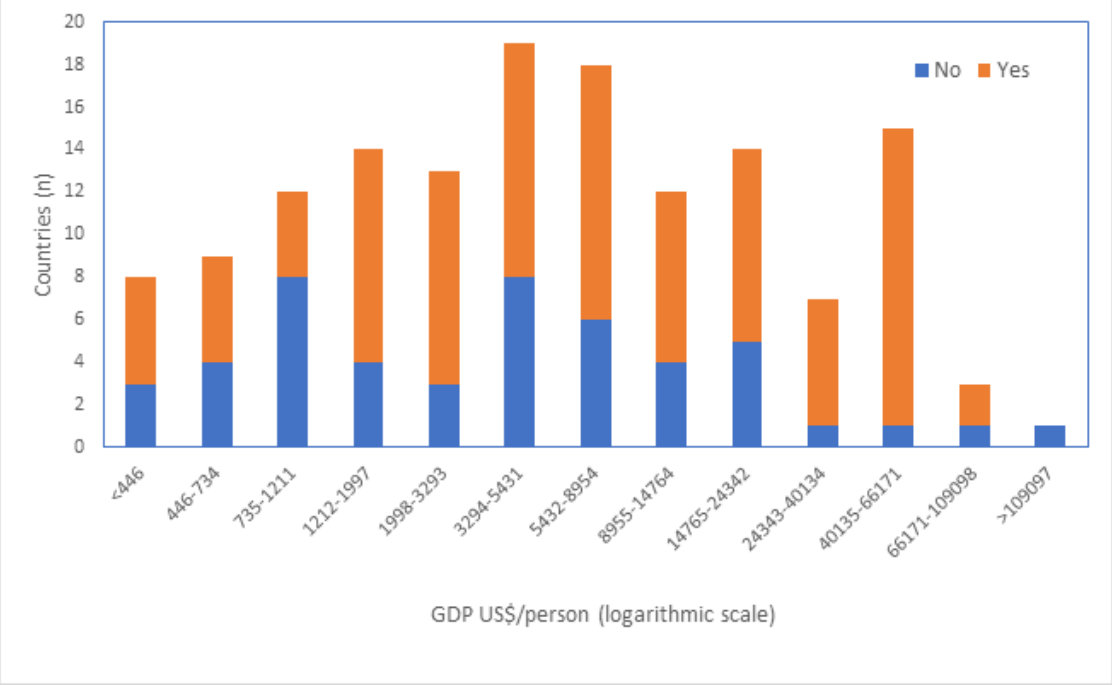

FIGURE 1.1
Numbers of countries reporting to have a gene bank (Yes/No) according to level of economic development (Gross Domestic Product per person)

Although it can require a substantial initial financial investment, many countries have decided to invest in gene banks despite their overall level of economic development. Figure 1.1 shows the number of countries reporting to FAO the presence or absence of an animal gene bank as a function of the size of its economy, expressed in terms of Gross Domestic Product (GDP) per person (in US$). No strong economic influence on the presence of gene banks is shown. Although most high-income countries report gene banks, in general, a majority of countries report having gene banks across all levels of GDP.

Cryoconservation and *in situ* conservation are complementary methods to manage animal genetic diversity. A cryoconserved collection of genetic material can be invaluable for improving management of an *in vivo* population. Small populations that are economically viable will usually have a low risk of extinction from competition with international transboundary breeds. However, their small population size implies that they still risk loss of genetic diversity, due to genetic drift, demographic factors, or sub-optimal breeding programmes. Banked material can be used to maintain or increase the genetic variation by effectively increasing the number of animals used as parents in each generation (Eynard *et al.*, 2018).

Cryoconservation activities can also contribute to sustainable breed use and development, even for breeds that have well-functioning breeding programmes. For example, alterations in the production environment (e.g. climate change) and economic forces that may alter the market are circumstances that require a change in the selection programme. The additional genetic diversity available in a gene bank would be crucial in these situations, even for populations that are not at risk in terms of census size.

Having multiple goals for a gene bank implies a variety of stakeholders interacting with the bank, both as providers and users of material. As a result, the interests of these stakeholders must be taken into consideration when planning for gene bank operation and management.

National gene banks should formally establish a gene banking strategy to address a range of goals. Once developed, gene banking strategies should be reviewed periodically to ensure their continued relevance and appropriateness.

1.2 ISSUES AND CHALLENGES TO BE CONSIDERED IN A GENE BANKING STRATEGY

Gene banking is not simply a technical exercise of identifying a sufficiently large number of donor animals and collecting and cryopreserving quantities of genetic material from these animals. Various factors need to be considered when developing the gene bank policy and strategy. These factors may influence the breeds and types and quantities of material to be gathered, complementary data to be collected along with the material, and even whether cryoconservation will be adopted within a given country as part of the overall approach to management of AnGR in the context of a national strategy.

1.2.1 Role of cryoconservation in dynamic AnGR management

A first step in developing a gene banking strategy is to develop or review national policies and management plans for AnGR in general. This step will help establish the context for gene banking and ensure that elements of the strategy are in harmony with the overall National Strategy and Action Plan

for AnGR (FAO, 2009) or similar policies and strategies developed for specific livestock sectors, species, or breeds.

Management of genetic diversity usually considers one or more of three basic goals:

1. Preventing the loss of breeds;
2. Maximizing or optimizing the amount of overall genetic variation maintained within and among breeds of a species; and
3. Ensuring the maintenance of important phenotypes and thus the genetic variation that underlies these phenotypes.

These basic goals are not independent from each other. Various breeds may have important phenotypes that are unique with respect to other breeds. Maximizing overall genetic variation should in general capture the alleles responsible for important traits. Emphasis placed on each of the goals may differ from one species to another. Cryoconservation can contribute to any of these goals.

1.2.2 Ethical issues

As mentioned previously, gene banking cannot be solely regarded as a scientific or technical activity. Sociological and ethical issues can be considered when developing a gene banking strategy, especially for banks that are supported by public funding (Zomerdijk *et al*., 2020). For example, assuming resources are not available to conserve all breeds within a country, ethical issues may arise in the process for choosing the breeds to be conserved. Also, cryoconservation relies heavily on utilization of biotechnologies, some of which (e.g. invasive germplasm collection techniques, or cloning) may be opposed for ethical reasons by members of the general public. On the other hand, even controversial biotechnologies may be considered acceptable if their application can be justified as the best, the most cost-effective or practical option to preserve a certain breed.

1.2.3 Cost effectiveness

Gene banks must spend their funds in the most effective manner possible. Therefore, the cost-effectiveness of gene-banking activities must be considered and accounted for in the gene banking strategy. Section 4 of these guidelines provides more detail on the economic aspects of cryoconservation programs.

Many public gene banks provide stored material free of charge to users who can demonstrate a justifiable need. However, banks may also decide to charge for services to generate revenues from the use of gene bank material (Albert *et al.*, 2014; Van der Stijl and Eijdems, 2019). Whereas gene banks are usually expected to act as not-for-profit entities, they may need to cover some or all of their operating costs to be sustainable. Such costs include staff salaries, housing, liquid nitrogen and other consumable items, electricity and other utilities and equipment maintenance (see section 4).

1.2.4 Information management

Recent advancements in computing and communication technology have greatly increased the value of data and information. That trend will only continue in the future. Cryoconservation is not exempted from this trend. Any stored material has value if it can contribute to maintenance of genetic diversity, but its value will increase in proportion to the degree with which it has been characterized and the resulting knowledge has been catalogued. Data and information about stored material should be regarded as an integral part of the resource maintained in the gene bank and accounted for in the gene banking strategy. For an increasing number of gene banks, the “resource” consists of the biological

material together with its associated data. Efforts should be made to collect as much information about gene bank samples as possible. The decreasing costs of genomic analyses in recent years have increased the feasibility of genotyping donor animals for gene bank samples.

Likewise, information systems to make gene bank data available to stakeholders have increased in importance, as have information systems to link data collected by the gene bank with other relevant sources of information available on the internet. Section 8 of these guidelines addresses information systems for cryoconservation.

1.2.5 Gene banking objectives

The general aim of gene banks for livestock species is to effectively and efficiently conserve the existing genetic diversity of AnGR over time. Several plausible uses for cryoconserved material have been discussed briefly above and objectives for gene banking were presented in the previous FAO guidelines on cryoconservation (FAO, 2012), but merit a review here. They include the following:

1.2.5.1 Reconstitution of an extinct breed

This objective is usually considered to be one of the most common for animal gene banks, especially for publicly funded banks. Although the continued existence of a breed is likely to be of primary importance for the livestock keepers of the breed, animal genetic diversity is generally considered a public good and breeds are the usual conservation unit. The previous FAO guidelines (FAO, 2012) generally emphasized this objective and Indicator 2.5.1b of the Sustainable Development Goals (SDG; UN, 2020) is based on quantities of stored material that are sufficient to achieve this objective.

There is a potential for significant animal losses due to pandemics or environmental disaster to occur. To address such threats, a major goal of gene banks will be to ensure that the livestock industry loses as little time as possible in recovering from the loss. This means that gene banks may have to store significant quantities of germplasm from the most important populations, and do this on a regular basis to keep up with genetic trends.

A special situation with experimental lines used in research is the choice between keeping a live population or conserving the line as a collection of cryopreserved germplasm. The population can then be regenerated when needed for research, as illustrated by Silversides *et al*. (2012). While this is routinely done for model organisms used in biomedical research, it is not common for farm animals, where fertility of cryopreserved material is variable and a source of uncertainty.

1.2.5.2 Support for in vivo conservation

Breeds that are subject to *in vivo* conservation are usually small in terms of both real and effective population size. A collection of material in a gene bank can be used to help maintain genetic diversity, such as by alternating the utilization of parents across generations when using gene bank material (e.g. Sonesson *et al.*, 2002) or extending the generation interval. Stored material may also be used to safeguard against the accumulation of deleterious recessive alleles or to redirect a breeding objective.

1.2.5.3 Development of new lines or breeds

As mentioned previously, breeds are the usual conservation unit for management of AnGR and cryoconserved material can be used for the management of the sourced breed. However, this is not absolute. For example, cryoconserved germplasm from one breed may be used to introgress its valuable traits into another breed. Stored material from one breed may also be used to introduce genetic variation into a genetically similar breed to help prevent its genetic erosion. This approach

may be especially valuable in cases where the population size of the targeted breed was dangerously small before reasonable quantities of its own genetic material could be cryoconserved. Material from multiple breeds may also be used to create a new (composite) breed (Paim et al., 2019).

1.2.5.4 Improved management of not-at-risk breeds

Breeds with sufficiently large population sizes that suggest they are not at risk (see FAO (2013) for information about breed risk criteria) may still benefit from cryoconservation activities. The earlier that a material collection programme starts, the larger and more diverse it will be in the future, which will be highly beneficial should unforeseen circumstances provoke a large decrease in population number or genetic diversity. In the meantime, the stored samples can be managed judiciously for other objectives, such as development of new breeds. Gene bank collections have also been proven to be a valuable resource for building reference populations for genomic selection and tracking and tracing purposes (Blackburn, 2018; Rexroad *et al.*, 2019). Gene bank collections are also a resource to re-introduce diversity and/or re-orient breeding goals.

1.2.5.5 Research

Gene-banked material is also a valuable resource for research, such as genomic analyses to help understand the biological basis for a given breed's distinct traits. Storage of material other than germ cells (DNA or tissues) is recommended as a complement to standard gene banking of reproductive material. Such material can be used for both characterization of the stored samples and for independent research.

Cryopreservation allows germplasm and other tissues to be stored indefinitely. Therefore, the storage of material in gene banks should not necessarily be limited to material that can be utilized now but also consider possible future developments in genetic and reproductive technologies.

1.3 GOVERNANCE

Effective development and implementation of a gene banking strategy will rely on the existence of a system of governance. This system will ensure that the goals of all relevant stakeholders are appropriately represented in the strategy and that the critical elements of the strategy are implemented through the operation of the gene bank.

1.3.1 Stakeholder identification

Management of AnGR involves a wide range of stakeholders. In general, all stakeholders will share the basic goal of maintaining access to the widest collection of genetic variation possible. However, different stakeholders will have different reasons for wanting future access to this genetic diversity when stored in a gene bank and how to utilize it. Different stakeholders will also play different roles, as some may contribute financially to its operation, some will provide material and others will have an interest in utilizing the material. Many stakeholders will play multiple roles.

Stakeholder buy-in is a key step in the development or implementation of a gene-banking strategy. An analysis of stakeholder needs should be part of a quality management system of the gene bank. According to a recent survey on quality management of gene banks (Zomerdijk *et al.*, 2020), few banks have undertaken formal stakeholder analyses.

The first step in engagement with existing and potential stakeholders is to identify and take an inventory of the most relevant stakeholders. For countries with existing gene banks, some stakeholders will already have regular interaction with the bank. In this case an additional step is recommended to identify potential future stakeholders. Key stakeholders usually include the following.

1.3.1.1 Government

Livestock gene banks are most often government institutions or government funded institutions. In general, the actions of the government should represent the interest of the general public including ensuring that the gene bank is operated in an ethical manner. A national gene bank can support the government's efforts to address international pledges and obligation with respect to international agreements for management of biodiversity (e.g. the Convention of Biological Diversity, the SDGs). The government may also mandate other public organizations to carry out this mission on its behalf.

1.3.1.2 Breeders and breeder associations

Livestock breeders and organizations that represent them will often be among both the most important providers and users of material stored in gene banks. Therefore, garnering the support from these groups is essential for gene bank success.

1.3.1.3 Breeding companies

Interests of breeding companies will be similar to those of breeders and breeder associations. Their focus will primarily involve the populations they have under their ownership. Therefore, gene bank governance will need to accommodate the organizational structure of each company.

1.3.1.4 Other nongovernmental organizations

Organizations that are not formal breeder associations may provide support to specific breeds or groups of breeds through advocacy and capacity building, operation of *in vivo* conservation programmes and provision of assistance in marketing, among other activities. They can often serve as a direct link to breeders of various local breeds.

1.3.1.5 Research and teaching institutions

Universities and other research or teaching institutions may wish to utilize the gene bank and its collections to support their activities. They may also provide services to the gene bank, such as data analysis and development or refinement of cryopreservation protocol. In general, these institutions and the gene bank will have similar issues involving governance.

1.3.2 Institutional commitment

The success of a gene banking programme will depend strongly on the commitment of stakeholders to its establishment and operation. In most cases, this will start with a strong acceptance by the government of the importance of gene banking and a commitment to provide substantial financial support, both a large initial investment for the establishment of a gene bank and then continued funding for its long-term operation. Many governments have agreed in principle to develop and implement a gene bank strategy but have difficulties in allocating funds to build the gene bank or sustain its operations. However, as shown in Figure 1.1, many low-income countries have a gene bank, suggesting that political will is at least as important as the available budget.

Because construction and establishment of a gene bank usually requires a costly initial investment, this activity is a government responsibility and requires the appropriate government ministry to allocate funding. Regardless of the source of the funding, the government must also be prepared to commit to

and plan for continued funding to support the development of collections, the maintenance and operation of the gene bank in the long term.

For success, non-government stakeholders must also be committed. They must be convinced about the benefits of providing access to their AnGR for the development and subsequent enhancement of collections and of regular and wise utilization of the material in the collections.

A firm commitment by international financial institutions may also be reasonable. Although many developin countries have already established gene banks, the initial investment can be substantial and many countries would benefit from financial assistance.

1.3.3 Governance structure and decision-making process

Most gene banks will have a manager who is responsible for the management and day-to-day decisions on operation of the bank but may consult with various stakeholders for long-term decisions. The establishment of a multi-actor stakeholder committee to provide input and support to the gene bank is recommended.

This stakeholder committee could have several responsibilities, in particular assisting in developing the gene banking strategy, monitor its implementation and update it as needed. As appropriate, the committee might also advise on annual budgets, capital, and infrastructure advancements.

Regarding the composition of the committee, FAO guidelines recommend the establishment of a National Advisory Committee for AnGR (FAO, 2011). Given the need for synergy between the gene banking strategy and the overall national strategy and action plan for AnGR, the potential exists for the National Advisory Committee to also participate as the gene bank's stakeholder group.

1.3.4 Data policy

As mentioned previously, information about samples in the gene bank collection should be regarded as an integral part of the gene bank. Therefore, a data policy must be developed as part of the overall gene banking strategy. This topic will be elaborated in more detail in Section 8. The following are potential issues that may be addressed in a data policy:

- Types of data to be collected and managed;
- System for organization of data and standards for documentation and metadata;
- Protection of privacy, security, confidentiality, intellectual property or other rights;
- Access to and sharing of data: how and when to share data and with whom; and
- Data storage: where are they maintained, how are they secured?

1.4 ELEMENTS OF THE GENE BANKING STRATEGY

Gene banking of AnGR is a comprehensive and dynamic process where flexibility in collection development is key. It is a long-term process that spans decades of continued sample curation and evaluation of the collection, while projecting future needs to the extent possible. The ultimate goal of the gene bank is to provide society with a broad range of genetic options for different types of future use. In the formulation of their strategy gene bank managers should consider aspects of sampling, storage organization, documentation, utilization, rationalization, as well as communication and awareness raising.

The motivations for developing a gene bank are broad and dependent upon country needs and long-term strategies. The industry is often assumed to maintain sufficient genetic diversity for their future use, but this is not always an accurate assumption. Therefore, in deciding the scope of gene banking activities it is essential to have comprehensive discussions with all stakeholder groups.

In essence, development of a gene bank's collection follows a hierarchical approach consisting of identifying motivations, deciding which species of livestock are important at the country level, deciding which breeds to collect, and then which animals within a breed might be selected for collection. The following provides a basis to start exploring and formulating a collection strategy.

1.4.2 Sampling

The gene bank may review the state of its collections as compared to its objectives and expectations from stakeholders to identify needs for additional sampling. The main steps are listed below.

1.4.2.1 Species

The strategic decision about which species to collect for the gene bank will govern the activities conducted by the gene bank, as methods and approaches for sampling and cryopreserving genetic resources will vary among species. This decision has major implications for the gene bank, as new species may be added or even deleted in specific instances depending upon the stakeholder and policy perspectives. The importance of the species to the country's economy or wealth of genetic diversity or its heritage value may all be considerations for inclusion but the availability of appropriate cryoconservation methods will have a critical influence (see Section 6).

A wide range of approaches have been taken in deciding the species and breeds to target for collection. National gene banks often consider collecting from all livestock species present in the country. A recent survey on gene banks showed that six species (i.e. cattle, sheep, goat, horse, pig and chicken) could be found in many gene banks whereas others (e.g. rabbit, turkey) were found only in a few countries (Zomerdijk *et al*., 2020).

1.4.2.2 Genetic information

Breed is an important determinant for both the global livestock sector and collection development, and countries have taken various approaches. Regardless of species, some type of genetic assessment at the breed level will be necessary in executing whichever within-species collection goals have been established. For example, in North America and Europe several gene banks have set a national goal of collecting all livestock breeds. Conversely, Brazil's gene bank initiated their collection efforts by focusing upon acquiring samples from rare breeds.

Due to the importance of breed as an indicator of diversity, assessments for collection purposes might include unique phenotypes or important production characteristics (e.g. milk, meat, or fiber), distinctiveness from other breeds, historical perspectives, genetic isolation based upon geography, and well recognized breeds at the national or international level (Blackburn, 2018). While some attempts (e.g. Weitzman, 1998) have been made to use genetic markers to develop subsets of breeds to capture a broad array of genetic diversity per species, such approaches do not account for the need to service the broader community of stakeholders and have generally not been implemented because of a lack of consensus of stakeholders on the subsets (Boettcher *et al*., 2010).

Molecular genetic studies suggest that the total variation accounted for by breeds within a species is usually less than the variation among animals within breeds (Paiva *et al.*, 2011). Some gene banks have successfully captured within breed genetic diversity by using different tactics, and over time the gene bank collections contained greater diversity than the *in-situ* population (Danchin-Burge *et al.*, 2011). By using molecular tools such as single nucleotide polymorphism (SNP) gene banks have developed collections that have captured up to 98 percent of the variation within a breed (Wilson *et al.*, 2019) and have identified subpopulations within breeds (Hulsegge *et al.*, 2019a).

Gene banks can use a number of approaches to capture genetic diversity based upon information on hand. Box 1.1 shows how simple random sampling of donor animals can be effective in capturing a wide array of diversity.

BOX 1.1
Random sampling of animals for the gene bank collection

Early on it was recognized that large portions of genetic diversity could be captured in collection development through random sampling of animals (FAO, 1983; Smith 1984). This fact can be demonstrated by using the equation below that calculates the probability (P) of capturing a rare allele:

$P = 1-(1-p)^{2N}$ for semen;
or
$P = 1-(1-p)^{4N}$ for embryos.

For an allelic (p) frequency of 0.05 and with an N of either 50 bulls or 25 embryos collected from a breed for the gene bank, there would be a 92% chance of capturing the allele. Utilization of this equation is a cost-efficient approach for building collections.

When a breed is dispersed among production environments with no or little migration, it may be desirable to sample within and among the environments. Geographic coordinates of collection locations should be recorded and stored in the gene bank database, as they provide links to extensive information in environmental databases. Gene banks have used pedigrees to develop sampling strategies and prioritize individual animals or families, to maximize representativeness and avoid losing the less-represented families. For example, pedigrees can be used to cluster animals within a breed based upon their genetic relationships (Blackburn, 2018; Wilson *et al.*, 2019) leading to prioritization of animals. Animals within each cluster are then selected for entry into the gene bank. An advantage of this approach, relative to targeting specific individuals, is that if germplasm from a selected animal cannot be obtained, another animal from the cluster can be collected. Within breed, optimal contribution methods have been developed (Meuwissen, 2002) and used in the Netherlands in an effort to build and optimize core collections. Such an approach is useful in ensuring that the gene bank collection is genetically balanced but requires sufficient organization and budget, and data availability can be a limitation.

Genomic information can be used in the methods previously mentioned, either instead of or in conjunction with pedigrees. In addition, genomic data can be used in other ways to select animals within a breed, including absence of known genetic abnormalities, ex-post assessment of the collection

versus *in situ* population diversity, and targeting animals for collection that have genotypes of interest for traits such as adaptability to climate change.

Utilization of estimated breeding values or phenotypes for traits of interest can also play a role in identifying animals for the collection. For instance, the gene banks in France and the United States of America (USA) have been collecting samples from animals that represent extremes for traits of interest outside the major production objective, to ensure a wide range of genetic variability has been captured and to keep the option of reorienting selection objectives. Whichever approach is used to select animals, it must be flexible (e.g., no fixed lists of animals to collect) and robust to accommodate breeding sector dynamics and time constraints.

Intensively managed breeding populations held by the private sector are also in need of the security gene banks can provide and they represent a special case in terms of how populations might be sampled. These collections become increasingly important as company populations undergo the pressures of genetic drift and selection intensity. Approaches for these populations may range from collecting substantial numbers of males (and females in the case of poultry) from a specific generation, so that an entire cohort of individuals may be rejuvenated from cryopreservation, to collecting "snapshots" of every new generation.

1.4.2.3 Continual management and updating of collections

In situ populations are continually changing so gene banks need to periodically collect new samples to ensure stakeholders have a range of genetic material. This step is useful to capture genetic changes, particularly when breeders want access to genetics to correct a recent problem, such as restoring fertility or recovering a lost trait. Having access to material from varying times can accelerate the process. The time interval between sampling depends on how rapidly a population is changing. Since the optimal interval for sampling may be difficult to determine *a priori*, a regular snapshot approach is probably the easiest to implement since it also helps to maintain awareness about gene banking on a regular basis. Furthermore, the onset of genomic selection has led to considerable acceleration of genetic progress, particularly in dairy cattle, so that regular sampling is recommended. For rare breeds undergoing low selection pressure with limited use of frozen semen, where a gene bank has collected at least the minimum quantities of germplasm and animals, the sampling interval can be increased to 5 to 7 generations, considering the specific collection effort to be set-up. There may be reasons to sample these breeds more frequently, such as to mitigate genetic drift or capture unique phenotypes.

1.4.2.4 Tissue types

As stated in the previous cryoconservation guidelines (FAO, 2012), reproductive efficiency varies among tissues, species and even among breeds within the same species. These factors will determine the minimum quantity of germplasm needed to perform any sort of reconstitution. How well gametes or tissues can be cryopreserved and thawed and how many live offspring are produced are all considerations in determining the types and numbers of gamete or tissues to be sampled and stored in the gene bank (see Section 6). Reproductive efficiencies vary among gametes and tissues, and some gene banks have found it useful to collect a variety of sample types that provide them with more flexibility in utilizing a collection. For example, due to the homogametic male in poultry semen, ovaries and testes have been collected, cryopreserved, and used to create chicks (Silversides *et al.*, 2012). More recently, cryoconservation of primordial germ cells has been developed and validated to revive a chicken breed (Woodcock *et al.*, 2019).

1.4.2.5 Sanitary status

Sanitary issues apply to animals and to collection facilities, that may impact their suitability for gene banking. This will also vary depending upon national legislation and gene banks will need to operate within the guidelines and regulations. Common venues for collecting germplasm include on farm collection, private sector artificial insemination facilities and research institutions, which may differ in sanitary status and regulations. Section 7 of these guidelines provides more information.

1.4.3 Storage organization

The key objective of gene banks is to guarantee safe long-term storage of genetic material and associated data.

1.4.3.1 Centralization and distribution

The size and capacity of a gene bank depends on its objectives, the range of species and breeds to be conserved, the financial resources available, the types and amount of genetic material to be stored, and the location of populations to be sampled. There is an optimum to find between exclusive centralization, which may increase the costs of collection and distribution, and wide distribution, which increases the total investment in equipment and may lead to underutilization of local storage capacity. Development of species-specific locations could increase competition among locations for financial resources from the same funding sources. Section 4 addresses economic optimization of gene banking.

In selecting the main location and facility for the national gene bank, several logistical issues should be considered including: physical safety of the collection, easy access for receiving and distributing samples by commercial carriers, accessibility and continuous access to liquid nitrogen, consistent and dependable electricity, a physically secure building, a secure room with controlled access for storing the collection, and closed circuit television or other system to record entry and exit of people from the secured room.

Animal health concerns and other potential hazards (e.g. floods, earthquakes, fires, tornados) of a geographic area might also be criteria in determining where a gene bank should be located. Location outside an area with endemic disease issues will facilitate the entry and exit of germplasm from the gene bank.

Human resource availability may also be a consideration in choosing a location. Incentives should be in place to attract and retain qualified staff. Health, sanitary aspects and physical security must be considered when identifying the site of the gene bank.

1.4.3.2 Duplication and back-up

Material stored in a gene bank is a highly valuable resource and must therefore be safeguarded against loss. It is strongly recommended to maintain two separate storage facilities in different geographical locations.

A minimum of two storage locations should be identified at national level, for primary and duplicate collections. If the gene bank is already organized with distributed sites, duplication of collection is easy to organize, either by specializing one location as a duplicate according to species, or by taking advantage of existing AI centers to host mirror collections for long-term storage. In that case, distribution will take place from the central site rather than from the mirror site, which acts only as a safeguard. National representatives making such a decision about back-up sites need to consider the

long-term (15 to 20 years) ramifications of such agreements, particularly when contracting with privately held companies to ensure the safe keeping of samples.

Exchange of duplicate material between countries may also be considered to reduce costs or promote transboundary collaboration. However, such arrangements are vulnerable to changes in national laws or disease outbreaks that may later make it difficult for a country to repatriate their samples. In addition, both locations should use the same database so that inventories can be appropriately managed, or at least agree on the same descriptors to be stored so that information can be easily exchanged between sites.

1.4.3.3 Storage of material

Gene bank managers will need to decide how to distribute samples across their liquid nitrogen storage tanks. Much of this decision will be made by the size of the liquid nitrogen tanks. The primary consideration is whether to have multiple species in a tank or to maintain separate tanks for each species. Sanitary status of samples may also impact the approach taken. Government health regulations may play a role in this decision (see Section 7). Storing multiple species per tank is usually more cost-effective and efficient, so that the liquid nitrogen tanks can be used to their maximum capacity.

1.4.3.4 Associated data

The database is essential for managing routine gene bank operations and to support management decisions. The database serves as the primary tool for receiving, storing and exchanging information about samples in the collection. Therefore, proper and accessible documentation is vital for the operational management of the gene bank, for optimization of gene bank collections, and for future use of any stored gene bank material. Gene banks need to develop and implement a database for this purpose. Basic information about gene bank collections should be easily accessible without the need for any additional information from outside the database (section 8).

1.4.4 Utilization

Gene banks are more powerful when used by a wide range of stakeholders. Stakeholder requests for gene bank samples are varied. Potential requests for gene bank samples include adding genetic variability to an in-situ population, corrective mating for any breed, reconstituting research populations, and genomic evaluations. In addition, there is the overarching long-term objective to be able to reconstitute breed(s) in time of national crisis.

1.4.4.1 Conditions for access

Access to national gene bank's collections requires policies that ensure that all users are treated equitably, sample use is non-trivial, and access to gene bank material does not infringe upon private sector business activities (see Section 9). Depending upon country laws, gene banks may or may not be able to charge fees for service or germplasm. Gene bank managers need to define criteria for access and use, and may find it useful to develop a committee, comprised of persons knowledgeable about a specific species (or breed), to review requests. In situations of short supply of requested samples, the potential gain achieved by releasing samples must be weighed against potential future demands for use and whether and how the genetic resource can be replenished.

The following are decision points to consider when releasing samples:

- Are there any specific conditions defined in the agreement between the gene bank and the original provider of the material? If yes, then the original provider may need to be consulted.

- What is the intended use of the sample? Is it beneficial to the breed, industry, or research?
- Can or should the request be met by the private sector instead of the gene bank?
- Does the requestor have sufficient experience to make successful use of the sample?
- Does the approach proposed lend itself to successful generation of live animals (if appropriate), for example using *in vitro* vs *in vivo* fertilization?
- Is the proposed mating beneficial (e.g., in reference to genetic relationship)?
- What does the gene bank get in return when samples are released (e.g. get germplasm from the progeny to replenish the gene bank collection, genomic information and/or progeny phenotypes to document the remaining collection, cost recovery fees if permissible by law)?
- If the requested sample is used for genotyping, will the gene bank obtain a copy of the resulting genotype/sequence information?

1.4.4.2 Usage scenarios

As pointed out previously, gene banks can serve a range of objectives. Box 1.2 illustrates the usefulness of long-term preservation of germplasm for highly selected populations, beyond the classical objective to be able to reconstitute breed(s) that may become threatened or extinct.

BOX 1.2

Using banked material to reconstitute lost Holstein sire lineages

Yue *et al.* (2015) reported that the genomes of Holstein cattle in the US had only two different familial Y chromosomes in the in vivo population, both tracing to two important 1970's sires. Evaluating the collection in the national gene bank, the same researchers determined there were two additional paternal line Y chromosomes that could broaden the genetic diversity. However, the identified bulls were descended from the population of the 1960s and thus had relatively low genetic merit. It was decided to introgress the Y chromosomes from the two repository bulls (Dechow *et al.*, 2020). Semen from the gene bank was used to create in-vitro embryos from seven elite (upper 70th percentile in performance) Holstein cows. The embryos (12 and 15 per bull) yielded seven male offspring (3 and 4 per bull). At one year of age the bulls were transferred to a commercial AI center. Genomic evaluation of the bull calves showed that one generation of mating with an elite female would be sufficient to produce offspring with approximately the breed's current average for milk production and other economically important traits. One sire's bull calves were actually higher than breed average for net merit and milk production. Semen from the bull progeny was repatriated to the gene bank and is also commercially available to producers. The bulls produced will be mated to highly productive cows and it is anticipated that their progeny will be competitive with other top AI bulls. Additional studies are planned to evaluate semen differences, resequencing the bovine Y chromosome, and monitoring lifetime performance of the daughters. This experiment demonstrates that by combining advanced reproductive biotechnologies and genomic information, reintroducing gene bank genetics into a population can be done much more quickly and efficiently than previously thought (Leroy *et al.*, 2011).

1.4.4.3 Tracking sample use and impact

Tracking the utilization is important for gene banks. Data on utilization are evidence of the value of banks and can be used in funding activities. They are also useful in planning for the future. Figure 1.2

shows the yearly utilization of samples from the US gene bank, according to birth year of the donor. The gene bank has released samples from more than 11000 animals since 2004. The data also shows that samples of animals of all ages are being continuously used by the various stakeholders.

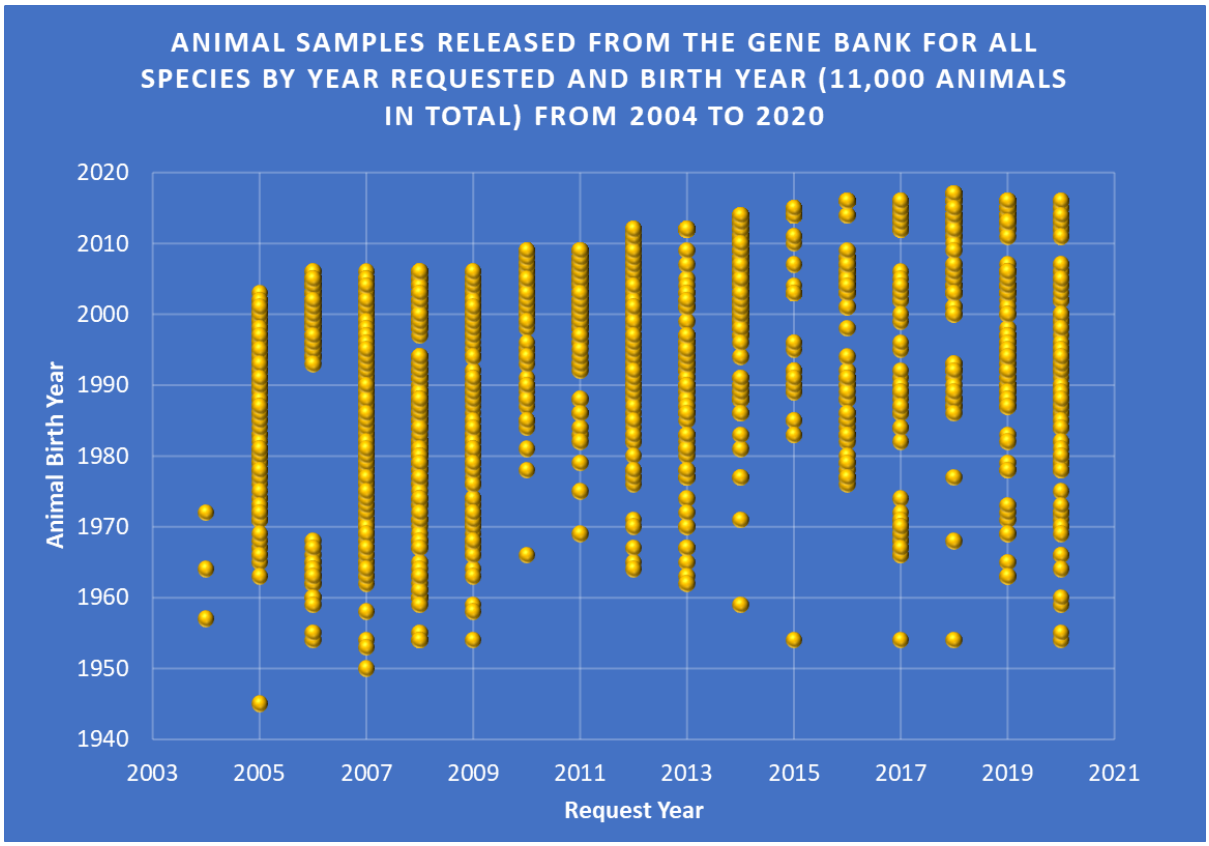

FIGURE 1.2
Samples released from the U. S. gene bank between 2000 and 2020 among species and germplasm types. Note among request years, samples with animal birth years ranging from 1960 to the most current year have been requested by stakeholders

If samples are used for genotyping, the gene bank should require the user to provide the data and other results and inform the bank about how the genotyping helped solve a problem (Box 1.3). Furthermore, such examples are extremely useful in articulating the value of gene banking to stakeholders, administrators, and the public at large. One option to facilitate tracking is to use a doi for a gene bank collection, so that each user of gene bank material should refer to this doi in any publication. Terms for material utilization and information sharing should be outlined in a material transfer agreement (See Section 9).

BOX 1.3
Genomic use: Experience from the US and The Netherlands

In the U.S. a major breed acquired a semen sample from an important 30-year-old sire to determine if he carried a lethal mutation. The bull was not a carrier and as a result over 29,000 animals did not have to be genotyped, saving breeders over $2.2 million in genotyping costs. Also samples from more than 400 repository dairy bulls were used to develop the first genomic breeding values for

dairy cattle, this technological advancement added over $4 billion per year to the U. S. dairy industry (Rexroad *et al.*, 2019).

In the Netherlands the Holstein and MRIJ gene bank and DNA collections have shown to be very valuable in the initial phase of implementation of genomic selection by Dutch cattle breeding industry. Moreover, 50k SNP data of all gene bank bulls of native Dutch cattle breeds was used to establish a breed specific DNA reference population for the purpose of identifying pure-bred animals without or with incomplete pedigree data and to confirm breed purity (Hulsegge *et al.*, 2019b).

1.4.5 Rationalization

Gene banks should regularly evaluate and rationalize their scope, policies, implementation strategies and protocols to optimize and further develop the gene bank strategy. By doing so, countries will contribute to Strategic Priority 12 of the Global Plan of Action to ”establish and strengthen national institutions, including national focal points, for planning and implementing animal genetic resources measures, for livestock sector development”. A rationalized strategy can be defined as the most cost-effective way to reach the gene bank objectives. Gene bank collections must be “fit for purpose”. For example, the notion of “sufficient” material in SDG Indicator 2.5.1b is motivated by one purpose, restoring a lost breed, but a smaller amount of material may still be useful for another purpose. As part of this rationalization analysis, gene banks should be mindful that the collections they are building might be of greatest use after 50 or 100 years.

Rationalization of livestock gene bank strategies should be done both *ex ante* and *ex post* and involves both supply and demand related elements. Demand side elements of a rationalization framework typically include future societal and stakeholder needs related to the use of gene banks and thus requires anticipation of drivers of change. The future demand for gene bank collections cannot be fully defined due to the unknown vagaries of the future. Gene banks may wish to evaluate the potential impact of different scenarios when developing, revising and implementing strategies to be as efficient as possible in building a collection that can meet a range of possible future demands. Supply side elements typically relate to genetic aspects, technical options, and cost effectiveness of gene bank operations.

1.4.5.1 Priority setting

Strategy informed by characterization data. Genomic and phenotypic characterization and storage of results in the database greatly facilitate future development and use of collections. Characterization data can also be used to compare collections with those already existing in other gene banks and determine uniqueness of material to be stored to avoid duplication (e.g. when collecting material from transboundary breeds).

Vibrant gene banks will offer stakeholders a range of services by expanding collections to include blood, DNA, or tissue samples for further research on livestock genetic diversity. The largest use of the Dutch (over 1000 animals of local breeds) and US collections (over 6000 animals) has been for DNA analyses.

Economic optimization. For rationalization, costs of collection decisions should be optimized against their benefits. Due to the long-term horizon gene banks deal with, routine cost-benefit analysis is difficult. Given the unknown status of future collection usage, benefits are particularly hard to

estimate. However, costs can be accounted for with a relatively high degree of accuracy. Gene banks can perform a full cost analysis of collection development, maintenance, and future regeneration steps. Section 4 details how gene bank operations can be optimized for a given objective.

From another viewpoint, gene bank collections represent a source of societal benefits. The advantages and disadvantages of five methods for documenting such benefits from scientific collections have been recently reviewed by Schindel *et al.* (2020).

1.4.5.2 Continuous monitoring

Continuous monitoring of the status, development and use of the collection is critical. The genetic profile of material already collected in the gene bank should be assessed in the context of genetic diversity of *in situ* populations. Allelic frequencies within commercially vibrant breeds are in a continuous process of evolution and, therefore, a gene bank needs to keep abreast with these genetic changes to keep the collection viable.

Regular gap analysis based on genomic and other relevant data can be used for adapting the strategy and its implementation. The gene bank may wish to budget for regular genomic analyses of its key collections or should develop partnerships with relevant stakeholders (i.e. breeders and breed associations, research institutes, breeding companies) to obtain such data.

1.4.5.3 Projections toward the future

Quantifying the different attributes of a gene bank strategy is difficult, especially in a long-term perspective. First, stakeholders' strategies are evolving (Box 1.4). Moreover, future scenarios have a higher or lower uncertainty, while conservation decisions must be made in the short term, taking into account budget constraints. Gene banks should anticipate changes in future demand, e.g. motivated by possible changes in climate, production systems, markets, consumer preferences and possible calamities (diseases, disasters, etc.). Implementing surveys among stakeholders at defined intervals may be a good way for the gene bank to keep up to date with user's preference and strategy. Alternative strategies and future scenarios should be compared before final strategy selection.

BOX 1.4

Gene banks serve local and mainstream breeds

The livestock breeding industry is constantly changing. Old breeds give way to new, as has been the case since Roman times (Wood and Orel, 2004). The process of change is especially evident in the poultry and swine industries. The elite populations of those industries are pedigreed and the genetics are intensively managed. While many pig breeding companies have similar breeds (e.g., Duroc, Pietrain, Landrace) and breeding goals, the finite populations mean that genetic drift will separate those populations over time, leading to unique sub-populations. The situation is similar for poultry. Gene banks should therefore engage these stakeholders to provide genetic security for these important food producing sectors. The needs of these corporations may be quite different than the normally practice for local breeds. Gene bank strategies will have to accommodate the needs of stakeholders for local breeds and commercial breeding companies. For example, maintaining in vivo populations is costly, so breeding companies may want the gene bank to store large numbers of animals of a unique but little used line so that the entire line can be quickly re-established if needed. In such scenarios, the distribution of costs and benefits across the public and private actors must be duly considered.

Technology breakthroughs and innovations will likely influence future use. Innovative reproductive technologies could change the value of different types of genetic material stored in gene banks. For example, genome editing could increase the value of gene bank collections as a resource base for research and development but could ultimately also result in less use of the germplasm in gene banks for breeding. Thus, the gene bank should continually monitor advances in cryopreservation and reproductive technologies and maintain a connection to research, for instance through a scientific advisory board.

Different objectives may compete in terms of budget allocation and prioritization. When developing future strategies, gene bank managers or stakeholder boards may employ a SWOT approach, identifying the strengths and the weaknesses from inside the gene bank, and the risks or opportunities pushed by external trends. The SWOT methodology is quite helpful to mitigate external risks and to identify opportunities that should be taken into consideration to revise the strategy about building, updating and using the collections. Regular revision of the gene bank strategy goes together with a gene bank policy for training its staff and informing its users, (those providing material or asking for material) on latest methods and techniques

1.4.6 Communication and awareness raising

Because gene bank deals with a large number and range of stakeholders, there is a need to ensure that these stakeholders are regularly informed about the activities of the gene bank and its future plans. Therefore, a communication plan should be included as part of the overall gene banking strategy. Such a communication plan will contribute to a country's implementation of Strategic Priority 18 of the Global Plan of Action, to "raise national awareness of the roles and values of animal genetic resources". Regular communication will raise awareness about the gene bank and the importance of its activities, increasing appreciation of its importance in maintaining agrobiodiversity and sustainability of livestock production systems. Users of the gene bank will be kept abreast of the new services.

1.4.6.1 Targets

The various stakeholders will have different reasons for interaction with the gene bank and will therefore be interested in different types of information. The communication plan should account for this fact and identify the expectations and topics of most interest to each of the various stakeholder groups. The stakeholders will vary in their amount of background knowledge and understanding of the context of gene banking, which will impact the type of language and terminology to be used.

1.4.6.2 Message

Although the explicit goal of communication will typically be to inform the various stakeholders, the implicit objective will be to produce a beneficial outcome for the gene bank. The information targeted for each stakeholder needs to be chosen by considering what they currently believe, what they need to know and how they are expected to react to the communication.

1.4.6.3 Media

In addition to different information, the various stakeholders will likely differ in the way they would like to receive the messages. The communication plan should also consider the delivery method through which the message will be transferred most effectively. For government policymakers, concise formal reports on outcomes achieved by the gene bank relative to the resources used will be of most importance. Researchers may have more confidence in material published in peer-reviewed

scientific literature. Breeders and breeder associations may appreciate an online catalogue documenting the material stored in the gene bank, its characteristics and availability for access.

1.4.6.4 Frequency

The stakeholders, message and media will all influence the frequency of communication. Some forms of communication with the governmental stakeholders may have a fixed schedule. Ad hoc communication with policy makers may be strategically planned to coincide with times at which major decisions are taken. Users of the internet generally want to see updated information each time they access the gene bank's website.

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SECTION 2

Quality management for improved organization and implementation

Quality management for improved organization and implementation

2.1 INTRODUCTION

Development and implementation of a formal livestock gene banking strategy is a critical step in ensuring that a country's cryoconservation programme can address the needs of multiple stakeholders involved in the management of animal genetic resources (AnGR) for food and agriculture. However, it is important for the strategy to have flexibility to accommodate the vagaries of the real world. Once the implementation of the strategy starts, complementary actions should be undertaken to establish an enabling environment for achieving the strategy, to ensure that the strategy is being implemented as planned, and to document progress toward the achievement of the strategy's goals.

Implementing a quality management system (QMS) is a useful approach for a gene bank to deal with the complexity and wide range of management and technical implementation issues. A QMS may be useful to both stakeholders and gene bank management. A QMS oriented towards user satisfaction will build trust with stakeholders and help the gene bank to implement its strategy. Globally, several gene banks have chosen to adopt some form and/or elements of a QMS (Zomerdijk *et al.*, 2020; IMAGE, 2020).

2.1.1 What is a Quality Management System?

A QMS is a formalized set of policies, processes, procedures, protocols, and responsibilities to be undertaken to achieve an organization's goals, with an emphasis on satisfying the demands of the organization's clients and/or stakeholders, while identifying and mitigating the key sources of risk.

The core elements of a QMS can include (Fenton, 2019):

1. **Quality objectives.** The gene bank should define goals that reflect improvements in the services it provides to stakeholders. Objectives should include an expected date of achievement and a framework for assessing progress in achievement.
2. **Quality manual.** The quality manual is the first document to be prepared for a QMS. It should explain the quality objectives, outline the scope of the QMS, indicate any formal quality standard being followed (e.g. ISO 9001), refer to quality control procedures and policies being followed.
3. **Organizational structure and responsibilities.** An organigram should be drafted that illustrates the organizational structure, including personnel and governing bodies, and explain the responsibilities of each. Flowcharts or other visual aids may be used to demonstrate specific roles, such as showing what each person does when processing a sample of materials to be gene banked.
4. **Data management.** Organizations must outline how the information associated with establishing and implementing a QMS is prepared, stored and routinely utilized.
5. **Key processes.** All activities, procedures and equipment associated with optimal operation of the gene bank must be documented.
6. **Stakeholder satisfaction**. Measuring stakeholder satisfaction with the service received from the gene bank is an important indicator helping to ensure that the gene bank is having the desired result and to identify opportunities for improvement.

7. **Opportunities for continuous improvement.** Approaches to address the shortcomings noted in customer satisfaction must be documented and possible solutions must be proposed.
8. **Instruments for measuring quality.** Any tools being used to monitor quality must be identified and plans for regular control and calibration must be specified.
9. **Document archiving.** All documentation showing evidence of quality management, including communication with stakeholders must be maintained in an organized manner.

The most important elements of QMS for livestock gene banks will be described in this section.

2.1.2 Benefits of a QMS for livestock gene banks

Gene banking is a complex and long-term endeavour. For example, samples acquired in 2000 may be of utility in 2050, whereas many gene bank staff will have come and gone, and new equipment and processes will have emerged in the interim. Ensuring that those early samples remain in the bank and are still viable is crucially important. A QMS helps ensure and document the integrity of samples maintained in the bank, as well as the standing of the bank itself to its many stakeholders.

Livestock gene banks are generally responsible to an array of stakeholders, the most immediate being the government and the livestock sector. Therefore, it is important to demonstrate that the gene bank is effectively and efficiently operated and has a positive impact on the conservation and sustainable use of AnGR. This in turn may result in greater willingness by the government and other stakeholders to maintain or increase financial or in-kind support for the gene bank.

A QMS should yield direct benefits to the gene bank. These benefits may include the following:

- Increased efficiency and cost effectiveness;
- Prevention of errors that may result in loss of material or decreased viability;
- Continual improvement of expertise, both technical and organizational;
- Greater staff safety;
- Improved risk management;
- Increased job satisfaction and performance of staff;
- Enhanced identification of staff development needs and opportunities; and
- Improved communication both internally and with stakeholders.

2.1.3 Trends in QMS among livestock gene banks and other biobanks

Countries and gene bank managers around the world have recognized the benefits offered by quality management and many of them are in various stages of either implementing or developing QMS or adopting some of its elements.

In 2019, FAO undertook a global survey of quality management procedures and plans among livestock gene banks (Zomerdijk *et al.*, 2020). Ninety gene banks responded, representing 62 countries. Approximately 30 percent of these banks reported having a QMS. Around 60 percent of the remaining banks were in the process of developing a QMS. In other words, more than 70 percent were at some stage in adoption of QMS. In particular, the gene banks were concerned with quality management of processes associated with technical aspects of cryoconservation such as processing and freezing of genetic material. Less emphasis was placed on interaction with non-governmental stakeholders (IMAGE, 2020).

The global interest in quality management for gene banks and other types of biobanks has led to the development of formal standards for evaluating the competence of biobanks (See Box 2.1).

BOX 2.1
Development of an international standard for biobanks

In 2014, the International Organization for Standardization (ISO) Technical Committee for Biotechnology (TC276) initiated a working group on biobanking in general. This working group developed a new ISO standard for biobanking activities, covering all biological domains including animals, humans, plants, and microorganisms. The standard thus recognizes the common processes underlying any biobanking activities, including animal gene banks. The document is targeted toward gene bank managers, users, regulatory authorities, and accreditation bodies. The new standard, ISO 20387, (ISO, 2018) is now considered the international reference document for quality management of gene banks. The standard covers the various gene-banking processes from collection or reception, preparation and preservation, storage, and validation. Technical and human resource requirements are addressed, as well as requirements for QMS.

As noted in Section 1, gene banks can support research activities. Therefore, gene banks (and other biobanks) can be considered part of a country's overall research infrastructure. In Europe, the concept of a research infrastructure has been formalized and adoption of QMS is a recommended process within this formal structure (See Box 2.2).

BOX 2.2
Gene banks, biobanks and QMS within the European Union's research infrastructure framework

The European Commission defines a national research infrastructure as "facilities that provide resources and services for research communities to conduct research and foster innovation". This definition includes research equipment and instruments, collections of material and data (e.g. gene and biobanks) and computing systems and communication networks. The European Strategic Forum for Research Infrastructures (ESFRI) has developed a roadmap that includes a list of EU and cooperating research infrastructures and the strategy for their utilization and has encouraged EU Member States to develop national roadmaps. Inclusion within these roadmaps is based on various recommended criteria, including the presence of a QMS. The ESFRI roadmap includes biobanking infrastructures for medical research (BBMRI), marine research (EMBRC) and microbial research (MIRRI), thus recognizing the importance of gene and biobanks for research (European Commission, 2020).

2.2 QUALITY MANAGEMENT FOR GENE BANKING

2.2.1 Defining the quality policy

As a foundation for its QMS, a gene bank shall compile in a single document its main objectives, commitments and plans to reach its objectives, addressing issues described in Section 1 of these guidelines. This quality policy document should be brief, formally institutionalized and made freely

available to the public. Commitment of the leadership is particularly important to support the implementation of the strategy. The quality policy, once completed and approved, is a reference document for the gene bank that needs to be regularly updated.

2.2.2 Mapping key processes

The gene bank will map the different key processes supporting its strategy. In general, there are three main types of processes: (i) steering and management (decision-making, communication, monitoring), (ii) support (human resources, informatics, equipment), and (iii) technical (collection, processing, documentation, storage, distribution). Figure 2.1 provides an example of a map of key processes for an animal gene bank.

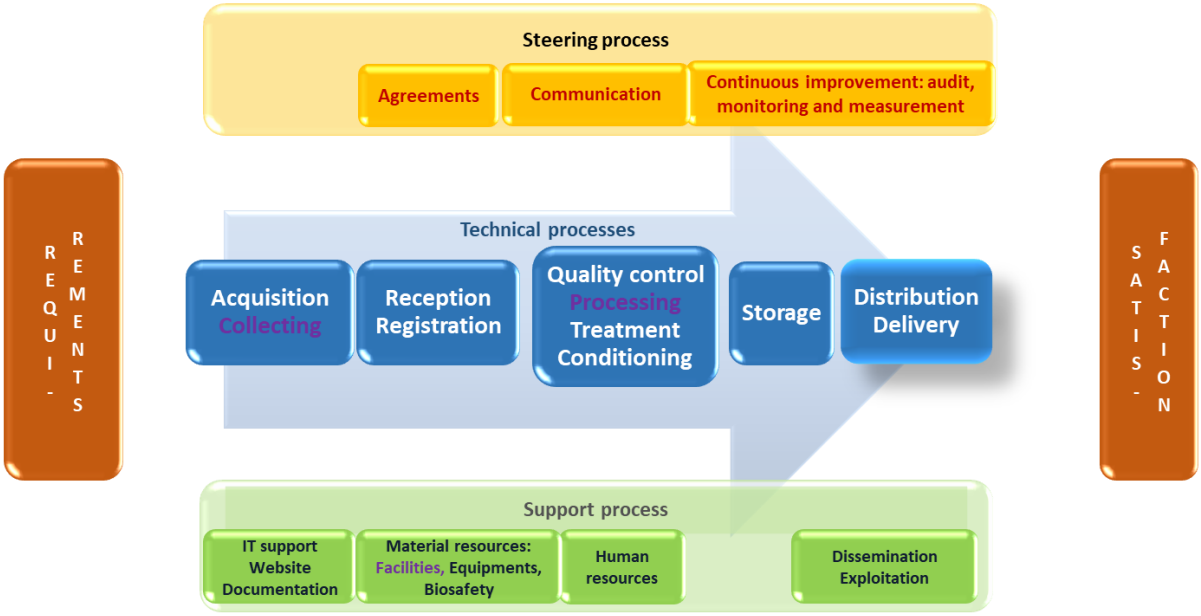

FIGURE 2.1
Mapping the processes of a gene bank Quality Management System

The gene bank will at the same time identify and refer to the relevant public policies and regulations for the type of biological material to be collected, stored, and distributed. These policies and regulations are discussed in more detail in Section 9 and may include the Global Plan of Action for Animal Genetic Resources and the national strategy and action plan, sanitary regulations, animal breeding regulations, access and benefit sharing, intellectual property, at the national and international level.

2.2.3 Stakeholder involvement

As shown in Section 1, the gene bank will cooperate with a range of stakeholders. The steering process of a gene bank should prepare a document that identifies all stakeholders, sometimes called ”interested parties” in QMS terminology, list their expectations and match these to the strategic objectives of the gene bank, and identify the pathway to fulfil these expectations. To this end, the gene bank may set up one or several committees to support stakeholder involvement. The document should describe the operating rules of the committees and the calendar of their activities. Organizing interactions with stakeholders is the best method to reach consensus, maintain trust in the gene bank,

and analyse trends across time. Ideally, the gene bank will have a decision-making body and one or several advisory committees, such as a users' committee, a scientific advisory board, or a strategic steering committee.

The general public may be engaged in many ways but one of the most effective is through the popular press (e.g. newspapers, television, documentaries produced by government). This activity must not be overlooked since the support by the general public is important for policy makers.

2.2.4 Risk assessment

Risk assessment and preventing risks associated with the gene bank collections and operations are central elements to its management. Table 2.1 shows the main risks and mitigation measures according to the main components of the gene bank activities.

TABLE 2.1
Sources of risk to gene bank operations and possible preventive measures

Component	Risks	Preventive measures
Equipment	Failure or breakdown leading to loss of material	Alarms; maintenance of equipment; secured access; electricity supply; duplicate storage
Human resources	Insufficient manpower or lack of expertise leading to mistakes and degradation of resources	Human resources management plan; replacement policy; training program; cross-training in the staff
Malpractice	Intentional damage to collection	Security clearances for personnel; logs monitoring date collection accessed and by whom
Hygiene and safety	Staff injury or death; Release of potentially dangerous material or chemicals in the environment; Specific animal health risks	Adapt organization to prevent accidents; safety plan; controlling sanitary status of animal material; waste management plan
Biological material	Insufficient quality	Perform regular quality control; request quality test results before entry into collection
Information system	Loss of data	Back-up system
Budget	Suspension or discontinuation due to lack of funds	Regular budget monitoring; monitoring and allocating funds derived from fees or extraordinary funding sources
Catastrophic events (e.g. floods, earthquakes, fire, internal disease transmission)	Destruction of or damage to facilities, resulting in loss of collections	Back up or duplicate collections Selection of storage sites
Customer relations	Dissatisfaction of users or stakeholders	Establish a multi-actor board Satisfaction scores and complaints Corrective measures in case of complaints

2.2.5 Evaluation framework

A gene bank must regularly undergo evaluation to confirm if quality control measures are up to standard. Three approaches can be taken: (i) self-evaluation; (ii) external assessment within the field of activity; and (iii) external audit by an accredited body.

2.2.5.1 Self-evaluation

The gene bank can start by implementing a self-evaluation test with the following actions:

- establish a Strength, Weakness, Opportunity, and Threat (SWOT) analysis for all or part of its activities, particularly those considered critical and update the SWOT at regular intervals. The Strengths and Weaknesses are internal, whereas the Opportunities and Threats come from external origin;
- use an external reference document, such as ISO 9001 or ISO 20387, and check whether the operations of the gene bank comply with the requirements of these documents; and/or
- use a self-diagnostic tool like the one developed by the Horizon 2020 EU project IMAGE to help gene banks in the development of their QMS (Appendix 2.1).

2.2.5.2 External assessment

Then, an external assessment can be used with the following actions:

- perform regular surveys to check satisfaction of its users and analyse the general trends as well as potential specific messages, this can be done periodically as deemed necessary; and/or
- use cross-evaluation among a set of other gene banks, which could include a peer review system.

2.2.5.3 External audit

Finally, independent evaluation with an external audit (also called third-party evaluation) is recommended to get an external and impartial analysis of the internal operations of the gene bank. This independent evaluation is a requirement of most official certification processes. This action can be performed by persons accredited with audit standards, who preferably have technical expertise in gene banking. Their report can then be used to strengthen gene banking processes and to make higher level administration aware of current and future gene bank needs.

These approaches have comparative advantages and disadvantages. The self-review can usually be expected to be the simplest and lowest cost option but is also the least impartial and has only internal value. The independent review is usually the most complex and expensive but will be impartial and may be of value for external certification purposes if such certification is needed or desired. The independent evaluation is also likely to be more effective for building trust with potential new users or stakeholders that are familiar with the evaluation procedures.

2.3 KEY PROCESSES

The QMS shall include the preparation and maintenance of a library of documents that list and describe the key processes involved in successful operation of the gene bank.

2.3.1 Management

Gene bank management has a unique responsibility. It not only oversees the day-to-day operation of the gene bank; in many countries, management is the interface between the gene bank and its stakeholders. The QMS documentation should include a description of the roles and responsibilities of management. The following is an exemplary list of external and internal roles of gene bank managers.

Externally, gene bank managers may be responsible for the following:

- Organizing and conducting meetings with funding bodies and advisory groups;
- Raising awareness among stakeholder groups, such as breed associations or companies, of gene bank activities, interests, needs and concerns;
- Informing upper administration of the institution hosting the gene bank about the status and goals of the gene bank, and stakeholder response to gene bank activities;
- Securing long term support for gene bank operations;
- Providing stakeholders with information about the collection, tailored to their species and/or breed; and
- Implementing a technological watch and monitoring relevant regulatory developments.

Internally, gene bank managers are usually responsible for the following:

- Establishing and adhering to all factors impacting collection security;
- Ensuring implementation and overseeing day-to-day operation of the gene bank, including in-coming and out-going shipments of germplasm and tissue, processing and cryopreserving samples, checking data entry, evaluating genetic diversity acquired from various breeds;
- Performing gap analysis for the various species and breed collections;
- Managing human resources by providing direction for operations and hiring competent staff; and
- Managing financial resources, in particular funds needed to execute collection objectives.

2.3.2 Gene bank equipment

The QMS will include a support process to record mandatory information about equipment. As a minimum, this information usually includes the following: (i) manufacturer and commercial model; (ii) date of purchase; (iii) value at purchase; (iv) location in the facility; (v) maintenance operations, including calibration; and (vi) records of failure and repair. Records of both maintenance and calibration are needed as a cross reference when comparing various measurements over time.

Gene bank managers need to be aware that new equipment purchased to replace equipment that is becoming outdated may perform differently due to manufacturer improvements. As a result, historic data may not correspond with measurements taken with the new equipment. To document such differences the old and new equipment must be tested using the same set of samples. If differences exist, conversion equations, such as by linear regression, can be developed to enable the utilization of old and new data.

The intended size of the germplasm collection will govern the scope of equipment and physical space. Equipment can be partitioned into various gene bank functions. How many samples and from what species might be processed will need to be assessed so that equipment purchases will match the maximum number of samples to be processed in a single day.

2.3.3 Gene bank personnel

The QMS will include a support process dedicated to human resources that records mandatory information about each staff position. This information will usually include (i) job description, (ii) training programmes and (iii) expertise on technical processes. The gene bank may want at least two persons trained for each critical activity, to avoid interruption of those activities.

Fully functional gene banks require combining the disciplines of genetics, cryobiology, and information systems. Like gene bank equipment, gene bank personnel can be scaled to match the size and requirements of the gene bank. At a minimum, a gene bank manager and a technician are required, and these may be part-time roles. Under sparse staffing conditions, gene bank managers may seek outside support from other branches of government or academia to fulfil short-term needs as well as to establish long term collaboration with scientists. At the other end of the spectrum, gene banks may employ full-time scientists and technical support for each of the disciplinary areas mentioned above. If countries should choose to have a distributed gene banking system, with more than one location, the various sites should each harbour the core set of expertise for gene banking (i.e. manager and technician), while sharing staff with expertise in a specific discipline. Close coordination among sites will be critical for maximum efficacy and efficiency.

2.3.4 Genetic material database

Section 8 details the issues related to data management, but the following issues are critical for quality management:

- Databases are indispensable infrastructure for efficient operation of the gene bank.
- The gene bank's database shall make publicly available a set of data defined in agreement with material providers and complying with regulations. This means they need to be accessible via the internet, according to FAIR principles (where applicable, see section 8). The database will generally also have a private section to keep information needed for internal use, such as sample storage in a specific tank location, or privacy-sensitive information.
- Spread sheets are not sufficient to efficiently and reliably run gene banks, i.e. they are not a substitute for a formal database.
- Security of data is a critical issue that merits maximum attention. One or more levels of redundancy should be maintained on machines in different physical locations or units of cloud storage. If a country has multiple gene banks all locations must be connected to a common portal or utilize the same database, to facilitate data sharing.
- The QMS documentation should describe all of the pertinent features of the database, including software utilized, data fields stored, security procedures and access rights.

Information about the samples is as important as the samples themselves (see also section 8). There are two data streams per sample that gene banks should be acquiring (see Figure 2.2 below). From outside the gene bank, information such as: species, breed, breeder, animal ID (which should follow a standard, as much as possible, since there may be more than one ID), date of birth, pedigree, date of sampling, sampling protocol, phenotypic measurements, genotypic information, environmental descriptors or potentially GIS coordinates for where the animal was born, and management system (extensive, mixed crop-livestock, or intensive) the animal was produced. Within the gene bank the data stream focuses upon various processes associated with sample handling. For example, date of arrival, sender name and address, state of arrival (fresh or cryopreserved), the temperature, pH, and sperm movement analysis of fresh semen at arrival. For samples already cryopreserved, information includes data on the straw itself, noting if there were any peculiarities about the shipment, etc. Once samples are frozen the storage location will also need documenting. This usually includes a hierarchy from the largest storage space to the smallest. For example, tank number, pie within a tank, canister, goblet, and cane/visotube (the level where no individual animal samples are mixed).

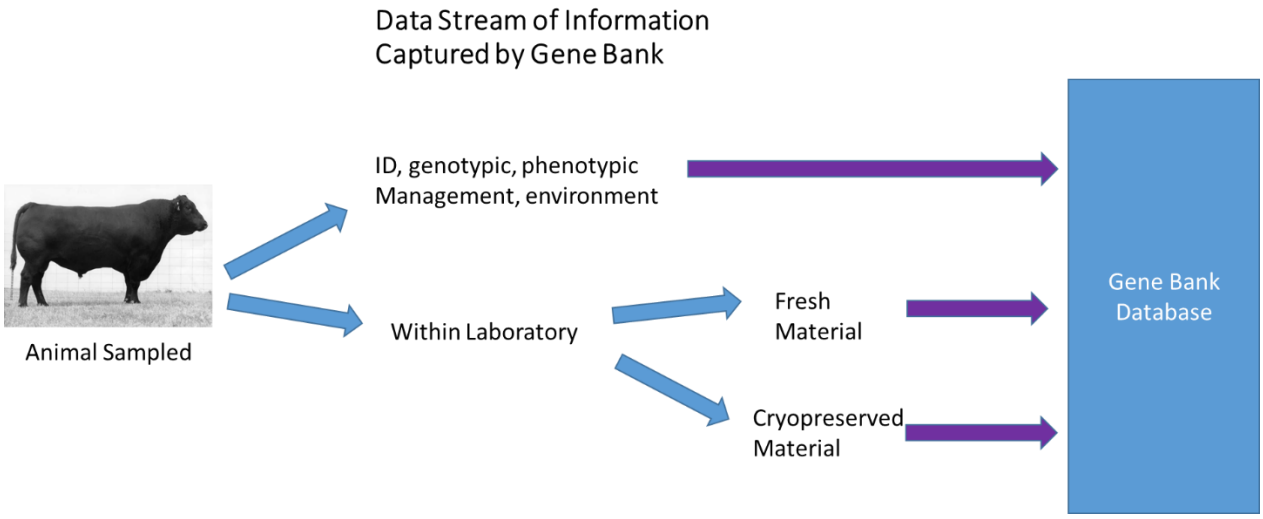

FIGURE 2.2
Data stream of information captured by a gene bank

2.3.5 Genetic material acquisition

The QMS documentation should describe the process of acquisition and specify the conformity criteria applied for collection and acceptance of the material. The conformity criteria will include both strategic (such as prioritization) and technical elements. These criteria should address standards for collecting material of a particular donor animal of a certain breed, as well as for individual samples of material and related data. Following the decision to acquire genetic material, a system must be in place to trace the acquisition between the provider and the gene bank. Generally, an agreement between animal owner and gene bank will be signed and will serve as the chain of custody as the sample moves from the farm or collection centre to the gene bank (see Section 9).

Within and among countries there is no "one size/approach fits all" scenario. The acquisition of germplasm and tissue is highly variable based upon the species and how functional technologies are for a species. Many gene banks have demonstrated that acquiring cryopreserved semen from industry sources is an efficient mechanism to capture a wide range of breeds and animals within breed. In addition, acquiring already cryopreserved samples can be a mechanism for obtaining biological samples that have been cryopreserved for a long period (>40 years), particularly in the case of beef and dairy cattle. Movement of these samples can be relatively easy with commercially available courier services and using liquid nitrogen shipping tanks. All acquisition options that are utilized must be recorded in the QMS documentation.

For some species cryopreserved semen is not typically used. For example, the swine industry mainly uses fresh semen in their artificial insemination programmes, relying on semen extenders that maintain the semen viable for as long as a week. Extended semen can thus be shipped to the gene bank, where it can be processed and cryopreserved in the gene bank's laboratory. Such innovative approaches have not been implemented in the poultry industry, where frozen semen almost never is utilized commercially as rooster sperm needs to be cryopreserved within a short time after collection (see Chapter 3).

Gene banks often acquire cryopreserved material that has already been fully processed by a third party. The bank must therefore define and document criteria for acceptance or rejection, be it at the

level of quantity, quality, or documentation. The receiving bank must obtain a technical description of the cryopreservation procedure used, which will not only help confirm the quality of the material, but also define how the material can be eventually utilized. To better control external processes, the biobank may include in its QMS a list of recommended material processing and cryopreservation protocols to be used by all providers of genetic resources.

In the case of frozen semen, the monitoring of the cold chain is critical, and the gene bank must define a threshold temperature above which it should not accept frozen semen for future reproductive use. In case of any doubt, the gene bank may use an aliquot for quality assessment, for instance by thawing semen and measuring the proportion of alive sperm cells and their motility (see Section 3).

2.3.6 Material collection

As will be seen in Section 3, animal germplasm may be collected and cryopreserved in various ways that are dependent upon technical expertise and on access to donor animals for collection. The gene bank should identify the anticipated use of a biological material, i.e. for reproduction, molecular studies, or health monitoring, in order to choose a collection procedure that will ensure fitness for purpose of the material collected. Also prior to collection the gene bank needs to have developed a unique identification system to ensure traceability of samples acquired. At the same time, the gene bank will define the information to be collected at sampling, also referred to as the ”minimum data set”, in order to properly document the material at the time of entry into the collection.

The gene bank must list the technical steps to be followed by the collection procedure in order to control the risk of degradation of the biological material at sampling. The gene bank should collaborate with researchers for the development of new methods or to remain informed of the latest technological progress. Before starting the collection, the gene bank should ensure it is complying with existing regulations, in particular regarding animal care, animal health and access-and-benefit sharing issues (See Section 9).

When collecting germplasm and tissue on farm, gene banks can either send staff or use commercial vendors. The samples collected on farm can be cryopreserved in the field or, as mentioned above, be shipped fresh and extended to the laboratory for processing. Gene banks should also take blood samples from the donor animals and perform a series of health tests to ensure the animal is free of any diseases transmitted via the germplasm (see Section 7 for further discussion). Depending upon national regulations gene banks may need to store material that has different health status separately. Standard operating procedures for on farm collection, including both technical and administrative actions, should be documented in the QMS.

Germplasm can also be collected at commercial AI or animal reproduction centres. Such centres have a controlled collection environment, including quarantine and health testing, as well as high levels of technical expertise. This may be the preferred route for semen collection, especially when the bank is acquiring material from an animal that the centre had already intended to collect. In such cases, the cost of obtaining a few extra samples may be low. On the other hand, if this scenario involves animals in which the centre has no direct interest, the costs of the services provided may be quite high.

2.3.7 Material processing

As discussed in Section 3 there are a wide range of cryopreservation protocols that can be used for each species. The QMS documentation should clearly outline the protocols being applied. In many countries there are professional organizations that establish standards and practices considering a wide

range of protocols. When such protocols are used, the QMS should refer to these organizations and protocols. Details on material processing are in Section 6 of these guidelines.

The gene bank's primary concern is to ensure viability of the stored material, to obtain pregnancy or a fertilized egg when needed. Procedures for monitoring cell damage through the preservation stages and performing post-thaw analysis on cryopreserved samples can be parts of material processing and listed in QMS documentation. Criteria for acceptable levels of success in processing biological material may be based upon literature values or experimentation by the gene bank (see section 6 for more detailed information about different types of biological material and associated criteria).

While recommendations from research and industry organizations, as mentioned above, may be useful, gene banks still need to formalize processes and testing procedures performed in-house. These processes and procedures need periodic review and in the case of cryopreserved semen samples to routinely withdraw a specified number of males from the gene bank and perform test matings to ascertain the level of fertility that can be achieved. If samples were collected and cryopreserved at a commercial AI centre, such a test may not be necessary because that entity will be selling samples from the same animal and any fertility problems will be known.

Often gene banks will be confronted with the challenge of dealing with poor quality semen samples from rare breeds. In these situations, gene bank standards for collection and post-thaw quality may have to be relaxed, while knowing that additional animals and samples will need to be collected for reaching collection goals.

2.3.8 Material storage

The key factors to consider for storage are traceability and safety. Each storage unit should be uniquely identified (such as with a barcode label, a printed unique identification number, or a colour code for semen visotubes) so that the identity and location of the biological material are unambiguously known and entered in the gene bank information system.

Duplicate storage is recommended. In addition, an empty storage capacity should remain available to rescue material in the case of failure of storage equipment.

Systems for continuous temperature control and control of access to the storage room must be implemented. The systems should record all fluctuations in temperature and entries of personnel into the room. These data are to be entered into the gene bank database so that long term trends can be documented during gene bank reviews.

1.

2.3.9 Material distribution

The gene bank should establish in its strategy and QMS the procedure for stakeholders to request material. Mandatory information to be provided by the applicant must be established and the decision-making process for access to gene bank material agreed with the governing board. Criteria to consider may include (i) the consistency of the request to the strategic objectives of the gene bank; (ii) the soundness of the request regarding to its objective (such as the number of semen samples requested considering the mean fertility of frozen semen in the species); (iii) the technical feasibility of the intended use; (iv) the quantity of material available, so as to maintain the minimal quantity of material the gene bank is committed to keep; and (v) opportunity to obtain the material from other sources.

From a technical viewpoint, distribution must be performed in such a way that quality of the material is preserved for the intended use. The gene bank should prepare a standard shipment procedure and identify reliable third parties in charge of transportation. Traceability of the shipment throughout the process is mandatory. The gene bank shall record all distribution events and update its database accordingly.

2.4 IMPLEMENTATION AND CONTINUOUS IMPROVEMENT OF QUALITY MANAGEMENT SYSTEMS

2.4.1 Reviewing indicators

Each process of a QMS must define its targets and establish indicators to monitor the achievement of the targets. For instance, regarding human resources, a target may be sufficient capacity for a given procedure, so a logical indicator would be the number of trained staff in that technique or the number of training events offered in a given period. Regarding storage, the target may be capacity to avoid loss due to equipment failure and the indicator the number of rescue tanks available or the rate of duplicated storage.

Indicators for the management process are usually related to risk control or to satisfaction of users but can also include more specific elements related to the gene bank strategy. An example is the percentage of endangered breeds for a given species with stored material in sufficient quantity according to SDG Indicator 2.5.1b. For user satisfaction, the gene bank may for example organize a survey among its stakeholders to check that their expectations are fulfilled.

Each process should regularly be reviewed to check indicators, confirm or revise objectives, update the SWOT, identify the need to update procedures or to add new procedures. Once a year, the management review of the gene bank will examine each process and its indicators; check for adequacy, effectiveness, and alignment with the objectives; identify priorities for actions, including any need for changes in the QMS; in order to establish an action plan for continuous improvement.

The action plan will include the organization of internal and/or external audits as deemed necessary by the decision-making board. The audits are aimed at checking that the QMS (i) is effectively supporting the objectives of the gene bank, (ii) complies with the requirements set by the gene bank, and (iii) is effectively implemented and maintained. The gene bank shall retain all information collected during the audit.

2.4.2 Recording operations

Recording all steps, both in management and technical procedures is mandatory. A QMS can be summarized as “writing what is done and doing what is written”. Gene banks should record and be able to trace any action associated with an objective of the gene bank. For instance, agreements signed between the gene bank and the provider of biological material are a key record for tracing the entry of resources, documenting its purpose and legal status, and identifying a contact person for any future needs. Another example is monitoring equipment, date of purchase and date of maintenance or repair.

2.4.3 Non-conformity assessments

Any deviation from normal operations or from achievement of the objectives, or any complaint from users, must be reported and classified according to its impact on gene bank operations. Impact can be minor, moderate, or critical. Critical non-conformities are those that prevent the gene bank from performing its operations. Examples include (i) long-term electricity blackouts without a safety

generator; (ii) interruption of liquid nitrogen provision; and (iii) computer failure preventing access to the database and blocking material distribution. Minor non-conformities are those that can disrupt an operation but not block it completely or those that do not affect technical quality of the material. Moderate non-conformities fall in between, such as those that may reduce technical quality of material but not cause it to be inviable or that may block some operations for only a very short period. The impact classification of a non-conformity should be reviewed to inform the establishment of necessary corrective and/or preventive measures.

Any non-conformity must be analysed by considering the possible causes involved. The potential causes are human resources, equipment, method, biological material and management or a combination of these. Then, the gene bank must evaluate the need for action to eliminate the cause(s) of the non-conformity to ensure it does not recur or occur elsewhere. It may update its risk assessment and mitigation plan to account for observed deviations or complaints.

2.4.4 Corrective and preventive measures

Corrections are actions taken to mitigate the risks and attenuate the impact of a non-conformity. They may consist of repairing an equipment, providing additional training to an employee, or adding a step in the monitoring process of an activity.

Preventive actions are steps to avoid that a non-conformity occurs. They may consist of replacing an equipment before it breaks down, hiring new staff, implementing a new protocol, or modifying a given decision-making process.

Corrective and preventive actions are examined at each process review and at the management review.

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SECTION 3

Choice of biological material to be preserved

Choice of biological material to be preserved

3.1 INTRODUCTION

The biological material preserved in cryoconservation programmes for animal genetic resources (AnGR) is expected to maintain and manage genetic diversity, and lead to its reconstitution if endangered or lost. Many routes are now available to reach this objective, and all of them have pros and cons. Determining which type of germplasm to preserve is a multifactorial consideration that must be performed so that the goals of a programme can be achieved while also identifying the maximum potential uses for any sample. Specifically, this means that a gene bank manager or curator of a collection must consider:

- Costs associated with supplies and equipment required for sample collection, processing, preservation, storage, and utilization;
- Sample storage capabilities such as liquid nitrogen tanks, freezers, locations that are impervious to the elements, and that are appropriate for the type of germplasm collected;
- The expertise of the staff and its complementarity with the complexity of the collection, preservation, and utilization methods;
- Success rates associated with the method of collection, the means of preservation, and the goal for utilization by the type of germplasm curated;
- The utilization goal, so that the sample is appropriately preserved for its intended use (such as reconstitution or expansion of the genetic diversity within a line/breed/species in response to genetic bottlenecks, to introduce genes to enable adaptation to climate change or production demands, research, evaluation of animal health evaluation - See Section 1);
- The implication of genetic background on the processes utilized, knowing that not all techniques work well, or in some instances at all, with all species or breeds (commercial, research, and local populations) of livestock;
- Physiological factors that determine the quality of a sample and apply to all types of germplasm, such as seasonality, health status, and age; and
- Whether a collection and preservation technique meet the ethical and animal care guidelines and if the animals/samples meet health and sanitation standards established by nation.

Therefore, this section will intend to present perspectives on the choice of germplasm to encourage preservation activities that meet the goals of a curator and result in a valuable collection with tremendous utility. Section 6 provides details about the procedures for cryopreservation and eventual utilization of the genetic material introduced in this Section. Box 3.1 provides a glossary of terms (methods and material types) and abbreviations that are discussed in both this Section and Section 6.

3.2 SEMEN

Semen cryopreservation is historically the first reproductive biotechnology developed and it is still frequently used across many livestock species because it allows for the conservation of a large number of gametes and often at a relatively low cost. Although cryoconservation based solely on semen will require initial mating with females of a different breed, followed by several generations of backcrossing to reconstitute a nearly genetically pure population, the practicalities of semen cryopreservation often outweigh this shortcoming. Furthermore, semen samples are perfectly

appropriate for other cryoconservation goals, such as managing the genetic diversity of *in situ* populations. Cryoconserved semen will also complement collections of other cells, such as embryos or oocytes.

Most countries have developed infrastructure to collect and freeze semen because cryopreservation protocols are an important part of the daily breeding and reproductive routines in almost all agricultural species. Consequently, collection and cryopreservation of semen should be a fundamental component within a germplasm collection because of the simplicity of collection, cryopreservation, and storage, the genetic variability which can be easily captured with this type of germplasm, and, although the techniques are variable, the relative ease of use for artificial insemination (AI), *in vitro* fertilization (IVF), intracytoplasmic sperm injection (ICSI), or for DNA extraction. Therefore, this sub-section will provide an overview of the current state of: (1) the advantages and limits of semen collection, cryopreservation, and utilization for the major agricultural species (2) address some improvements to aspects of these processes, and (3) discuss how these developments increase the value of this form of germplasm.

BOX 3.1
A glossary of abbreviations for key terms in the germplasm collections

Artificial insemination (AI) - deposition of semen or sperm, which was previously collected from the male, into the vagina, cervix, or uterus of the female.
Assisted reproductive technologies (ART) – the collection of methods for artificially improving the reproductive performance of animals, which may include such artificial insemination, *in vitro* fertilization, embryo transfer and their related procedures.
Cryoprotectant (CPA) – a substance added to media that is used to inhibit cellular damage during freezing and thawing.
Embryo transfer (ET) – placement of an embryo into the uterus of a female for the purpose of establishing pregnancy and producing live offspring.
In vitro culture (IVC) – the maintenance of cells, zygotes, or embryos in a culture to achieve specific stages of development.
Intracytoplasmic sperm injection (ICSI) – injection of a single sperm into an oocyte to achieve fertilization.
Induced pluripotent stem cells (iPSC) – somatic cells that are reprogrammed into an embryonic, pluripotent state.
In vitro fertilization (IVF) – the process of combining sperm and oocytes in culture to achieve fertilization resulting in embryos and offspring.
In vitro maturation (IVM) – a stage of the IVF or vitrification process in which oocytes are placed in culture to achieve a specific developmental state.
In vitro production (IVP) - the process of creating embryos via IVF and then performing ET to produce live offspring.
Mesenchymal stem cells (MSC) – stem cells from non-embryonic sources that are capable of differentiating into a variety of tissues.
Primordial germ cells (PGC) – an undifferentiated stem cell that is the precursor of gametes.
Ovum pick up (OPU) – transvaginal aspiration of ovaries with ultrasound guidance resulting in the collection of oocytes.
somatic cell nuclear transfer (SCNT) – deposition of a somatic cell into an enucleated oocyte resulting in an embryo which can be transferred to produce viable offspring.

Semen collection is a simple procedure. When performed correctly, for many species, it is minimally invasive (for example, by using an artificial vagina) and causes no apparent physical discomfort, thus usually resulting in the collection of high-quality samples with significant quantities of sperm. For example, some species including boars, roosters, and tom turkeys, can likewise be collected using non-invasive techniques such as the gloved hand (boars) or abdominal massage (poultry). When males cannot be trained for collection using non-invasive methods, and if permitted within animal care and use guidelines, electroejaculation may be a substitute. It is important to note that poultry and stallions should never be collected using electroejaculation and if possible, this technique should be avoided with goats (bucks) and used only following administration of sedation in boars, as they tend to be very sensitive to the electrical stimulation. If the use of an artificial vagina or electroejaculation is not possible, then alternative methods, such as transrectal ultrasonic-guided massage of the accessory sex glands) (Abril-Sánchez et al., 2017, 2018ab) or transrectal digital stimulation (Tekin et al., 2019) has recently been investigated with small ruminants. With these approaches for semen collection, fewer and potentially no electrical stimuli are used while a quality sample may be collected, which may mitigate animal welfare concerns. Likewise, procedures for pharmacological stimulation of stallions continue to be developed to enable semen collections from animals that may have idiopathic ejaculatory dysfunction (Cavalero *et al.*, 2020) or other issues with the collection process. Similarly, pharmacological agents are also being explored to increase the quality and quantity of semen samples when administered out of an animal's normal breeding season (Beracochea *et al.*, 2018, 2020) thus potentially acquiring semen samples more accessible.

While semen is traditionally collected via ejaculation, alternative methods may also be considered. In mammals, viable epididymal spermatozoa from excised testes can be obtained from castrated animals or from animals that experience an untimely death. For seasonal breeders, this technique is best used during the mating season, but seasonality may not be an issue for animals residing in equatorial regions. This method has been successfully applied to stallions (Cunha dos Santos, 2017), roosters (Villaverde-Morcillo, 2016), rams (Bergstein-Galan, 2017), bulls (Turri et al., 2012), bucks (Turri et al, 2014), boars, and other agricultural species.

The quality of the semen sample acquired also depends greatly on collection method and on the technical knowledge of the staff who perform the collections. For example, proper, gentle animal handling techniques usually result in the acquisition of a higher quality sample. The sample quality is also greatly influenced by the genetic background, the physiological state of the males (in or out of reproductive season), the frequency of semen collection (species specific), and many environmental factors such as photoperiod, food and water availability, and temperature. All these factors should be considered prior to sample collection and cryopreservation.

Once collected, the quality of semen samples is evaluated for motility, plasma membrane integrity, acrosomal integrity. In addition, a quality can be assigned by kinetics or cytometrics, which raises the issue of establishing a threshold below which a sample will not be preserved or utilized. It is wise for each gene bank to establish this threshold for acceptability of a sample, but it should also consider allowing flexibility if the animal is from a transboundary, local, or research breed or line, the ability to acquire additional samples, and potential uses for the semen sample (AI, IVF, ICSI). When considering these factors, it may be obvious to preserve a high-quality sample. However, when some or all of these factors are substandard, preservation of a lower quality sample may still be warranted if there is limited opportunity to acquire a second collection, or if it is from a rare breed or research line. Furthermore, the type of preservation method should also be considered. Livestock semen is frozen using a variety of freezing rates specific to each species and following the addition of internal and

external cryoprotectants (CPAs, substances added to media that is used to inhibit cellular damage during freezing and thawing). The samples are then stored in liquid nitrogen (see Section 6). The goal is to have the sperm recover their fertilizing ability after the freeze-thaw process so that they can be used to generate progeny. However, not all samples are of a high enough quality to merit freezing. In such cases, a semen sample of extremely low quality may still be useful for DNA extraction and genetic analyses and thus may have value for storage. In this instance, it may be appropriate to store the sample at -20 to -80 °C in freezers rather than in liquid nitrogen. However, the latter is always a viable option regardless of the intended use. Depending on storage space, access to liquid nitrogen and the associated expenses, the species, as well as the expertise of staff in assisted reproductive technologies (ARTs), dehydration or lyophilization of samples (with ICSI being the intended use) may also be a viable option in specific circumstances in mammal species (Saragusty *et al.*, 2020). Recent reports have documented the financial savings of storing samples in this format and acceptable cleavage and blastocyst development rates demonstrating that optimization of these techniques may come to fruition in the future (Keskintepe and Eroglu, 2021).

As previously noted, the fertility rates with frozen-thawed semen are affected by the species, cryopreservation medium, cryoprotectant, the male, the breed or genetic line (especially if there has been significant selection pressure as is the situation with some highly inbred poultry research lines), factors related to the female of the species that is inseminated (such as age, health, synchronization protocol, and parity) and the type of ART utilized such as AI, IVF, and ICSI. Moreover, the interaction of these influences makes optimization of ARTs for some species challenging, and this is particularly observed in non-mammalian species, such as poultry and honeybees, where low post-thaw sperm quality, coupled with extended periods of storage in the female following insemination, results in highly variable, and often low fertility rates. For many agricultural species, analysis of these factors should result in standardization of methodologies so that consistent fertility rates are achievable with known insemination doses (Spencer *et al.*, 2010). If fertility rates are lower than expected, the sperm dose, number of inseminations, and the model used to generate progeny (such as through backcrossing, and the number of backcrosses needed to regenerate a line or breed) can potentially be increased, or adjusted, to address the deficiency (Amann and DeJarnette, 2012). These factors should be considered, by species and breed/line, to ensure that enough samples are acquired based on the intended application of ARTs to meet the needs of utilizing the genetic resources encompassing recreation of populations and genetic analyses.

3.3 OOCYTES AND EMBRYOS

Oocyte cryopreservation is now commonly performed with many species, which makes this a powerful tool when conserving AnGR because it enables selection of a male (semen) and female (oocyte) from a collection to create offspring that will meet a current need (such as research goal or market demand) at the time of use. However, like semen, because they are gametes, reconstitution of an extinct breed would require the use of males from another breed followed by generations of backcrossing if semen of the extinct breed were not also cryoconserved. Cryoconservation of exclusively oocytes would also result in the loss of the breed's Y-chromosome in mammalian species. Therefore, semen should always be conserved along with oocytes, if possible.

When cryopreserved embryos are considered, their preservation captures the diploid genome of an animal and can therefore be immediately utilized to meet a particular need in the future. However, the challenge with embryos is that the reanimated animal may not be able to meet the average level of

production in the future if a significant amount of time has passed and substantial genetic gains were achieved for that breed or species. This is particularly relevant for high performing breeds where selection is actively being applied.

3.2.1 Oocyte cryopreservation techniques

Slow freezing or vitrification are both options for cryopreservation, depending on the species and developmental state as described in Section 6 (cattle, Do *et al.*, 2019, buffalo, Parnpai *et al.*, 2016, Liang *et al.*, 2020, goats, Wahyuningsih and Ihsan, 2018, sheep, Menchaca *et al.*, 2016, Mogas, 2018). Success rates are often low due to the physical structure of the cell (e.g. surface to volume ratio) and non-optimized protocols that do not consider many critical factors such as the water or CPA permeabilities (Díez *et al.*, 2012, Mogas, 2018). Improvements in post-warming oocyte development and IVF rates are being achieved by considering the state of the cumulus oocyte complex at the time of vitrification with cattle (Zhou and Li, 2013, Ortiz-Escribano *et al.*, 2016) and sheep (dos Santos-Neto *et al.*, 2020) and favourable post-warming expansion rates have been attained (> 50 percent, Romão *et al.*, 2015). Still, conservation of these germplasm types may be expensive for some programmes because the costs of the technical expertise needed for collection and preservation and of the media (reagents, serum, hormones) may be considerable. Moreover, collection of these types of germplasm may be challenging and expensive as well if superovulation or embryo flushing is utilized, and when the low success rates for producing live animals from these germplasm sources are considered, regardless of species, then the cost of these activities may not be justified for all programmes.

Equine oocytes can be harvested from live mares using ultrasound-guided Ovum Pick-Up (OPU). Mares do not respond to superovulation like other animals, so either one *in vivo* matured oocyte, or a dozen immature oocytes can be collected by OPU per cycle from the dioestrous mare. The latter technique is more efficient in terms of blastocyst production (Jacobson *et al.*, 2010). Equine immature oocytes can also be acquired post-mortem from excised ovaries, but it is critical to begin the process immediately following collection of the ovaries. The ovaries should be slowly cooled to room temperature and then maintained at 12 °C (Hinrichs 2018). Following collection, immature equine oocytes can be held or shipped overnight at room temperature without affecting developmental competence.

Oocyte vitrification results in greater post-vitrification quality in the horse than slow freezing and has resulted in live offspring (De Coster *et al.*, 2020). Currently, vitrification of *in vivo* matured oocytes provides the best results, with the first foal reported after oocyte transfer in 2002 (Maclellan *et al.*, 2002), and a blastocyst rate after *in vitro* embryo production of 33 percent resulting in live offspring (Maclellan, *et al.*, 2010). However, collection of *in vivo* oocytes and the current efficiency of the vitrification technique is limited (De Coster *et al.*, 2020).

Immature oocytes can either be vitrified upon collection in the immature state or after *in vitro* maturation (IVM). Vitrification of immature equine oocytes has resulted in blastocyst production (Ortiz-Escribano *et al.*, 2018, Angel *et al.*, 2020) and the birth of a foal (Ortiz-Escribano *et al.*, 2018), although efficiency was ten times lower than with fresh oocytes (Ortiz-Escribano *et al.*, 2018).

As with other mammalian species, the primary means of gene banking of pigs is by cryopreservation of male germplasm, particularly semen. Cryopreservation of oocytes and embryos is also possible, but the practical use of oocyte and embryo cryopreservation in pigs has been limited by the sensitivity to cryopreservation and the difficulty of embryo transfer (ET) technology. Various protocols for the

vitrification of porcine oocytes and embryos have been reported and the development of a standardized (optimum) protocol would be beneficial for each. To date, oocyte cryopreservation for gene banking has only been reported in two local breeds (Varga *et al.*, 2008; Somfai *et al.*, 2019).

Porcine oocytes are most frequently collected by the aspiration of follicles from ovaries removed after slaughter. However, the harvest of oocytes from live animals is possible by endoscope-assisted OPU (Brüssow *et al.*, 1997) and ultrasound guided OPU (Yoshioka *et al.*, 2020). Porcine oocytes are very sensitive to low temperatures and do not survive conventional slow-freezing methods, but they respond well to vitrification (Somfai *et al.*, 2012) and an optimized protocol for the efficient, rapid, and inexpensive vitrification of oocytes in large groups has been developed by Somfai and Kikuchi (2021). Oocytes vitrified at the immature stage have a higher competence for development than those vitrified at the mature stage (Egerszegi *et al*., 2013) and this consequently results in the production of live piglets using *in vitro* embryo production (IVP), technology of vitrified-warmed oocytes (Somfai *et al.*, 2014) and surgical ET (Kikuchi *et al.*, 2016). Another approach was reported by Gajda *et al.* (2015) who vitrified oocytes at the mature stage using the Open Pulled Straw method and obtained piglets by the surgical transfer of warmed oocytes into recipient pigs followed by insemination.

3.2.2 Embryos Cryopreservation Techniques

Embryo vitrification has had more promising results, but additional research is still needed in order to improve the success rates with cattle, buffalo, goats, and sheep (Díez *et al.*, 2012; Menchaca *et al.*, 2016; Parnpai *et al.*, 2016; Wahyuningsih and Ihsan, 2018; Mogas, 2018; Do *et al.*, 2019). While the efficacy of this ART is improving with these species, it still requires the use of some form of *in vitro* processing (such as fertilization, maturation, co-culture, grading or cloning) which makes it more expensive (labor and resources), and more invasive (requiring ET) compared with semen preservation and AI.

Equine embryos can either be collected *in vivo* by uterine flushing or they can be produced *in vitro*. Equine *in vivo* embryos enter the uterus 144-156h after fertilization and are characterized by rapid expansion and the formation of an acellular glycoprotein capsule. Both slow freezing and vitrification of small embryos (<300 µm) generally result in pregnancy rates of 50 to 60 percent (Squires, 2020). In contrast, cryopreservation of expanded equine blastocysts has been perpetually problematic. Reduction of the blastocoel fluid by aspiration through micromanipulation, followed by vitrification, has provided a breakthrough in this field, and results in pregnancy rates comparable to those of fresh embryos (Choi *et al.*, 2011).

The IVP of equine embryos has substantially grown in recent years with the worldwide clinical application of OPU, followed by IVM, intracytoplasmic sperm injection (ICSI), and *in vitro* culture (IVC) The advantage of IVP is that the embryos can be selected for cryopreservation upon blastocyst formation when they are still small. Consequently, cryopreservation of equine IVP blastocysts is very successful, with pregnancy rates like those obtained after fresh transfer when either vitrification (Choi and Hinrichs 2017) or slow freezing (Lazzari *et al.*, 2020) of the embryo is used. Cryopreservation of IVP embryos is routinely performed in the horse, which allows IVP outside the breeding season, and facilitates the selection of recipient mares. Equine IVP embryos should only be cryopreserved for transfer when they have reached the blastocyst stage, but that will occur between day 6 and day 10 following fertilization, which adds to the complexity of the techniques. Early developing blastocysts result in higher pregnancy rates and should be transferred on day 4 post-ovulation (Cuervo-Arango *et al.*, 2019). By comparison, using the current techniques, cryopreservation of IVP embryos is more efficient than cryopreservation of oocytes for conservation of equine genetic resources.

Similarly, success rates when using porcine embryo technologies have also recently increased. Porcine embryos can be obtained either *in vivo* (by flushing out from the reproductive tract after AI) or by IVP. Although *in vivo* produced porcine embryos at the blastocysts stage can be preserved by slow freezing (Fujino et al., 2007), much higher survival rates are obtained by vitrification (Cuello *et al.*, 2004). In the last decade, highly efficient vitrification protocols have been developed for morula and blastocyst stage porcine embryos employing chemically defined media (Maehara *et al.*, 2012; Mito *et al.*, 2015). Also, a pathogen-free closed system without the direct contact to liquid nitrogen (thus eliminating the chance for cross-contamination) has been developed (Misumi *et al.*, 2013). Although early cleavage stages are not optimal for the cryopreservation of porcine embryos (Sanchez-Osorio *et al.*, 2008), porcine zygotes (at the 1-cell stage) can be vitrified with high efficacy (Somfai and Kikuchi, 2021).

A promising component within this area of germplasm preservation is the focus on the development of new devices that either automate the process (Arav *et al.*, 2018) or aid in minimizing the volume of media during the oocyte and embryo vitrification process (Paul *et al.*, 2018; Chinen *et al.*, 2019; Olexiková *et al.*, 2020). Additional advantages with the latter devices are that they are inexpensive and enable the preservation of large numbers of oocytes or embryos at a single time, thus saving time, money, and resources for a laboratory.

3.4 GONADS, TESTICULAR AND OVARIAN TISSUE

3.4.1 Poultry gonads

Historically, most of the biotechnological methods of genetic conservation with poultry have focused on semen preservation. Female gametes were neglected due to the difficulty of oocyte collection, manipulation and handling, and because of the impossibility of cryopreservation due to the large size of the oocyte (from 1 to 10 cm in diameter depending on the species) and because of the volume of the yolk which constitutes more than 95 percent of the oocyte content.

To circumvent this problem, Song & Silversides (2006; 2007a, b) explored the preservation of the immature ovary of young chicks at a stage where the oocytes do not yet contain yolk. They successfully demonstrated that chick ovaries can be transplanted (by allograft) into recipient chicks of a similar age and, following puberty, the hosts will ovulate mature oocytes from the transplanted tissue. These oocytes may be fertilized and produce non-chimeric chicks within the first generation. Similarly, Song & Silversides (2007a) also demonstrated that testes of a young chick can be vitrified and transferred, and at the time of puberty, the host will produce fertile sperm. More recently, Liptoi *et al.* (2020) demonstrated the successful production of chicks from fresh and frozen-thawed donor ovaries using both commercial and heritage breeds of chickens. A variable amount (8 to 100 percent) of frozen-thawed gonads of a donor were accepted by the host and produced viable offspring (9 percent). These results depended on the donor x host combination and the type of gonad transplanted (testis or ovary). To maximize the probability of a successful outcome, the authors encouraged testing combinations of breeds prior to full experimentation or reconstitution of populations when using limited supplies of vitrified germplasm because of the incompatibility of some breed crosses (Liptoi *et al.*, 2013; 2020). The acceptance of testicular tissue was slightly greater than that of ovarian allografts, although both types could be successfully transplanted.

Although matching donor and host genotypes is certainly a challenge, the greatest obstacle to use this type of germplasm is acquisition of and proficiency with the surgical skills. Prior experience with local and general anesthesia, particularly with poultry, is essential, and experiences with fine surgical techniques is immensely helpful. Strict attention to the procedures listed in the Appendices for the acquisition and utilization of this type of germplasm are critical for achieving success.

3.4.2 Non-avian species

Like the cryopreservation of whole gonads, cryopreservation of excised testicular or ovarian tissue pieces has recently emerged as a viable means of preserving germplasm from prepubertal and sexually mature animals. Methods have been developed to slow freeze or vitrify whole, hemi-, pieces, or follicles from ovaries as well as whole, hemi-, or pieces of testes (Devi & Goel, 2016). Preservation of those tissues in those formats has been undertaken in human, wildlife, non-human primates, domesticated pets, rodents, and aquatic species (Dupré *et al.*, 2016; Pšenička *et al.*, 2016), insects (Fukumori *et al.*, 2017; A. Rajamohan, USDA ARS, personal communication, 2020), and agricultural species (Devi & Goel, 2016). However, the success is highly variable and influenced by the type of tissue (e.g. whole, hemi-, pieces, follicles), the species, and the breed/line within a species (Portela *et al.*, 2019), the type of cryopreservation process (slow freezing or vitrification), the post-thaw method of utilization (xenografting, allografting, tissue culture, organ transplantation, ICSI) and the interaction of these effects (Devi & Goel, 2016).

While births of mice have been commonly reported using frozen-thawed testicular and ovarian tissue pieces or whole gonads, this is not yet the case for other species. Still, it is important to note that success with these techniques has resulted in promising blastocyst development rates (Kaneko *et al.*, 2019) and the birth of live offspring (Kaneko *et al.*, 2013) with pigs, thus demonstrating the promise that this germplasm preservation method holds. Whole porcine ovaries can also be cryopreserved by slow freezing (Imhof *et al.*, 2004) and small segments of ovarian tissues can be cryopreserved by both slow freezing and vitrification (Gandolfi *et al.*, 2006). Furthermore, tissue segments have been xenografted into immunodeficient mice to harvest mature oocytes that have the ability for normal fertilization (Kikuchi *et al.*, 2016), however, to date live piglets have not been produced from samples cryopreserved using that technology.

For both avian and non-avian species, cryoconservation of gonads shares the same challenge as with semen and oocytes with respect to chromosomal content. At least under today's technologies, storage of either only ovarian or testicular tissue would require generations of backcrossing to restore an extinct breed to a nearly pure state. Therefore, gene banks that conserve gonadal tissue should either store both ovarian and testicular tissues or store the complementary gametes or embryos.

3.5 GERM, STEMS AND SOMATIC CELLS

3.5.1 Preservation of diploid cells for cloning or the in vitro formation of gametes

Cryopreservation of stem cells, embryonic and adult cells, induced pluripotent stem cells (iPSCs) and mesenchymal stem cells (MSCs) will effectively preserve the DNA/genome of any species. Currently, not all somatic cell types and techniques are effective for regenerating live animals for all species. Somatic cells can be cryopreserved and used in somatic cell nuclear transfer (SCNT) for reproductive cloning. Cloning of mammalian livestock species has been successful for many domestic mammalian species (cattle, sheep, horses, pigs, goats) by replacing the oocyte nucleus with a somatic cell nucleus (Wilmut *et al.*, 1997; Cibelli *et al.*, 1998; Baguisi *et al.*, 1999; Polejaeva, 2000; Galli, 2003). However,

health and development problems have been observed in many of the clones, which demonstrates the need for further refinement of this methodology. In some countries, cloned livestock may be an ethical issue that could prevent eventual utilization or require a legal exception. Nevertheless, the ease and low cost of somatic cell banking may justify their current collection and storage, while waiting to address ethical issues in the future, when and if they are ever needed.

The hope for future utilization of embryonic stem cells, iPSCs, and MSCs is that techniques will be developed to directly differentiate these cells into either primordial germ cells (PGCs) or functional gametes (Pieri *et al.*, 2019) which can then be transplanted, or used for artificial insemination, IVF, and ET. Certainly, substantial progress has been made in recent years in the ability to collect and culture stem cells and to induce pluripotency, but because of species differences there is a lack of understanding of the essential elements necessary to routinely apply this approach in livestock. Furthermore, because of suboptimal culture conditions to maintain pluripotency, reprogramming of somatic cells is often unsuccessful (Pessôa et al., 2019, Pieri et al., 2019) and consequently not practical for conserving genetic diversity. In addition, iPSCs have been used to create live, chimeric offspring in sheep (Liu *et al*., 2012) and pigs (West *et al.*, 2010; 2011, Su *et al.*, 2020), which may attest to the efficacy of the procedures used to generate the iPSCs and the quality of the cells (Sartori *et al.*, 2012), but to date these somatic cells have not been used to generate true clone (non-chimeric) animals (Pessôa *et al.*, 2019). Still, in the mouse it was demonstrated that naive pluripotent stem cells can differentiate *in vitro* into functional sperm (Hayashi *et al.*, 2011) and oocytes (Hikabe *et al.*, 2016; Hayashi *et al.*, 2017), which could be used to produce viable offspring. Potentially, functional gametes may be generated with iPSCs derived from cryopreserved somatic cells from domestic species and be used to produce embryos that can develop into healthy animals after uterine transfer.

3.5.2 Primordial germ cells and derivatives

Perhaps some of the most promising recently developed techniques involve the use of PGCs to preserve chicken germplasm and recreate live non-chimeric chicks. With this technique the PGCs are typically collected from stage 16 HH (Hamburger and Hamilton, 1951; approximately 2.5 days of embryo incubation) embryos and they can then be successfully frozen (cryopreserved or vitrified, Nakamura *et al.*, 2011) or cultured prior to transplantation to increase the quantity of viable cells (>50 percent success rate, Whyte *et al*., 2015; Nandi *et al.*, 2016; Wang *et al.*, 2016; Tonus *et al.*, 2017). Transplantation of the PGCs can be performed to generate chimeras, but these are randomly created, result in about a 4 percent transmission rate of offspring containing the desired genotype from transplanted PGCs, and will require multiple generations of back-breeding to recreate a specific breed or line. While use of the technology in this way is viable, it is currently inefficient and would require significant amounts of labour and chickens to attain the desired offspring. Recently, using a knock-out chicken (DDX4-) created as a result of TALEN-mediated gene targeting, Taylor *et al.*, (2017) demonstrated that these inefficiencies can be overcome if enough PGCs are transplanted into a host embryo and the gonad of that host is devoid (sterile) of native PGCs. Then, when a DDX4- rooster is created he can be used as part of an AI program to produce sterile female embryos for use as sterile hosts. PGCs from a donor (fresh or frozen-thawed) that can be injected into the sterile host (hen) embryo, where they migrate to the embryonic gonad, and multiply, making the ovary functional (Taylor *et al.*, 2017). At sexual maturity, the recipient hen is inseminated with sperm of the same line/breed as the transfected PGCs and the progeny will be fertile and capable of progenerating the line. This method is significantly more efficient than creating live chimeric offspring and employing multiple generations of back-breeding, and results in the production of genetically pure offspring in the first generation (Woodcock *et al.*, 2019).

A technique similar to the poultry PGCs model has recently been developed for use with mammalian species. Ciccarelli *et al.,* (2020) demonstrated that spermatogonial stem cells isolated from testicular parenchyma can be cultured, transplanted into sterile hosts, and those hosts (goat, mouse, and pig) will produce sperm from the donor. Bulls were also tested in similar experiments and, although donor sperm were not acquired in that research, the prospects are high for applying these technologies to that species. While transplantation of spermatogonial stem cells and the creation of chimeras has been demonstrated in other animals (e.g. quail, trout, salmon, zebrafish), the novelty to the research reported by Ciccarelli *et al.,* (2020) is that this is the first-time sterility was induced using CRISPR/Cas9 editing of the NANOS2 gene (knock out), rather than through chemical, environmental or radiological sterilization. It resulted in azoospermic hosts that are capable of maintaining donor-derived spermatogenesis. Moreover, this is the first report of this combination of techniques successfully producing sperm using livestock species. This combination of results supports the current preservation of spermatogonial stem cells, even though improvements in the efficiencies of the techniques are needed. In contrast, research with oogonial stem cells and regeneration of live offspring has not progressed as rapidly or produced live offspring. Still, Hou *et al.,* (2018) reported that germline stem cells from porcine ovaries can be isolated, purified, cultured, and induced to differentiate into oocytes when injected into tissue grafts. However, the existence of oocyte stem cells in mammals is a controversial topic, and many reports demonstrate that they do not exist; thus validating this finding is requisite.

3.6 STRATEGIC CHOICES OF STORED MATERIAL TYPE TO FIT NATIONAL NEEDS

The wide range in the types of tissues and cells available for cryoconservation offer a practically infinite number of possible strategies for countries to choose from when addressing their gene banking goals. Each type of biological samples has its advantages, disadvantages and outright limitations and these vary depending upon the species, the cryoconservation and the technical capacity of the country. No single solution will be appropriate for all countries. In a series of case studies, gene bank managers from Brazil (BOX3.1), Canada (BOX 3.2), China (BOX 3.3), France (BOX 3.4), Thailand (BOX 3.5) and the United States of America (BOX 3.6) describe their choice of stored materials in the context of their most important livestock species and national goals for management of AnGR.

3.7 RECOMMENDATIONS

3.7.1 Semen

For nearly all livestock species, semen collection, and cryopreservation are an inexpensive and effective option for preserving AnGR. Consequently, based on the easy access to this type of germplasm, the simplicity of preservation, and the minimal costs associated with these techniques, a gene bank should endeavor to make semen an integral part of their collection. The gene bank should also spend a significant amount of time identifying the appropriate species, breeds, and males to ensure that their collection contains the suitable quantities and individuals to meet the country's AnGR conservation goals (Sections 1 and 5). When feasible, gene banks should aim to cryoconserve some complementary material (embryos or oocytes), if they wish to avoid the generations of backcrossing required for reconstitution of breeds based exclusively with semen.

3.7.2 Oocytes and embryos

The costs associated with preservation of oocytes and embryos (labour, laboratory facilities, reagents) are considerable and may be prohibitive for some gene banks. As with most types of germplasm, the success when using cryopreserved-thawed or vitrified-warmed oocytes and embryos for regenerating live animals is highly dependent on the species, the state of the germplasm (the developmental state of an oocyte or embryo) when collected or produced and should be considered when determining the quantity of samples needed for each species and breed.

3.7.3 Gonads, testicular and ovarian tissue for avian species

Documentation of the success with this technique with vitrified-warmed gonads, rather than with freshly excised samples, was a critical step in the acceptance of this technique. Research to understand the genetic components and immune response of the donor and host will further improve the success rates. Utilization of these methods should be considered if a gene bank has, or has access to, appropriate surgical expertise and can afford the related expenses. Even if that option is not currently available it may still be prudent to preserve gonads from chicks, as the costs of the collection and preservation activities are minimal, the future benefit is that the offspring derived from the transplantation are non-chimeric, meaning the desired chicks are created in the first generation thus eliminating the need for backcrossing.

3.7.4. Gonads, testicular and ovarian tissue for non-avian species

Once optimized, cryopreservation and subsequent grafting of testicular or ovarian tissue could provide a potentially inexhaustible source of germplasm for the preservation of AnGR. However, the methods to utilize these types of germ plasm in all species have not been optimized but should be considered a viable and inexpensive means of preserving the genetics of a breed, line, or species. Current preservation of this material is based on a presumed high probability that the methods for its utilization will be developed in the future (Kaneko *et al.*, 2013, 2019) and issues regarding morbidity and ethics will be resolved.

3.7.5. Germ, stem, and somatic cells

Undifferentiated germ cells such as PGCs, especially from poultry, and intermediate germ cells such as spermatogonial stem cells represent legitimate opportunities that should be pursued. Nuclear reprogramming strategies that can transform germ, stem, or somatic cells into germ cells or gametes are developing rapidly, but significant improvements are needed. Further research and development are especially needed in cell culture methods to allow a user to differentiate many of these cell types into functional gametes. Still, preservation of these cell types, and specifically fibroblasts or general tissue samples in the form of ear notches, should be pursued with the understanding of their potential value and utility for the future, while monitoring the technological status of these methods.

3.8 SUMMARY

Successful collection, preservation, and utilization of germ plasm depends on numerous factors. However, even though variable results should be expected when utilizing any assisted reproductive technology, a secure, genetically diverse collection of AnGR can be assembled for all agricultural species. Moreover, the collection, preservation, and utilization techniques can be chosen to match a country's or gene bank's budget, level of expertise, and the ethical perspectives of its nation. A comparison of these factors, by species, is presented in TABLE 1 to aid with decision making.

TABLE 1.

A comparison of germplasm types according to various factors influencing their utility

The types of germplasm are rated on a relative scale from 1 (lowest/easiest) to 5, (highest/hardest) within each category. The genetic profile of the resulting offspring is also reported.

Type of germplasm	Ease of acquisition	Cryopreservation expertise	Collection costs[a]	Utilization expertise	Utilization costs	Proportion of desired breed in 1st generation offspring (%)
Semen	2	3	1	1 to 3[b]	1 to 5[b]	50 – 100
Oocytes	5	5	5	4	3	50 – 100
Embryos	5	5	5	4	2	100
Gonads	2	2	2	5	3	100
PGCs[c]	4	1	1	5	3 to 5[d]	<10 – 100
Somatic cells	1	1	1	2 to 4[e]	5[e]	50 – 100

[a]Collection costs can vary greatly depending on who performs the collections and cryopreserves the samples. For example, when semen is considered, the costs of obtaining a sample for a gene bank will vary depending on whether the semen is acquired from a stud and is already cryopreserved (< $0.50/straw), if a sample is collected and then sent to a gene bank for cryopreservation (~$1.00/straw), or if the gene bank travels and performs the collection on-site and cryopreserves the samples ($15.00/straw). These factors can be applied to any type of germ plasm and therefore this category considers the inherent costs associated with each when making the comparisons.

[b]Dependent upon if semen will be used for artificial insemination, IVF, or ICSI.

[c]Only successfully demonstrated with chickens and conditionally with other avians.

[d]Variable depending on whether the laboratory is in possession of DDX4- hens.

[e]Requires cell culture and transfer of the cells into a host organism, and these methods have not currently been optimized.

3.9 EXAMPLES OF THE CHOICE OF GERMPLASM BY COUNTRY

BOX 3.1

The Brazilian Animal Gene Bank

The Brazilian Animal Gene Bank for Animal Genetic Resources was created in 1983 and is located at Embrapa Genetic Resources and Biotechnology (Cenargen) in Brasilia. It is one of the 42 research centres of Embrapa (Brazilian Agricultural Research Corporation), which is under the direction of the Ministry of Agriculture, Livestock and Supply. The gene bank is part of the country's conservation programme, which also has an *in situ* component that is spread nationwide in research centres, universities and private farms. The gene bank collection includes semen, embryos, and fibroblasts as well as biological material such as DNA, hair, and blood samples. The collection includes samples from approximately 17 000 animals and represents 87 breeds and 12 species. Real time data can be accessed through the online information system *Alelo Animal/ Animal GRIN* (Embrapa, 2021– see also Section 8). The repository has been mainly used for molecular biology research studies.

Current and future efforts include establishing infrastructure to fast-track legal agreements and implementing quality standards of the International Organization for Standardization to attract more interest from farmers, research institutions and industry (see Section 2). In addition, improvements to boost gene bank management and utilization are also underway. The first improvement will change the focus of the collection from breeds to species. The majority of the current gene bank samples are composed of rare local breeds but going forward collection activities will also include cryopreservation of specialized and commercial breeds to enhance the food security of the national livestock production system. The second improvement to the collection is that all semen samples currently in the repository have now been genotyped by medium density DNA chips (tens of thousands) and this data will be available through Animal GRIN. The third improvement is implementation of a conservation index to verify if the amounts of germplasm stored are sufficient to recover a breed, to carry out experimentation, and to enable AnGR exchange with institutions and breeders. All these changes will be monitored and evaluated over time to adapt the strategy, comply with the needs of the Brazilian population, and fulfil the main Strategic Priority Actions defined in the Global Plan of Action for Animal Genetic Resources (FAO, 2007).

Source: Samuel Paiva

BOX 3.2

The Canadian Animal Pedigree Act and cryoconservation activities

Production of Canadian livestock used for food relies on the producers and industries. To assist them with breed improvement, Canada's government (1988) adopted the Animal Pedigree Act (APA) to regulate the establishment of livestock associations and maintain breed registration records/pedigrees. One of the advantages of the APA is that the status of a breed can be monitored to identify those breeds at risk or endangered, and then actions can be taken to conserve Canadian livestock genetics. In addition, a conservation programme, Animal Genetic Resources of Canada (AnGRC – Government of Canada, 2021), is available for livestock associations or industries with mission to preserve germplasm or gonadal tissues from Canadian purebred animals. Typically, frozen semen or embryos are sent to AnGRC's facility at no cost to the donor and when frozen germplasm of a purebred animal is not available, AnGRC can collect and freeze semen samples on-farm. Electro-ejaculation is the preferred method to harvest sperm cells. This collection process does not require the training of an animal, and the genetic material can be rapidly retrieved and analysed. Each step of the germplasm preservation process is demonstrated to producers for their education, which also serves as a tool to promote the preservation programme. Each produced dose must contain enough viable sperm cells to be used for artificial insemination to generate a new progeny. Sample acquisition requires that the owner complete consent forms which release the doses to the AnGRC group (see Section 9). Then half of the semen doses preserved by the AnGRC group can be returned to the donor to improve its herd or flock's breeding management while the other samples become the property of the Canadian government. All information regarding the animal and the collection are entered into the Canadian Animal-GRIN database, which can be consulted by the public. Only livestock associations or industries may request access to the doses stored in the Canadian repository, but they must demonstrate that the genetic material is not available on the Canadian market and the release of doses will benefit the breed. Consequently, AnGRC is a tool for Canadian producers and industries and strives to preserve their animal breeds.

Source: Carl Lessard

BOX 3.3

The Chinese animal germplasm collection

The Chinese animal germplasm collection (China, 2021) provides a means to address the current reduction in genetic diversity of domestic livestock and poultry genetic resources, as well as to diminish the risk of resource destruction caused by the deterioration of ecosystems. The collection activities will allow for the rescue of endangered species, the recovery of populations, and maintenance of the diversity of domestic animal germplasm. These activities will lead to a better exploration and utilization of potential germplasm and genetic resources, which can then be used for the creation of new breeds or lines to meet the need of sustainable farming development.

Semen is the main form of germplasm preserved in China across all species. Because of the utility of cryopreserved semen (AI, ICSI, sperm-sexing technology) high-quality sperm can be used to breed targeted females or oocytes to manage the genetic diversity of a breed or create new breeds.

The successful application of *in vitro* embryo technologies to 16 livestock species in China makes embryo conservation very appealing. This form of germplasm is especially attractive because embryos contain the complete nuclear and mitochondrial genome of their parents. Then, when utilized, they enable recreation of a founder population for a breed in one generation. Similarly, the use of oocytes is considered practically equivalent to embryos, but when coupled with frozen semen, *in vitro* fertilization, and semen sexing it enables more precise and timely selection of the quality of germplasm that is used and the sex of the offspring.

Somatic cells are considered to be most effectively preserved in conjunction with gametes and embryos. Somatic cell nuclear transfer technology has already been used to restore species. Somatic cells can also be used to produce IPSCs, which can be differentiated into gametes for preservation and used to create new breeds by gene-editing. Although both somatic cells and stem cells can be used to generate animals through nuclear transfer technology, which can regenerate populations of endangered animals and preserve genetic resources, stem cells are considered superior to somatic cells because of their greater potential for cellular differentiation.

DNA (tissue) analysis assists in improving gene bank management and population restoration, can be used to identify potential germplasm samples, functional gene exploration to discover superior genetic variation, and as a tool for creation of new breeds or lines. The tissue is mainly used for identifying physiological and biochemical indicators, intestinal flora, and other quality traits, as well as for the acquisition of somatic cells, DNA, RNA, and protein. While creation of animals exclusively using DNA is not currently possible, the gene bank preserves somatic cells, nuclear DNA, and tissue for long-term preservation and scientific utilization, especially when semen and embryos are difficult to obtain from rare or endangered breeds, wild relatives, and breeding groups across China.

A forward-looking type of information that is also utilized in China is via digital preservation. The structure of the physical animal body is digitized through the integration of information technology and biotechnology. This kind of new "germplasm" can be used to meet diverse requirements such as digitization of the information in multiple dimensions to provide virtual reality services and experimental animal models for future use.

Source: Xueming Zhao

BOX 3.4
The French National Cryobank of Domestic Animals

The mission of the French National Cryobank of Domestic Animals is to manage the constitution, the conservation, and the distribution of domestic animal germplasm collections. More specifically, the mandate states that the gene bank is responsible for restoring rare lines/breeds following catastrophic events, reintroducing genetic variability in populations in combination with *ex-situ*/*in situ* conservation, aid in genetic selection activities, create new resources, and develop tools for research.

At this point in the programme's history, the main use of the bank has been via the semen collections. These collections have enabled the reintroduction of genetic variability in small populations of rare breeds and lines of pigs (Blanc de l'Ouest, Bayeux, Cu noir, Limousin, Basque pigs), sheep (Avranchin, Berrichon de l'Indre), horses (Cob normand, Trait Poitevin Mulardier), and chickens (INRAE R+ experimental lines). Reintroduction of genetic variability was also achieved using the goat embryo collection (Caprin Créole).

Increased use of the collections is foreseen in the future and the cryobank has consequently developed a specific interface through our web portal which is a consortium of French gene banks for animal germplasm (CRB-Anim, 2020). This portal offersthe opportunity to view the contents of the collections, provides a tool to identify germplasm that should be stored, and includes a simulation tool to assess the impact on performance and inbreeding when a particular sample of germplasm is used.

Source: Elisabeth Blesbois

BOX 3.5
***Ex situ* conservation in Thailand**

The Department of Livestock Development (DLD), Ministry of Agriculture and Cooperative is required to follow Thailand's 20-year National Strategic Plan. Specifically, this operational plan for biodiversity management includes the policy to conserve the genetics of animals, plants, and microorganisms, using *in situ* and *ex situ* conservation.

The *ex situ* conservation of animals includes the following materials:

1. Frozen semen from dairy and beef cattle, native cattle, and buffalo that have been developed at semen production centres serving the farmers. DLD facilities include a frozen semen storage centre that preserves samples for commercial entities and for preservation of livestock genetic diversity in general. The cryopreservation of pig semen is currently being explored.
2. Frozen embryos are conserved from dairy and beef cattle, native cattle, buffalo, and goats for genetic improvement and maintenance of genetic diversity.
3. Tissue and DNA samples are collected for both research and biodiversity needs from all livestock species. DLD also co-operates with the Office of Natural Resources and Environmental Policy and Planning, Ministry of Natural Resources and Environment for the development of a database for banking genetics (Biotec, 2021). The permanent DNA storage facility is managed by the National Science and Technology Development Agency.

Source: Rangsun Parnpai

BOX 3.6

The National Animal Germplasm Programme of the United States of America

The National Animal Germplasm Programme (NAGP) is the programme of the government of the United States Government of America (USA) charged with the conservation of AnGR. Since the programme's inception in 1999, it was realized that an active gene bank would be required to acquire and store a variety of tissue types for current and future use -- where future use may include presently unthought-of options. A key element of the USA programme is that germplasm samples and tissues are given to NAGP by small and large livestock producers and corporations with no compensation and owners generally forego their rights to the germplasm. Because the many components of the livestock industry openly sell semen, either in a fresh or cryopreserved form, choosing semen as a primary germplasm type for the repository was viewed as a cost-effective approach. In addition, embryos for cattle, sheep, and to a lesser extent swine, have been acquired, again, at no cost to the programme. For chickens, the tissue types collected have evolved over time. Initially, semen was collected and cryopreserved, but the homogametic nature of avian males underscored the need to seek other paths for conservation. This has included collection and cryopreservation of primordial germ cells, and ovaries and testes for transplantation. Oocytes for swine have been acquired experimentally, which upon use may minimize the problem of genetics becoming obsolete over the course of decades. While the above tissue types have been collected as a means to reconstitute populations or reintroduce lost genetics, other tissues have been collected. For example, blood has been collected and stored for health tests. Genotyping animals in the collection is accomplished by using semen, blood, or various tissue types (such as heart); thereby making the repository a one-stop shop for stakeholders (USDA, 2021).

Source: Harvey Blackburn

BOX 3.7

The Cryobank of Animal Genetic Resources in the Philippines

The Cryobank of Animal Genetic Resources (AnGR) was established in the Philippine Carabao Center (PCC), Science City of Muñoz, Nueva Ecija, the Philippines, in 2012. This supports the existing genetic improvement program for livestock and the response to the threats posed by climate change, through the Department of Agriculture of the Philippines and the support of the Korean International Cooperation Agency.

Strategies to run the Cryobank consist of various components, including the collection of animal genetic resources, preservation, data banking, provision of access to stored samples, and implementation of information, and dissemination campaigns. Adherence to these strategies is closely monitored to attain the objectives set for this purpose.

Cryobank aims to preserve the diversity of animal genetic resources (AnGR) of indigenous species and introduced breeds that have economic importance and exhibit adaptable and resilient traits towards diseases and environmental elements in the country. To accomplish this, Cryobanking focuses explicitly on (1) securing the biodiversity conservation of animal genetic resources, (2) establishing the phenotypic and genotypic characteristics of animals to be cryoconserved, (3) establishing and maintaining inventory and records of the genetic materials being cryoconserved, and (4) continuing the state-of-the-art cryopreservation techniques.

Cryobank facility is capable of long-term storage of frozen semen in storage tanks applied and maintained with liquid nitrogen. This is being done for future utilization in sustaining the supply of buffalo genetics to farmers, livestock breeders, and other interested clients. AnGR germplasms came from superior breeds of buffaloes managed with good nutrition and were identified with good physiological and reproductive qualities. As of January 2021, the collection of cryopreserved semen and oocytes are from various species, which are 91.31% buffaloes, 8.41% cattle, 0.28% caprine, and 0.01% swine. DNA samples and cryopreserved whole blood cells from various species are also preserved as genetic sources for molecular analysis in genetic diversity studies, species identification, and other research outputs.

However, the number of species and breeds of animals is still meager to represent the animals' genetic diversity in the Philippines. As such, the Cryobank is moving forward to pursue advancements in documenting, promoting, and strengthening linkages and information relative to Philippine livestock breeds, including their cultural heritage and social importance. This is feasible through the initiatives on (1) duplication of the facility with AnGR stores in other sites in the country or international gene bank through international collaboration, (2) provide necessary training of more researchers and technicians with technical knowledge in cryopreservation and molecular techniques, (3) provision of online access to the AnGR Information System to AnGR providers and other stakeholders. and (4) strengthening legal policies, pertaining on Cryobanking activities.

The AnGR banking programme envisions establishing a national repository of germplasm and biological samples from diverse livestock species and breeds, threatened and wild animals, and indigenous species that will sustain the genetic diversity animals in the Philippines. With this, the cryobank will pave the way to sustain various animals' genetic diversity in the Philippines in the future.

Source: Lilian P. Villamor

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SECTION 4

The economics of gene banking

The economics of gene banking

4.1 INTRODUCTION

The costs of gene bank programmes and facilities are an important consideration for any institution managing gene bank collections. FAO (2012) reviewed the basic functional requirements for typical small, medium and large facilities and the corresponding costs for operation and maintenance. Basic requirements regarding infrastructure and physical design of gene-banking facilities have not changed substantially since the previous guidelines. This section draws attention to the economics of genetic collections in which the cost element is one variable in a broader objective of designing efficient collections. Other elements to be considered are the benefits derived from the eventual future use of stored material and minimizing overlaps and redundancy in genetic resources that may be held elsewhere, either nationally or globally. This rationalization process involves careful planning of material collection to minimise cost for maximum benefit. Methods for formal economic evaluation and mathematical optimization are presented and the institutional needs and barriers that can facilitate or hinder rationalization efforts are discussed.

4.2 REVISITING THE COSTS OF GENE BANKING

The costs of gene bank operations and facilities are country and context specific and depend upon the strategy and the objectives of the gene bank and of the general management of animal genetic diversity within the country. Several preservation parameters need to be determined, for example: how many species and breeds need to be preserved, which type of germplasm, which preservation protocols are to be used for each species, and the number of doses per animal to be stored in liquid nitrogen. Answers to these questions will help to determine the long-term investments and the annual operational costs. The purpose of this section is to provide a summary of relevant cost categories or items to be considered when planning a budget to implement an animal preservation program. Specific cost categories may require *long-term investment* and can be considered as *fixed* or *variable* costs (Table 4.1). As a general principle, maintaining infrastructure and staff can be considered *fixed* costs. Costs directly implied when collecting new doses or when upgrading equipment are *variable* costs.

4.2.1 Long-term investments

Long-term investments are the foundation of an animal preservation program, which should target at least twenty years of operation. The physical plant's location needs to be carefully chosen in relation to the animal genetic diversity in the vicinity, the environment, the biosecurity, potential hazard risks, and the proximity of public services (liquid nitrogen supplier, fast courier companies, airport, etc.). Cryopreservation laboratory and long-term storage rooms are the most critical infrastructure for an animal gene bank. A cryopreservation laboratory must be designed to facilitate the different steps of the preservation of germplasm. It must include areas to prepare, evaluate, and freeze germplasm. Also, a computer workstation is needed to record the different information on the preserved genetic material. Long-term storage rooms require enough space for the cryotanks and liquid nitrogen dispensers. To ensure safety of personnel and prevent loss of the collection, the environment of these rooms should be carefully monitored with specialized equipment to detect liquid nitrogen spills, humidity and oxygen levels, and smoke.

Key staff to operate an animal gene bank will determine the success of an animal preservation program. A curator or gene bank manager will supervise the strategy and different operations of the gene bank, including material acquisition, material processing, storage and distribution This person will be the primary contact for stakeholders interested in providing or having access to animal gene bank material. Skilled technicians should be available for collecting the genetic material from animals in the field or in-house. A lab technician will be in charge of cryopreservation processes. Finally, a database manager will ensure that all information on the donor animal (such as species, breed, line, registration number, pedigree information, phenotypic and genomic information) and germplasm (such as viability, number of doses, location in the cryotank) are correctly digitally recorded (see Section 8). These human resources should be a minimum to operate an animal gene bank adequately and to efficiently provide services to stakeholders. Flexibility in the availability of the human resources and their responsibilities would help gene banks to anticipate variability in workload during the year.

Basic equipment to collect and process germplasm is essential in a cryopreservation lab (Table 4.1). These instruments would allow the handling of germplasm and produce doses for storage in liquid nitrogen. However, it is strongly recommended to invest in specialized processing equipment if the preservation program's mission and objectives are growing in terms of number of donor animals and doses. For instance, a computer-assisted sperm analyzer can rapidly evaluate the motility and viability of sperm cells collected from several animals in a day. Another example is a straw filler and sealer capable of handling an extensive collection of germplasm. For the storage of the doses in liquid nitrogen, the number of tanks is proportional to the number of species to be preserved; preferably one tank for one species. These tanks should be equipped with an alarm system to monitor the level of liquid nitrogen. Quality of equipment should not be compromised to respect the investment target of twenty years.

Technological and practical advances since 2012 have on one hand increased options to cryopreserve reproductive material for a range of species, which also may result in higher total operational costs for a gene bank. For example, cryopreservation of embryo or ovarian tissue is generally more expensive compared to semen. On the other hand, technology development may contribute to enhanced effectiveness or efficiency of gene bank operations, thus increasing the value of potential benefits or decreasing the cost per unit of genetic variation conserved. For example, better cryopreservation protocols may allow the gene banking of new species or improve the viability of material from certain breeds. Genomic information can be used to optimize the genetic variability in collections (see Section 5) and thus reduce costs associated with duplication.

4.2.2 Annual operational budget

When planning an operational budget, categories/items should be classified as fixed or variable costs. Fixed costs could be defined as a fixed expense required every year, regardless of the collection and cryopreservation activities in that year. It is important to have long term commitments of relevant stakeholders to cover the fixed costs and to avoid compromise of the investments done in the previous years. Major items/categories classified as fixed costs are annual costs of cryopreservation lab facilities and cryo-storage rooms, salary and overhead of the staff, basic equipment, database, and long-term storage tanks. Gene bank infrastructure requires preventive or corrective maintenance every year. Annual collection and preservation activities hardly influence the salary and overhead of human resources. Basic equipment needs to be replaced or fixed regularly regardless of the amount of genetic material handled in a year. Regular maintenance and security of the informatics system ensure the protection of the data and the continuity of the services to stakeholders. Cryogenic tanks require a

constant supply of liquid nitrogen. Usually, the number of tanks in storage rooms is set for several years following long-term investment in acquiring these cryo-containers. Thus, the annual amount of liquid nitrogen should not vary substantially over the years, facilitating the estimation of this cost when preparing a multi-year budget.

Variable costs in the operational budget are influenced by the quantity of genetic materials to be collected and processed in a year. Frozen germplasm needs to be shipped to the animal gene bank, and the cost will depend on the size of the required (dry) shipper. When the genetic material needs to be collected from an animal, the cost of preserving the material depends on the preservation strategy. For instance, a producer can transport his animal to a specific handling facility, and an independent veterinarian/technician would perform the harvesting of the genetic material.

Another instance is field collection trips organized by gene banks themselves. Staff will need to travel to the collection area, requiring a budget to cover different costs such as transport, accommodation, meals, consumables, storage liquid nitrogen tank, etc. In general, a field trip duration could extend to several days if many farms need to be visited in a larger country. So, it is essential to have clear objectives for the preservation program when planning a budget associated with variable costs.

A basic framework for evaluating full costs of gene bank operations is presented in Table 4.1. Examples of how to budget gene bank costs in the context of national programmes and objectives are provided from Canada (Box 4.1) and the Netherlands (Box 4.2).

TABLE 4.1
Cost structure and cost evaluation framework for gene bank operations

Cost category / item	**Fixed costs** (annual costs, 20-year horizon)	**Variable costs** (per donor animal or per dose)
Key staff – labour costs (salary and overheads) • Gene bank manager / curator • Field technician/animal housing / collecting • Lab technician • Database manager	X	
Wet lab (costs/m2) • Receipt of material • Preparation and evaluation • Processing • Packaging • Freezing • Computer work station	X	
Long term storage rooms (costs/m2) • Main storage • Duplicate storage • Monitoring and security	X	
Equipment Maintenance and repair Processing equipment (microscope, centrifuge, spectrophotometer, counter	X	

chamber, haemocytometer, pH meter, osmometer, water bath, straw filling equipment, straw printer, styrofoam box, programmable freezer, quarantine tank)		
Database Maintenance Security	X	
Staff (additional or temporary) • Animal facilities • Collecting (in field, on farm, on station) • Processing (in lab)		X
Genotyping		X
Animal collecting facilities • Quarantine period • Collecting period		X
Veterinary costs and diagnostic tests		X
Transport • Animal • Genetic material • Shipping tanks		X
Collecting material • Consumables, disposables • Reagents • Portable equipment		X
Processing material • Consumables and disposables • Reagents		X
Long term storage • Tanks • Liquid nitrogen		X

BOX 4.1

Investments of Canada to preserve its animal resources

Canada launched the Animal Genetic Resources of Canada (AnGRC) in 2006 (previously called Canadian Animal Genetic Resources). The industry principally owns Canadian livestock, and AnGRC is a preservation program to sustain Canadian animal production development. Its mission is to ensure the genetic diversity of the Canadian livestock by acquiring, evaluating, and cryopreserving germplasm. A genetic representation of each animal breed used for food and agriculture should be stored in the Canadian national gene bank located in Saskatoon, Saskatchewan, Canada.

The objectives:

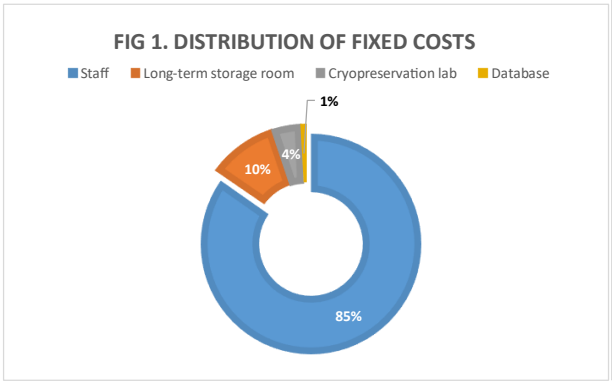
1. Collect sperm or embryos from donations from producers or industries. These donations can be frozen germplasm, or field collections can be organized to capture Canada's animal resources;
2. Determine the genetic diversity of each Canadian breed; and
3. Develop a database to record the animals' information, doses, and location in the gene bank. Information contained in the database should be accessible to the public.

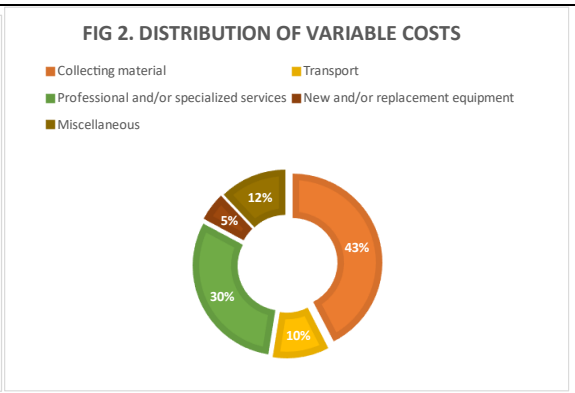
Long-term investment:

- **Infrastructure:** The animal national gene bank is located in a refurbished room at an existing federal centre in Saskatoon (Saskatchewan, Canada). This room has all the monitoring equipment to detect liquid nitrogen spills or a low level of oxygen. In addition, an industrial dehumidifier ensures that a proper level of humidity and temperature is maintained, which improves the environmental conditions to handle and store germplasm in this room. The cryopreservation laboratory is located at the Western College of Veterinary Medicine (University of Saskatchewan), located near the animal national gene bank. Having a physical presence at the Veterinary College allows the AnGRC group to train future veterinarians in the field of animal genetic diversity. Thus, Canada did not build new infrastructures for its animal preservation program but used existing facilities to accommodate the needs of AnGRC.
- **Equipment:** AnGRC acquired CASA (computer-assisted sperm analyzer), a flow cytometer, a genetic sequencer, a straw filler and sealer, a straw printer, a programmable freezer, and several cryotanks for the adequate preservation and quality analyses of the donated germplasm.
- **Database:** AnGRC joined an international group (Brazil, USA, and Canada) to build a shared database to record the different information. Investment on informatics system and security programs were made to ensure the protection of the data (see Section 8 for more information).
- **Staff:** AnGRC group comprises a curator, a field specialist, a genetic advisor, and a programmer. Graduate and summer students help to complete the different AnGRC's preservation activities.

Operational budget: In general, fixed costs represent around 80 percent of the operational budget, while the variable costs are 20 percent.

- **Fixed costs:** Staff salaries account for around 85 percent of the fixed costs (figure 1). A significant amount of liquid nitrogen is required to fill all the cryotanks located in the storage room, which 10 percent of the fixed costs budget is required every year, because the number of cryotanks to fill does not vary significantly. The remaining fixed costs budget covers the renting of space at the University of Saskatchewan for the cryopreservation labs and the security programs' updates or upgrade of the informatics system.
- **Variable costs:** Each year, several livestock producers and industries donate frozen germplasm to the AnGRC program, which shipping to AnGRC's facilities is free of charge. Animals can also be brought to the Veterinary College for collection. So, transport can represent 10 percent of the variable costs (Figure 2). Field collections are a major activity for the Canadian preservation program, and they can represent around 43 percent of the variable costs. Canada is a vast country, and the production of animals is geographically dispersed. AnGRC staff must travel long distances to reach important animal resources produced in Canada. The number of trips in a year depends on the interest of producers or the working capacity of the AnGRC group. Analysis of genetic diversity can be done in-house or by a third party. Veterinarian services can also be solicited to collect genetic material from animals when the AnGRC group cannot attend. These services can represent 30 percent of the variable costs every year. Finally, a small portion of the operational budget is kept to cover replacement or repair of equipment and unexpected contingencies.





BOX 4.2

The Dutch farm animal gene bank

The Dutch gene bank for farm animals is managed by the Centre for Genetic Resources, the Netherlands (CGN), of Wageningen University & Research. Development and maintenance of the gene bank collections for farm animals is part of the Statutory Research Tasks program of CGN, funded by the Ministry of Agriculture and supported by the Dutch livestock breeding sector.

The main objectives of the Dutch gene bank are:

1. To establish and to maintain gene bank collections of all native Dutch rare livestock breeds;
2. To facilitate regular back-up gene bank collections of all breeding programs; and
3. To stimulate the use of gene bank collections in breeding and research.

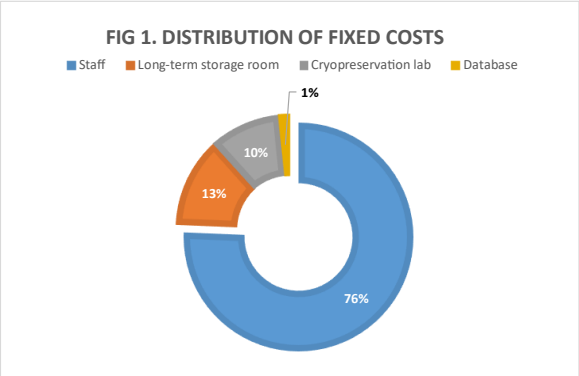
Long-term investment:

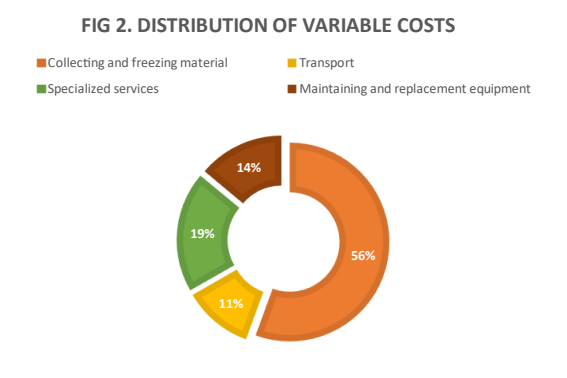
- **Infrastructure:** The national Dutch gene bank for farm animals is located at the Campus of Wageningen University and Research, and a duplicate collection is maintained at the Veterinary Faculty of Utrecht University. The gene bank consists of a cryopreservation laboratory facility and two storage rooms. The facility has monitoring equipment to detect liquid nitrogen spills or a low level of oxygen in the liquid nitrogen containers. The facilities are rented from Wageningen University and Research.
- **Equipment:** All relevant equipment for quality analysis and freezing genetic material is available to carry out cryopreservation for different species
- **Database:** CGN is using the gene bank database CryoWeb that was developed by a European consortium, funded by the European Commission. Genomic data is stored in a separate database.
- **Staff:** CGN staff involved with the gene banking activities include the following (part time) positions: (i) gene bank/project manager, (ii) database specialist and programmer, (iii) lab technician, (iv) field technician, (v) cryobiology specialist and (vi) a genetic advisor.

Operational budget: In general, fixed costs represent around 90 percent of the operational budget, while the variable costs are 10 percent.

- **Fixed costs:** Staff salaries account for around 75 percent of the fixed costs (figure 1). Around 10 percent of the fixed costs is related to the rental costs of the cryopreservation lab, and more than 10 percent of the fixed costs is associated with storage (main storage and duplicate storage).

- **Variable costs:** The major part of the variable costs is associated with collecting material and adding material to the gene bank collections. Around 10 percent of the costs are transport and travel costs, around 20 percent are costs of specialized services (in particular veterinary costs), and around 15 percent is associated with maintenance or replacement of lab equipment.





4.3 CONSIDERING THE BENEFITS DERIVED FROM GENE BANKING

Establishment or operation of a gene bank can be regarded as an investment. Funds are spent now with the expectation of obtaining benefits (or avoiding losses) in the future. With this in mind, the conventional economic approach to evaluating alternative options for establishing new or modified genetic collections is to treat them in the same way as any other investment decision (FAO, 2020). That is, to consider the private and economic rates of return (the benefits) to the investment (the costs) over the expected period of the investment (the period during which the materials are stored and eventually used).

This approach requires clarity in the initial "investment objective(s)" (the gene-banking goals), so that the expected costs and anticipated benefits can be identified and quantified as part of the analysis. A complement to the analysis is a consideration of who is incurring investment costs and who will receive benefits. This second consideration extends to the governance of the gene-banking process and the related institutional decisions around ownership and control of the genetic resources and the sharing of costs and benefits. While these questions will be clearly relevant to the ultimate configuration and management of *ex situ* collections, the purpose of this section is not to directly address or resolve these governance questions. Section 9 of these guidelines addresses these matters in more detail.

The assumed rationale for genetic collections is that the stored material will be used in future breeding, research or *in situ* species conservation or restoration activities. The variety of potential scenarios for use of stored materials is almost infinite, and the range of possible uses is usually summarized in terms of future option or insurance values (see Box 4.3). That is, genetic resources are usually cryoconserved to the possibility of presently uncertain future uses. These values may or may not be quantified explicitly, but public and/or private stakeholders implicitly express their perceived value of the stored material by their willingness to support collection and storage costs in anticipation of unknown future need.

BOX 4.3
Types of "values" for cryoconserved genetic material

Current use value – the value derived from immediate exploitation stored resources now or in future. Future value may be contingent on the emergence of new information about stored resources. The value of the information gained from that delay is the quasi-option value (see below).

Option value – the value of a potential benefit associated simply with the opportunity or need to use that resource in the future (even if the probability of its use is low). This is also known as insurance value and is of particular relevance when the objective of gene-banking is to protect against breed extinction or loss of genetic variation.

Bequest value – the value of potential future benefits to be obtained by future users that are different from the current investors or gene bank stakeholders. This type of value is of particular importance for public rather than private stakeholders.

Existence value – the value associated with simply knowing that a resource exists. This type of value is usually associated with natural or cultural treasures, such as an endangered wildlife species or livestock breeds that have a typical historical or cultural value. For livestock gene banks, it may be relevant if stored material helps to ensure the continued existence of a culturally significant breed.

Opportunity costs - the potential benefits a decision maker misses out on when choosing one alternative over another.

Modified from OECD (2018).

Being specific about use scenarios for stored genetic resources can help to identify multiple benefits and values that can be important as justifications of investment funding. Some scenarios are common to many gene banks, such as helping to maintain sufficient genetic variation to adjust to changes in market needs and avoiding extinction. Estimating the value of these benefits may be relatively straightforward, achieved by considering the economic consequences of losing current market share or by loss of a breed in its entirety. Determining the value of other uses is more uncertain and may depend on other variables. For example, quantification of value for scenarios involving improved breeding and productivity scenarios is potentially feasible, but speculative.

One important question is how gene bank stakeholders might adopt and implement new breeding innovations related to the use of *ex situ* stored material. Economic justifications can also be derived from less tangible categories of non-use-values that can entail elements of existence and bequest value - the value accruing to us through knowing something exists irrespective of location, and in securing a legacy to future generations (See Box 4.3). The maintenance of cultural (including environmental) heritage and the vitality of rural areas and communities that depend on a livestock economy is important to many countries. Gene bank material has value through supporting the maintenance of the *in situ* population. Such scenarios involve not only future use values to the breeders in these communities, but also bequest and existence values for other members of society. For example, iconic long-haired Highland cattle may be valued by many people beyond Scotland, even if they have never seen live animals. Benefit valuation in monetary terms for such nonmarket situations is challenging, but some approaches have been developed (Bishop *et al.*, 1997; Bockstael *et al*., 2000).

While obtaining an estimate of such benefits may facilitate a cost-benefit appraisal of gene bank investment options, such estimates may not always be necessary in the rationalization process. Simple awareness that gene banking can provide such types of benefits may be sufficient justification for gene bank managers, policy makers and other stakeholders to support a given cryoconservation programme or activity.

4.4 COST ANALYSIS CHALLENGES

Financial tools such as cost-benefit analysis (CBA) and cost-effectiveness analysis (CEA, see 4.5) are useful for evaluating public and private investment decisions (Riegg Cellini & Kee, 2015), and can be applied to investments in *ex situ* conservation programs. A CEA relates the costs of a programme to clearly defined outcomes or benefits. In the conservation context, the costs of achieving identical breed or species survival outcomes by using different *ex situ* facilities can be compared. Alternatively, the *ex situ* and *in situ* costs can be compared, provided both can grantee the same outcome. The outcome does not have to be expressed on monetary terms.

In contrast, CBA goes a step further and converts multiple outcomes (that is, the value of the benefits achieved) into monetary values for comparison with costs. CBA has advantages of using a common metric to compare outcomes that are sometimes not strictly identical. For example, a CBA applied to decide which livestock breed (materials) to collect might determine the monetary value of several potential benefits of each breed (cultural value, genetic gain that could deliver increased performance and productivity, climate change adaptability, etc.), and then compare the benefit-cost ratios to see which breed generates the highest ratio. Ultimately, this valuation exercise is far from straightforward when moving beyond productivity benefits.

Guidance on CBA (see EIB, 2013) defines investments as being either private or public decisions. This difference in perspectives describes who is incurring the cost of the investment and defines which costs and benefits are included for comparison. In most gene banking situations, it can usually be assumed that the relevant perspective involves a public resource allocation decision. Looking from a governmental perspective, the (hypothetical) investment considers how to minimize overall (public and private) costs and maximize total (or social) benefits related to the eventual configuration of *ex situ* collections. This decision can be conceptualised as being taken in a single region or country, or as a collaborative decision between several authorities. Configuration in this case refers to storage of which materials from which breeds and species, in which locations. In the CBA, the net-benefits (or benefit-cost ratio) of the current collection configuration are compared with alternative scenarios where collections are consolidated to save costs or to maximize benefits through use.

This type of economic CBA is routine for trained economists but is challenging when cost data are incomplete and when there are non-market benefits deriving from an activity such as gene banking. Appraisal is also complicated when costs and benefits are uncertain, and this uncertainty increases over longer time horizons as is often the case with gene banking.

These guidelines will not address in detail the principles of non-market valuation challenge (see Bockstael *et al*., 2000, for further information). Even without benefit valuation, gene bank managers, governments and other stakeholders can seek other ways to maximise the efficiency of their investment spending.

4.5 COST-EFFECTIVENESS ANALYSIS

As noted, CEA avoids the need for benefit scenarios and their monetary valuation by re-framing the economic problem as one where the aim is to maximise the diversity of genetic collections at minimum cost. Essentially this redefines the appraisal as an optimization problem requiring clear definition of the objective and selection of the least-cost investment option to deliver it. There may only be one technical option for conservation or several. The key point is that these cost alternatives relate to an identical outcome.

CEA thus defines an optimization problem that can be solved either by simple comparison of options or through more detailed mathematical methods such as linear programming (LP) (Dantzig, 1998). The LP, also known as linear optimization, is a method to achieve the best possible outcome of a planning problem, such as maximum profit or least cost. The rationale behind LP is that in real life problems, resources such as capital, labour, water, and storage capacities are limited and therefore an "optimum for use" should be identified. So, for example an LP could suggest ways to collect and store genetic materials, in terms of number of semen doses, collection regions and in which gene back to store, so that the current costs of operating gene banks could be reduced by up to 20 percent, see Annex 4.1 and De Oliveira Silva *et al*. (2019).

In a more complex modelling exercise, seeking to rationalize *ex situ* collections, LP models can be used to frame the problem in terms of minimizing collection costs and maximizing diversity. The latter can for example be defined as the maximum number of breeds that can have material in a gene bank network. The maximum number would be limited by several factors, such as collective budget, distance between gene banks and collection regions, gene bank fixed and variable costs, and cryotank capacity (De Oliveira Silva *et al*., 2019). Optimization can be used for efficient reallocation of existing collections (De Oliveira Silva *et al*., 2019) or for planning future collections, for example by considering projected extinction risks (De Oliveira Silva *et al*., 2021).

A focus on breeds is a simplification, as genetic diversity rather than the number of preserved breeds might be the more appropriate goal and can theoretically be addressed when genomic data are available. In the case of public conservation efforts, for example, in national policies incentivizing the conservation of local livestock breeds (MAPA, 2020), it is reasonable to consider a variable that relates to current and future status of the populations in terms of risk of extinction.

BOX 4.4

Cost-effective analysis (CEA) with optimization

1. Define objective functions (OF). A single or multiple OF should be determined, e.g., least cost O.F, consisting of fixed and variable collections costs. Multiple OF may be used to balance conflicting objectives (e.g., genetic gains vs genetic diversity).

2. Decision variables (DV). DV relates to collection and allocation of genetic materials across a set of gene banks. For example let $X_{t,gb,b,r}$ represent the number of semen doses of livestock breed *b* (in straws of 0.25 mL) collected in year *t* by gene bank *gb* in region *r*.

3. Collections constraints (CC). CC are presented by budget limitations (local or collective budget for gene banks network), geographic distribution of endangered animals, technological limitations (success rate, degradation), capacity (volume of cryotanks), labour availability, restoration targets, and expected economic returns.

4. Parameter uncertainty (PU). Considering PU is recommended for parameters with significant uncertainty within the timeframe of the analysis, for example future extinction risks should be added as stochastic parameters in the model.

5. Model outputs (MO). MO are generated to produce efficient (cost-effective) collection and allocation strategies of genetic resources (See Figure 1). MO allow for deriving cost-curves of diversity vs expected costs, or extinction risks vs costs for example.

The probability of endangerment can be estimated using census data and regression methods (De Oliveira Silva *et al*., 2020). As resources are limited and *ex situ* conservation is a relatively expensive technology, it may be rational to prioritize breeds that are more likely to be at-risk. In this case, CEA can be used to identify the trade-offs between costs (public or private) and extinction risks, genetic gain, or other attributes.

Box 4.4 illustrates the steps in terms of defining variables, constraints and data for a simple optimization model for cost-efficient collections. The final goal is to construct a model that is able to inform economically efficient *ex situ* collections across gene banks.

Figure 4.1 is an example of how information from LP can be used to inform strategic *ex situ* collections for a gene bank network with single objectives. The example illustrates how reallocating existing collections across European gene banks can save costs, which in turn allows greater resources for conservation of endangered breeds.

Applying the concepts of box 4.4, De Oliveira Silva *et al*. (2019) derived a CEA analysis of optimized collections and found that current costs across European cryogenic banks could be reduced by around 25 percent by reallocating genetic material to more efficient banks, allowing for collective budget sharing and avoiding overlapping collections.

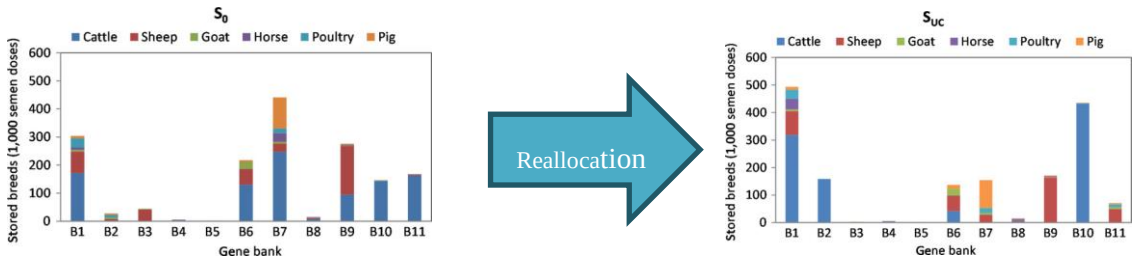


FIGURE 4.1

An example of a CEA (De Oliveira Silva *et al*. 2019) in which the reallocation of semen doses of livestock breeds in 11 European gene banks could reduce collection and storage costs by around 20 percent. S_o represents the current (2018) collections, while S_{UC} is the optmized (or least cost) collection strategies. Note how number of doses increases in some gene banks (e.g. B1, B2 and B10), but are reduced in others (B3, B6 and B11). The difference in collection costs is explained by a more cost-effective collection strategy that considers the relative breed costs (fixed and varible costs) across different gene banks.

Source: De Oliveira Silva *et al*. (2019).

4.6 RECOMMENDATIONS FOR COST ANALYSIS

From the work by De Oliveira Silva *et al*. (2019 & 2021) carried out as part of the Horizon 2020 EU project IMAGE, the following basic recommendations for performing a full cost analysis of collection enrichment, maintenance and future regeneration steps can be given (IMAGE, 2020):

- Collect cost estimates that are as accurate as possible. When undertaking an analysis that involves optimizing collections across multiple banks, cost collection should be standardized across banks and countries where possible. The data collection list provided in Annex 4.2 may be used. This helps prevent inconsistent cost data across gene banks, as gene bank managers tend to consider different components when estimating costs, and some costs, labour, electricity, documentation are not exclusive for managing the collections.

- Use mathematical modelling to estimate costs in specific scenarios but determine first whether modelling is required/beneficial and for what purpose. Mathematical modelling offers a flexible tool for rationalizing *ex situ* collections avoiding redundancy, at the same time providing a systematic approach to cost data collection and in relation to formulating conservation objectives including acceptable *in situ* extinction risks.

- Requirements for accurate modelling include the following: (i) consistent gene bank data; (ii) information on the quantity and nature of germplasm (e.g. number and volume of semen doses or goblets); (iii) real or potential cryotank capacity; (iv) breed census data (to link collection decisions with *in situ* populations and policy scenarios); (v) limits on the available or projected conservation budget; and (vi) conservation priorities for the formulation of conservation scenarios.

- *Ex situ* collections are generally costly, and resources are limited. Rationalizing collections through cost-efficiency analysis can prevent suboptimal collection strategies.

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SECTION 5

Developing and using gene bank collections

Developing and using gene bank collections

5.1 INTRODUCTION

As noted in Section 1, gene banks have value not only as a backup to recreate a breed in case of disasters but also to serve existing *in situ* populations, to develop new populations, and to support research. In contrast to live populations, a gene bank collection does not "evolve". Genetic drift and consequent loss of alleles does not occur, nor does adaptation to the environment. Consequently, a cryo-collection can play different roles in the conservation of a breed (Figure 5.1). An advantage of gene banks relative to live populations is that the gene bank represents genetic variation in the population at the time of sampling, which can be continuous. Consequently, alleles can be present in a gene bank that had been lost in the live population and gene banks thus can help to increase genetic diversity in live populations (Dechow *et al.*, 2020).

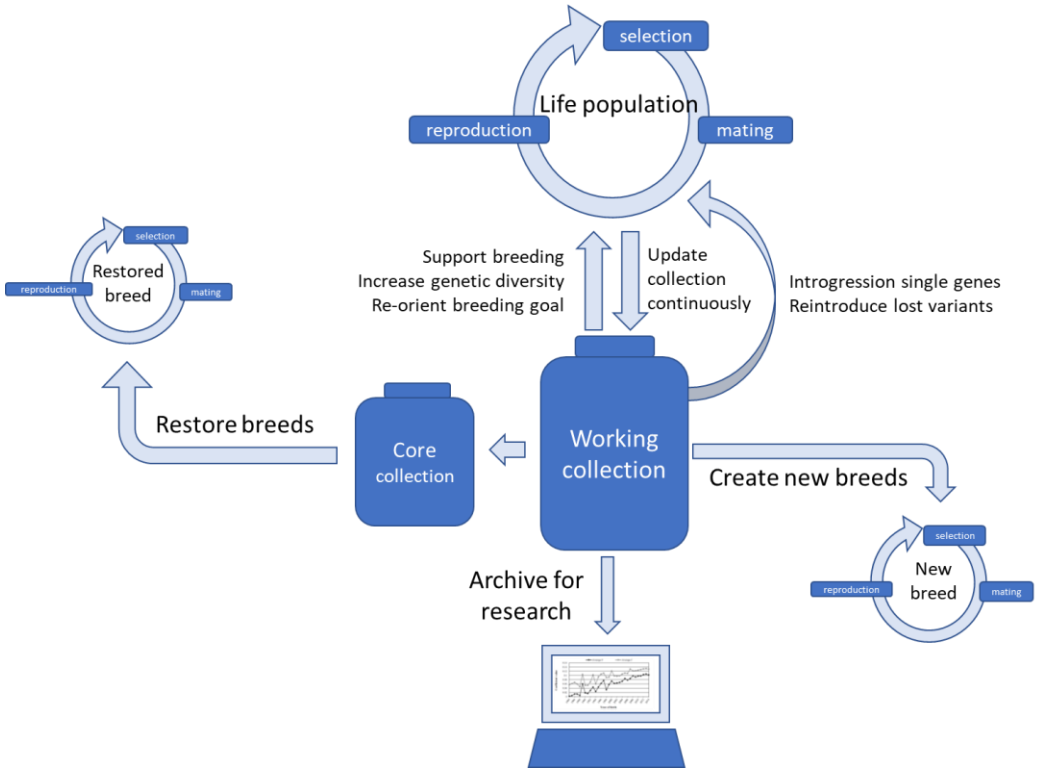


FIGURE 5.1
Uses of gene bank collection (= *ex situ* in vitro cryo-collection). The gene bank collection consists of a core collection serving as an insurance in case of disasters to enable restoration of breeds, and a working collection that can be used in the live populations or to create new breeds or for research purposes (adapted from Berg & Windig 2018, and Tixier-Boichard & Crooijmans 2019).

Figure 5.1 and the previous FAO guidelines (FAO, 2012) refer to the maintenance of "core" and "working" collections, for which the core collection is material kept for reconstitution of breeds in the case of a disaster, while the working collection is used for all other purposes such as research or breeding programmes. The previous FAO guidelines (FAO, 2012) contain detailed instructions on the size of the collection needed for the core collection. As time has passed and gene banks have become more and more utilized for purposes other than breed reconstitution, the distinction between these two

collections has become blurred. In many cases, scarcity of specimens available for storage may even prevent the creation and maintenance of separate core and working collections. Therefore, many gene banks no longer make such a distinction, but rather choose to develop a sufficiently large single collection and to manage it strategically to serve multiple purposes. Therefore, it is recommended to further develop exploitation of the core collection.

The size needed for the collection depends on how intensively the collection is used. A good practice is that when breeders use samples from the gene bank an effort is made to obtain samples from the resulting offspring. This ensures not only that the size of the collection remains large enough, but also that it keeps pace with changes in the live population.

With regard to supporting *in situ* populations, gene bank collections can be used to introduce valuable traits into live populations (see BOX 5.1), serve as an archive for research and improve breeding to better meet changing breeding goals. The latter may be because of changes in the market, due to environmental conditions or because of emerging diseases (Gandini & Oldenbroek, 2007).

Consequently, conserved genetic material can be used to enlarge the effective population size of the living population. Since endangered populations are small, there is a risk that a long-term viable effective population size of at least 50 animals cannot be maintained *in situ*. The population may then suffer from inbreeding depression and show a reduced selection response because of limited additive genetic variance.

When genetic material from the gene bank is available, the breeding population consists not only of the individuals from the current population but is augmented by individuals with genetic material in the gene bank. The impact of gene bank material on the effective population size can be estimated depending on the proportion of offspring with gene bank animals as parent (Sonesson et al. 2002). The inbreeding level of offspring from animals in the gene bank sampled *k* years will reduce to the level at that time and simultaneously the generation interval will increase to a maximum of *k* years.

Gene bank collections play an important role in the long-term conservation of animal genetic resources (AnGR). The *FAO guidelines for Cryoconservation of animal genetic resources* (FAO, 2012) contain recommendations on how to develop gene bank collections. These recommendations start with assessing the status of AnGR populations and determining which populations should be conserved in the gene bank. The latter contains aspects such as cultural, societal, and historical importance, genetic uniqueness, and economic importance. There is no single “correct” approach that is appropriate for all countries. Some countries will aim to conserve all breeds, whereas others will target breeds at the greatest risk of extinction. Other countries may target strategies to support the most commercially important breeds, even if they are not at risk of extinction.

Once the decision is made to conserve a population, one must decide the type of genetic material, such as semen or embryos should be conserved, from which animals and how much. The guidelines (FAO, 2012) provide detailed explanations on how to determine the amounts of material to be stored per breed in the gene bank. In most instances, these calculations were based on quantities of material required to reconstitute an extinct breed from material in the gene bank. These calculations remain valid using the most commonly available and reproductive technologies and especially for many local breeds. There are instances, however, where the estimates may be conservative, such as for countries with access to the most advanced reproductive technologies and for breeds for which such techniques have been developed and refined.

BOX 5.1

A practical application of a marker-assisted introgression of a specific trait

A genetic resource population conserved in a gene bank can be used to take a specific trait of this population and to transfer it into a contemporary breeding line. Within the EU project IMAGE (Horizon 2020), the marker-assisted transfer of a specific trait, the blue eggshell colour, from the donor population, the Araucana breed, to a commercial chicken breeding line was demonstrated. Researchers of the University of Göttingen and the Friedrich-Loeffler-Institut in Germany integrated this causal mutation into a commercial White Leghorn breeding line to induce production blue shell eggs.

An initial F1 generation, two marker-assisted backcross generations (BC1 and BC2) and a final intercross generation (IC) were generated, aiming at a high performing WL-like line that is homozygous for blue eggshell color. To achieve this, all birds of the study were genotyped with a custom-made 52 000 SNP array and 24 newly developed breed/line specific SNPs at the introgression locus. Genotyping results were analyzed for the detection of haplotypes at introgression locus. A recombinant animal contains a different combination of alleles from either of its parents. For the BC2 only recombinant BC1 cocks were used in order to reduce the Araucana genome content next to the introgressed insertion.

Selection criteria were heterozygosity or homozygosity for blue eggshell color, high similarity to WL and high genetic diversity. Marker-assisted selection increased the proportion of the White Leghorn genome in the BC2 generation by 4.4 percent more than expected on average (87.5 percent), thus reducing the donor genome of Araucana accordingly. In 2019, the IC population hatched, of which 188 animals were homozygous carriers for the blue shell colour allele. Preliminary results of performance tests for the IC population, yielded promising results. The laying rate was quite similar between the blue layer IC and commercial White Leghorn hens, while the mean egg weight was only slightly lower. The eggshell strength increased from generation to generation but was still lower in the IL compared to the White Leghorn line. Homozygous intercross hens and cocks are the basis for a high performing blue egg layer line that is highly similar to the White Leghorn.

Source: the H2020 EU project IMAGE.

As explained in Section 1, nowadays more attention is given to uses of gene bank collections other than the opportunity to reconstitute an extinct breed. Development and use of gene bank collections have received increased attention since the release of the previous guidelines. The possibilities of using genomics to characterize populations and gene bank collections have increased and software has been developed to better predict the impacts of and guide the use of genetic material from gene bank collections in live populations. This section provides updates on these methods and describes how gene bank collections can be used in management of *in situ* populations.

5.2 ANALYZING GENETIC VARIABILITY CHANGES IN A GENE BANK

5.2.1 Pedigree analysis

Analysis of the genetic variability based on pedigrees is a widely acknowledged method that can be used effectively to assess the variability of past and potential gene bank donors versus the live population. Numerous scientific publications (Maignel et al, 1996; Gutiérrez and Goyache, 2005) are available on indicators calculated for a wide array of breeds and species, with different methods described in the current cryoconservation guidelines (FAO, 2012). The main advantage of pedigree analysis is that assessment methods are simple, low cost, and robust. Pedigree analysis does not involve any additional cost other than labor time if pedigree data are available. A drawback of pedigree analysis is deviation from genomic data for inbreeding level. Various indicators of genetic diversity can be calculated. One of the most relevant indicators is the kinship of gene bank donors versus the live population. For instance, one could calculate the kinship of all males in the gene bank versus the living male population. The males in the live population with low average kinship with gene bank donors may then be targeted for collection. Another approach is to estimate the kinship of the donors with the *in situ* population's main ancestors (see for instance Boichard, 2002) and then collect from descendants of ancestors that are not yet well-represented in the gene bank.

Pedigrees can also be used to guide collection development and comparison to a breed’s in-situ population by computing genetic relationships followed by clustering the animals based upon their level of relationship to one another using statistical methods like Ward’s Minimum-Variance. Once clustered gene bank collections and in-situ populations can be compared for completeness (Blackburn, 2009).

5.2.2 Molecular analysis

Molecular analysis generates more accurate estimation than pedigree analysis and is recommended for any breed with unavailable and/or unreliable pedigree data. The disadvantage is the frequent scarcity of available samples in the living population and almost complete absence of samples from previously living populations. Also, when the collection of genetic material was not done in conjunction with DNA sampling, a dose of material from the gene bank must be planned for to obtain DNA and perform the analysis. Finally, there is an associated cost for DNA extraction and genotyping. In the case of breeds with no routine DNA analysis, a new field sample of the *in situ* population will also be required. Similar to pedigrees, the most common indicators that can be calculated are kinships between gene bank donors and living breeding males or the main breeding males at the time of the study (see Eynard 2015 for an overview of different methods). In addition, the effective population size, changes in allele frequencies and inbreeding over time also play a role. At present, there is no consensus on how to calculate the above-mentioned indicators from the molecular data, but most approaches provide similar results when applied to the same data.

BOX 5.2
Utility of genomic analysis for an existing collection

The French National Cryobank was set up in 1999 and currently (2020) preserves reproductive biological material from 250 breeds of 21 different species. For rare pig and goat breeds, a large collection has been established, which was set up mostly for long term conservation. The samples in the collection were gathered at the beginning of the conservation programmes and very little information about the male animals was recorded at the time. The recent development of genomic tools offers a unique opportunity to answer some very essential questions for the conservation programmes, such as whether the founder animals were related, if they represent the history of the breed as individuals, and how well they are represented in the *in situ* population.

Genetic variability indicators such as molecular kinship calculated from genotyped data to answer these questions and to aid the breeder associations. The results allowed the breeders to optimize the breeding of the next generation of males to be sampled for the gene bank. The new generation of male donors was chosen by targeting breed origins that had not been previously sampled for the gene bank, based on molecular analysis of both gene bank material and animals from the living population.

Source: the H2020 EU project IMAGE

5.3 NEW DEVELOPMENTS IN GENOMICS

Since the turn of the millennium, sweeping advancements have been made in the ability to study the genomes of organisms. Prior to this, molecular analyses were based on single loci or small sets of genetic markers on relatively coarse genetic maps. The advent of high-density SNP arrays has fundamentally changed genome analysis and insights into genetic diversity. With these arrays, genetic analysis is no longer restricted to individual loci or imprecise maps but could be extended to the entire genome (Mei et al. 2000). Among the most important applications that have emerged from this in the field of animal science is the estimation of the genetic value of an animal using thousands of typed markers and its application in efficient breeding and animal selection, better known as genomic breeding value estimation and genomic selection (Meuwissen et al 2001). It has also become possible to study the genetic architecture of important traits in more detail through so-called genome-wide association studies (GWAS). Last but not least, the realized relationships derived from the marker data (Van Raden, 2008) can be used for more sustainable management of genetic diversity (Sonnesson *et al.*, 2012). The *FAO Guidelines on genomic characterization of animal genetic resources* (FAO, 2021) provide more detailed information about the utilization of genomic data in the assessment of the diversity of animal genetic resources.

BOX 5.3

Two case studies of complementary use of pedigrees and molecular information to evaluate the genetic variability in several Holstein cattle gene banks.

A. Analysis of Holstein gene banks

France, the Netherlands, and the United States of America (USA) all maintain Holstein-Friesian (HF) gene bank collections. Genetic variability of the collections within and between countries was assessed and compared with active male populations in each country by using pedigree data (Danchin-Burge et al. 2011). Measures of genetic diversity such as probability of gene origin, inbreeding and kinship were calculated. The three gene banks have captured significant amounts of genetic diversity for the HF compared with the current populations. Although a substantial part of the USA, French, and Dutch collections seems to be genetically similar, the USA collection in particular represents an interesting reservoir of HF genes of the past which is not present in the current *in situ* population.

B. Change in genetic diversity in the Dutch Holstein population determined by pedigree and genomic data analysis of gene bank samples

A recent study in the Netherlands used pedigree and genotype data of more than 6 000 bulls to assess trends in of genome-wide inbreeding and kinship (Doekes et al 2018b). Gene bank samples contributed to the study. The study estimated inbreeding trends in specific chromosomal regions by detecting runs of homozygosity (ROH) and changes in allele frequency over time. Two major points of inflection were observed trends of genetic diversity. Around the year 2000, inbreeding and kinship both temporarily decreased. Then, from 2010 onwards, they began to steeply increase, with estimates of inbreeding rates up to 2.8 percent per generation, depending on the method used. The amount of inbreeding varied according to the genomic region. A large proportion of the marker alleles had changes in frequency that could not be explained by random genetic drift. Although cause and effect could not be proven, the decreases in inbreeding observed after 2000 corresponded to the introduction of optimal contribution selection and a shift in the breeding goal. The increases in rates of inbreeding and kinship occurring after 2010, on the other hand, correspond closely with the adoption of genomic selection. The observed trends in genetic diversity reflect major changes in the Dutch-Flemish HF breeding programme over the past 30+ years.

Source: the H2020 EU project IMAGE.

A drawback of SNP arrays is their limitation to a non-random set of SNP markers which were selected (ascertained) during the design process. This leads to underrepresentation of globally rare variants on SNP arrays compared to whole genome re-sequencing data, known as SNP ascertainment bias (Nielsen, 2004; Lachance & Tiskoff 2013). Some design schemes further increase this bias by intentional selection of SNPs with overrepresentation of common SNPs with regard to the natural background distribution. To a degree this can be addressed by constructing sub-panels composed of minor alleles (Blackburn et al., 2014 WCGALP). A direct implication for monitoring genetic diversity is that this bias towards common alleles increases heterozygosity estimates and populations therefore show an increased amount of genomic variation. Whole genome sequencing offer a mechanism to alleviate biases that are introduced with smaller SNP panels (Eynard et al. 2015).

Within this design process, such target breeds are screened for variant sites which are especially informative in the target breeds. Such sites may not necessarily be as informative in other breeds apart from the target panel and it is therefore inherent that important variation in such alternative breeds is not represented in the panel. This might be, for instance be the case for variants that were lost in highly selected commercial breeds. The limitation to variants of a small number of populations additionally introduces the problem that populations that are distantly related to the populations for which the array was originally developed show reduced estimates of genomic variability compared to WGS data (Malomane *et al.*, 2018). This population-specific of ascertainment bias therefore inflates genomic estimates of genetic distinction between populations. Ascertainment bias may therefore have a consequence for gene banking if breeds are prioritized for collection based on genomic estimates of genetic variation. Gene bank managers should be aware of this possible bias.

Although SNP technology is a widespread standard and has generated extensive knowledge, it also has other disadvantages relative to WGS besides the ascertainment bias. For example, the variants are usually limited to SNPs. Unfortunately, this class of variants can only explain part of the genetic variance of traits. Variants such as insertions and deletions of nucleotides and inversions of genomic regions may not be detected. Therefore sequencing (either WGS or specific sequencing of target genomic regions) may need to be applied if selection of donors is to be based on these other types of genomic variants.

Today, breeding programmes in most major livestock species incorporate the use of genomic data and genotyping with an SNP array is by far the most routinely used strategy to generate that data. This option also merits consideration for gene banking.

Fifty percent of genetic variation is inherited from the parents, and the other fifty percent is simply due to random Mendelian sampling. Consequently, full sibs have on average only fifty percent of additive genetic variation in common. This means that every additional animal, even if full sibs already have material in the bank, will contribute to variability and is therefore unique and potentially worth sampling.

To facilitate genotyping of animals in gene banks a multi-species array has been developed (see Annex 5.1). Animals can be selected on the basis of the real diversity they carry instead of just expectations made from pedigree evaluations. E.g. full sibs are expected to have fifty percent of additive genetic variation in common, but in reality this will vary around 50%. Also, a library of variation in the gene bank will be known and of immense importance in identifying the genetic originality of individuals when samples from the gene bank are used for *in situ* populations. Especially for the latter aspect, complete genotyping of all samples is valuable. Examples of the use of genotype information are presented below in subsection about software programs.

Also, genomic analysis has costs in terms of time and requires personnel that are capable of performing the required analyses.

BOX 5.4

A glossary of the most commonly used methods in genomics

Whole-genome shotgun-sequencing. Whole-genome shotgun -sequencing (WGS, short-reads/next-generation sequencing, NGS) is a technology that decomposes the whole DNA sequence of an individual into short fragments. Those fragments are subsequently sequenced to a defined length (often 50-150 bp), either from one side (single-reads) or from both sides, generating a connected pair of reads (paired-end sequencing). With such reads the assembly of the whole genome becomes possible by merging reads via overlapping patterns into larger segments, called contigs (shorter) and scaffolds (longer), and eventually chromosomes, to generate a reference genome.

Long-read sequencing. One of the latest developments is long-read sequencing, using single-molecule real time sequencing (SMRT) or nanopore technology. The output of both technologies consists of very long sequence reads, with a length up to several kilobases. Those technologies hold great promise in the successful detection of large structural variants, such as huge deletions and assembly of complex chromosomal regions (Miga et al. 2020).

Resequencing. The method aims at sequencing of an individual's genome in order to detect sequence differences between the individual and the standard reference genome of the species. Sequence alignment can detect many sites of variation in genes and intergenic regions for studying functional genomics or genetics differentiation.

SNP array. A single-nucleotide polymorphism (SNP) is a DNA sequence variation occurring when a single nucleotide in the genome differs among individuals, and can therefore be used as a marker for the underlying linked haplotype. These markers are normally bi-allelic SNP markers. With a SNP array, a high number of such markers can be typed from the extracted DNA, usually from several animals (typically 48 or 96), in parallel. Common SNP arrays contain marker numbers ranging from a few thousand to more than a million, depending on the species. The chips currently (2020) used in genomic selection belong to the middle category (around 50 000). The typing of an animal costs about 50 € with decreasing tendency. Naturally, these arrays contain a predefined set of variations, which are selected according to certain criteria from a reference population (Kranis et al. 2013). These criteria may include, for example, a certain distribution of the minor allele frequencies or an equidistant coverage of the genome.

Note that due to the technical characteristics of the methods, the results obtained may differ significantly between full sequences and selected panels of SNP on arrays. This is because genotyping arrays contain sets of pre-ascertained SNPs, which may result in bias.

5.4 SOFTWARE FOR MANAGEMENT OF GENE BANK COLLECTIONS

Today, interested animal breeders or conservation geneticists can for many species obtain high-throughput genotyping data at a reasonable cost and exploit it. Apart from the general tasks of data and herd book management and breeding value estimation, breeding programme design and strategies for managing diversity are major concerns of a more general interest. In utilization of gene bank

collections decisions on the use of collected samples can be supported by such approaches. The following section presents two software examples for management of animal genetic diversity. The first one is MoBPS, a flexible simulation framework to simulate breeding programs and thus evaluate the impact of breeding decisions on a population. The second example demonstrates how optimal contribution software can be used to manage genetic progress, while efficiently limiting inbreeding.

5.4.1 Modular Breeding Program Simulator, MoBPS

Breeding programmes aim at improving the genetic properties of livestock populations with respect to a given goal, such as increased productivity, fitness and adaptation. Progress towards the target is limited by the available resources, but also by negative effects of selection such as inbreeding depression, decreased fitness or loss of genetic diversity. These effects should be minimized if possible. Additionally, population history, such as fluctuating population size and selection pressure, has an impact on the current genomic architecture and thus the potential for future improvement. Hence, the allocation of resources to gene banks and design of a breeding programme are complex optimization problems.

MoBPS is an R-package to perform stochastic simulation of breeding programs and thus assist breeders to evaluate and optimize their breeding programmes (Pook et al. 2020). Similar to the gene-flow concept introduced by Hill (1974), MoBPS allows grouping of individuals into cohorts that have similar characteristics such as age, sex and genetic origin. Thus, MoBPS provides a highly flexible tool to allow detailed modelling of todays' complex breeding programmes, which may include cohorts of animals with material stored in gene banks, a variety of pre-implemented functions for common breeding practices such as optimum genetic contribution selection and estimation of genomic breeding values. MoBPS allows for the simulation of breeding programmes with millions of animals or population genetic studies with thousands of generations.

To make the MoBPS simulation framework accessible to a wider audience like breeders and scientists with limited experience in programming or as a tool for teaching, it also includes a front-end user interface that can be accessed via a web-browser at www.mobps.de (Pook et al. 2021) that includes most of the functionality of the R-package.

One example for the use of MoBPS is the simulation of a backcross breeding programme to recreate a breed from gene bank material, as was described in the previous guidelines (FAO, 2012). In such a breeding programme, only semen has been stored in the gene bank. Females from another breed are thus inseminated with cryoconserved semen of the breed to be recreate. The offspring are inseminated again with semen from the gene bank and this is repeated until the offspring is "pure" enough (4 generations) to be considered as the original breed. This scheme has been successfully simulated with the MoBPS program (Figure 5.2). Results showed that the purity of the recreated breed closely followed the expectation for each generation (e.g. 50 percent for BC1, 75 percent for BC2, 87.5 percent for BC3 etc.) with hardly any variance.

The input modules in MoBPS allow users to adjust parameters such as pregnancy and survival rates to account for differences among species, breeds and production systems. By doing so, gene bank managers can more precisely estimate the quantities of material to be collected for the prevailing circumstances in their country and even explore the impacts of changing these parameters on the probability of successful breed reconstitution.

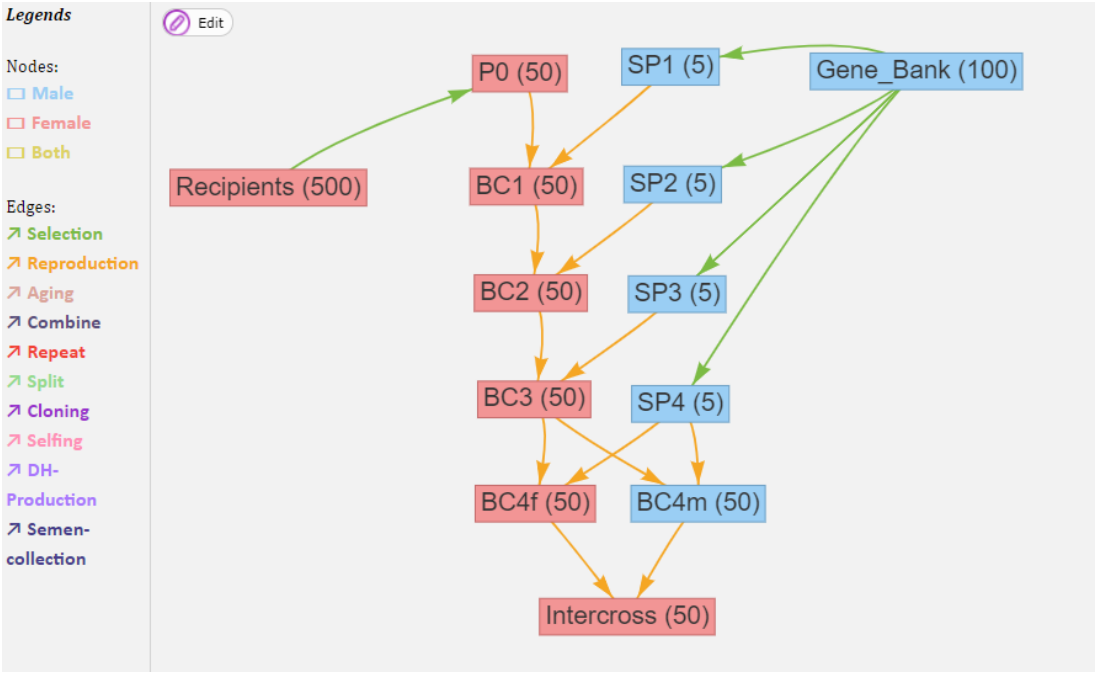


FIGURE 5.2

Set up of the MoBPS program to simulate reconstitution of a breed by backcrossing with semen from a gene bank.

Females are selected from another breed (Recipients) to be inseminated by sperm (SP) stored in the gene bank. Each resulting backcross (BC) is inseminated again with sperm from the gene bank until the 4th backcross generation from which males and females are mated to produce the final generation (intercross) that is considered as the recreated breed. In brackets the number of individuals used in the breeding programme.

Simulations can be executed directly from the web-interface as the R-package, the back-end simulator, is directly linked to it. After those simulations are done, a variety of functions to analyse and compare different breeding programmes with regard to breeding objectives can be performed (Fig. 5.3).

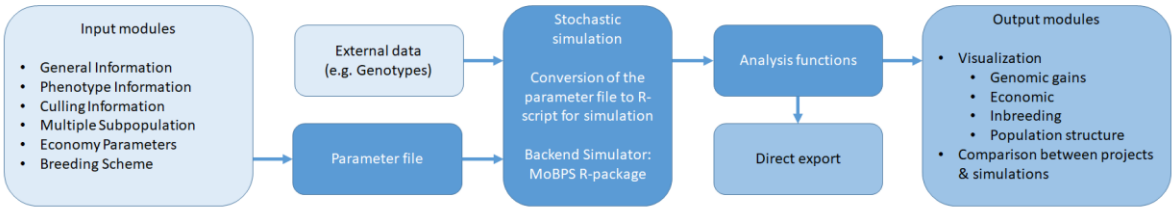


FIGURE 5.3

Setup of the MoBPS simulation framework

A key strength of a simulation approach lies in the fact that, in contrast to real-world experiments, far less time and money is needed. Also, potential harm to animals, such as adverse fitness effects, can be avoided. Furthermore, experiments can be repeated and modified, which leads to much greater statistical power when comparing scenarios. A huge advantage of stochastic simulations is that variation can be studied by repeating the process many times, which is very helpful for risk analysis. For example, by chance, low reproduction or unbalanced sex ratios may decrease numbers of offspring in a breed reconstitution programme. Simulation allows one to observe the likelihood of failure due to such problems. Even if the absolute value for estimated effects, such as genomic gain, inbreeding rate

etc., might be slightly incorrect due to simplifications of reality, these effects should usually affect all scenarios considered and thereby still ensure comparability.

5.4.2 Optimum contribution software

As discussed in Section 1, the genetic material stored in the gene bank can serve different purposes. Regardless of the purpose, it is essential that genetic diversity in the gene bank is maximised, given restrictions such as the number of animals that can be sampled. Optimum contribution theory and associated software can assist in achieving this goal.

Optimum contribution selection was originally developed to determine the optimum number of offspring of each breeding individual in a selection programme subject to certain constraints (Meuwissen 1997). The approach is equally applicable to management of gene banks. A common objective in a commercial breeding programme is to maximize the average breeding value while restricting the rate of increase in the average kinship and inbreeding. Another constraint in a breeding programme is that the total contribution of each sex must be 50 percent. For a gene bank, one logical objective may be to minimize the average kinship when sampling a fixed number of individuals from the live population.

With the development of genotyping, kinships can now be reliably estimated both by using genotypes and by using pedigrees. If pedigrees are used, care must be taken to ensure pedigree completeness because predictions of optimum contributions will otherwise be biased. If genomic kinships are used, missing genotypes can be problematic and methods that combine pedigree and genomic information may be needed. When using genotypes, the use of segment-based kinships, which can be computed from phased marker data, is recommended. This way, the segment-based kinship of two individuals equals the expected proportion of an offspring genome that is included in runs of homozygosity. Different programs are available for optimum contribution selection, which includes the free R package optiSel (Wellmann, 2019), the free software EVA (Berg, 2006), the free software Gencont (Meuwissen, 2002) and the commercial software TGRM (Kinghorn, 2011).

Constraints to sex and the rate of increase in kinship can influence the results. For example, conserved genetic material can be used for the recovery of “native” genomes of the local breeds that have undergone “upgrading”. Many local breeds have been subject to generations of occasional or systematic crossing with other breeds. Continued crossing with other breeds eventually leads to the genetic extinction of the local breed because the native alleles get lost. To prevent this, breeders (or other stakeholders) may wish to remove the introgressed genetic material from the genomes of the local breed as much as possible. Older individuals of local breeds not only tend to have less “foreign” genetic material, but the foreign haplotypes also tend to be longer. Long haplotypes can more easily be removed than short haplotypes. The previously conserved genetic material from animals born years ago is therefore of high value for a breeding programme that aims at removing the foreign haplotypes from the population.

The objective of such a selection programme could be to maximize the proportion of the local breed genome, while constraining the increase in the average kinship of native alleles below a certain threshold. An alternative is to reduce the frequencies of alleles associated with the “foreign” breed, while maximizing diversity in the rest of the genome. For both procedures, the R package optiSel can be used.

BOX 5.5

Using gene bank material to optimize selection in Creole cattle

Creole, or "Criollo" cattle are locally adapted cattle found throughout Latin America. Although they directly descend from breeds that originated in southwestern Europe during the latter half of the previous millennium, they are often considered native to the Americas due to their long-term presence there.

A recent study was undertaken to improve the management of the Creole cattle in Colombia by evaluating the active population, as well as the usability and usefulness of the gene bank samples. The breed Blanco Orejinegro, was studied in detail. The first step was to assess the genetic diversity of all available samples. In the follow up steps the gene-flow approach was used to model and optimize introgression and conservation schemes.

The optimal contribution method was used to develop a programme to maximize genetic gain for growth traits while constraining inbreeding in the Blanco Orejinegro breed, while including the use of semen from the gene bank. Around 50 females and 50 males are registered each year. The gene bank collection consists of semen of 104 bulls, of which 28 have genotype data and are no longer part of the active population.

The value of the bulls in the gene bank was assessed in terms of genetic merit and diversity, by simulating an OCS scheme with the 28 cryobank bulls as selection candidates, in addition to the current population. The MoBPS software was used. The simulation showed that the growth traits could be improved while controlling the level of inbreeding. Both the genetic gain and variability of the population were improved by applying OCS and using semen from the gene bank.

Source: the H2020 EU project IMAGE

5.5 CHANGES OVER TIME

Cryopreservation has been widely used in cattle since the 1960s. Therefore, the implementation of gene bank collections has a long history in some countries. According to a survey carried out during the H2020 IMAGE project about European gene bank collections (Passemard et al. 2018), the average onset of sampling for the collections is 2002, which is quite recent. However, the oldest collection was sampled in 1963 for the Meuse-Rhine-Yssel cattle (MRY) breed (in the Netherlands) and there are also samples for three other cattle breeds that were preserved in 1966 (Dutch Friesian, Polish Red and Original Austrian Brown cattle), all kept in the gene banks of their country of origin. Some collections are highly interesting since they are a unique representation of breed evolution over time. We will show in the following case studies (BOX 5.8) that gene bank collections differ from the live population (Danchin et al. 2011), and finally we make recommendations on how to take this into consideration.

BOX 5.6

European gene banks as gene archives

A survey was conducted during the H2020 IMAGE study about gene bank collections. Some managers gave detailed answers about their collections, which revealed that there are at least 92 breeds from five distinct species that are sampled every year: cattle (62 breeds); goats (7 breeds); horses (2 breeds); pigs (8 breeds); sheep (13 breeds). These collections are kept in eight different European countries (Austria, France, Germany, Hungary, Iceland, Italy, Poland, and Spain) and 14 different organizations, and the time of first sampling varies between 1966 and 2014, and the average year of first sampling is 1997. These data show that some collections are likely to be distinctly different from the current *in situ* populations. The gene pool of a breed changes constantly over time due to genetic drift (which affects rare breeds most due to their small numbers), selection and inbreeding. These changes do not affect all breeds and species in the collections equally. For instance, due to their slower generation interval, the cattle collections representing the most common collections will not change as rapidly as collections for pigs. On the other hand, the selection pressure in dairy cattle is stronger than e.g. in horses or sheep, but this may be compensated by a large census size such as in Holstein cattle. Therefore, the only way to assess if a gene bank collection differs radically from a live population is to assess its genetic variability.

Source: the H2020 EU project IMAGE

BOX 5.7

Assessing the impact of the use of an old cryopreserved bull on the genetic variability of the breed.

The aim of this study was to assess to what extent the use of old cryopreserved material can support reintroduction of genetic variability within a given breed. To do so, the impact of using cryopreserved material from a bull born in the 70s during the years 2004-2007 in a regional dairy cattle breed, the Abondance, was examined. Molecular data (50k and HD chips) as well as pedigree information were available for the cryopreserved bull as well as for recent reproducers (bulls, as well as a significant number of cows born over the last two years), including his descendants. Genealogical and molecular approaches were used in a complementary manner to evaluate the consequences of this reintroduction on neutral and genome-wide diversity of the breed. The results showed that there was a favourable impact for the genetic variability of the breed as well as some progress on specific traits (mostly functional ones) which counterbalanced the loss of genetic gain on production traits. However, one should bear in mind that this favourable result was obtained in a breed with genetic gains that are limited in comparison with large international breeds.

Source: the H2020 EU project IMAGE.

BOX 5.8

Two case studies where the samples over the years are used to evaluate genetic changes of a breed

A. A genetic investigation of Island Jersey Cattle, the Foundation of the Jersey Breed

The genetic difference between the founding population and non-Island Jersey cattle was analyzed by Huson et al (2020). Samples from Jerseys raised in the USA (n=49) and on Jersey Island (n=34) were obtained from the USDA gene bank. The cross-section of bulls were born from the 1960s to 2000s and were lowly related to one another. These data provided the first insights into the divergence of two subpopulations of the Jersey breed over decades of isolation between its place of birth, the United States.

Cattle samples by breed per birth decade

Population	**Decade of Birth**							
	1950	**1960**	**1970**	**1980**	**1990**	**2000**	**Unknown**	**Total**
Jersey_ISL		2	8	10	21	8	0	49
Jersey_USA	1	3	3	5	18	4	0	34
Total	1	5	11	15	45	17	1	95

B. Annotation of selection signatures in the bovine breed Asturiana de los Valles

This study was implemented to demonstrate the usefulness of gene banks for detection of recent selection and annotating signatures of historical selection. It was based on a Spanish autochthonous beef cattle breed, the Asturiana de los Valles, which is raised under semi-extensive breeding conditions. The gene bank collection enabled the analysis of evolution of genetic diversity along 35 years, from 1980 to 2015.

Generation	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
First year	1980	1984	1988	1992	1996	2000	2004	2008	2012
Final sample size	0	4	8	13	17	28	29	9	9

The genome data analysis detected selection signatures at different sites which appeared over time. It revealed candidate genes for meat or milk production, immunity and olfaction. The study showed that time series material stored in gene banks serves as rich information source in breed history and biology.

Source: the H2020 EU project IMAGE.

5.6 RECOMMENDATIONS FOR GENE BANK UPDATING

It can be concluded that reproductive gene banks are multi-functional. Since cryopreserved material retains its viability for many decades, some AnGR gene bank managers choose not to follow the recommendations and do not update the conserved material. This is unfortunate because living populations are constantly evolving and therefore gene bank managers and breeding organizations should regularly monitor and update their gene bank collections, especially the working collection (Fig. 5.1). As a rule of thumb, the faster the breed develops, the more often the gene bank collection should be evaluated and updated if necessary. Estimates how often this should be done have been made and indicate roughly every 4 to 7 generations (see BOX 5.9; Blackburn 2018).

In the case of endangered breeds, one goal is to store material from a sufficient number of animals needed to restore the breed's genetic variability. So far, a limited number of breeds have reached this goal (cf. Leroy et al., 2019). Eventually, this can become an obstacle in the utilization of gene bank material as farmers could be reluctant to use genetic material not corresponding to the breed's objectives. For example, an assessment was performed of dairy cattle breeds in French gene bank collections after 10 years of storage. Selected breeds are sampled on two principles, the first one is to gather a snapshot of the breed from a genetic variability point of view, for a given year, and the second is to sample extreme bulls for various traits (Verrier et al., 2003). The assessment showed that for breeds intensively selected for a trait, such as milk yield or protein content, extreme bulls for these traits were quickly surpassed by contemporaries. Therefore, the gene bank sampling goals were changed for these breeds: animals were sampled according to their breeding values with extremes that differed according to how fast a trait is selected. For instance, for heavily selected traits such as milk production or stature, Holstein bulls are included in the cryobank only if their genetic evaluation is superior to three square types more than the average (based on the AI bulls' evaluation) for the particular trait, on a given year. On the other hand, the threshold traits such as fertility which have a lower genetic progress, the threshold is lowered to two square types more than the average. Holstein bulls are sampled if they are three square deviations more than the average value for milk yield or two square types on fertility evaluation since this trait is not selected as steeply as milk. In conclusion, the following recommendations can be used for establishing and maintaining a gene bank:

- Gene banks should be established as early as possible for breeds, despite census size. It will allow breeders to select the most interesting males to be cryopreserved while maintaining a sufficient degree of genetic variability.
- The best individuals of the new generation should be deposited back into gene bank collections.
- Gene banks founder individuals can be used to generate improved offspring which will better meet recent breeding objectives and farmer expectations. The best ones should be sampled and join the gene bank collections.
- Compare the gene bank collections with the living population on a regular basis and update the gene bank collection with new material both based on genetic variability and genetic gain in breeding objectives, even if the collection has met FAO standards.
- Use new knowledge and tools for measuring fertility rate in cryoconserved material, since the current knowledge about fertility rate obtained with frozen semen is incomplete for many species, especially birds.
- For intensively selected breeds, assessment of the collection may also be done to evaluate specific changes in genetics of the breed, for example depending on (1) the level of intensity of the selection for a given trait, (2) the variability and extent of changes applied on selection

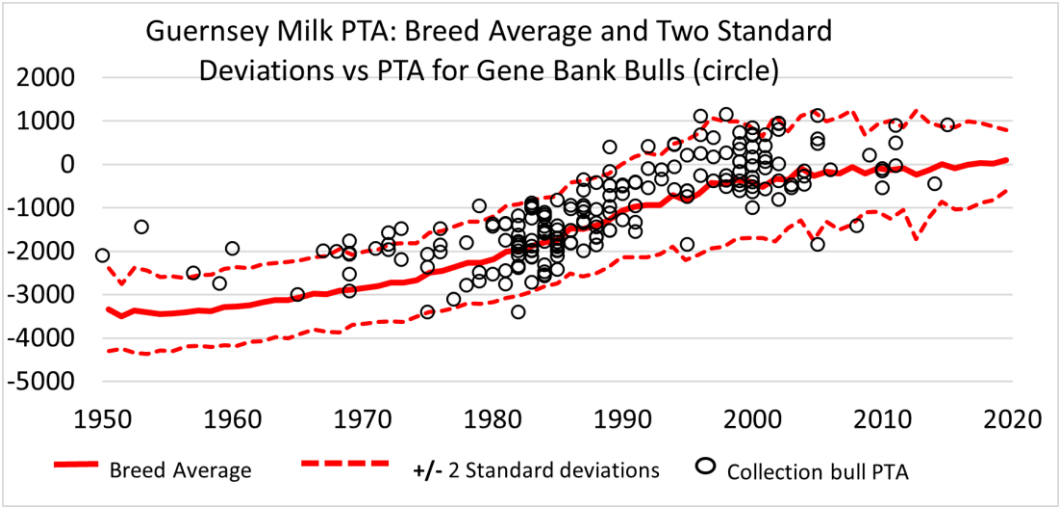
goals over time and (3) the existence of similar collections of the breed in other countries/sites.

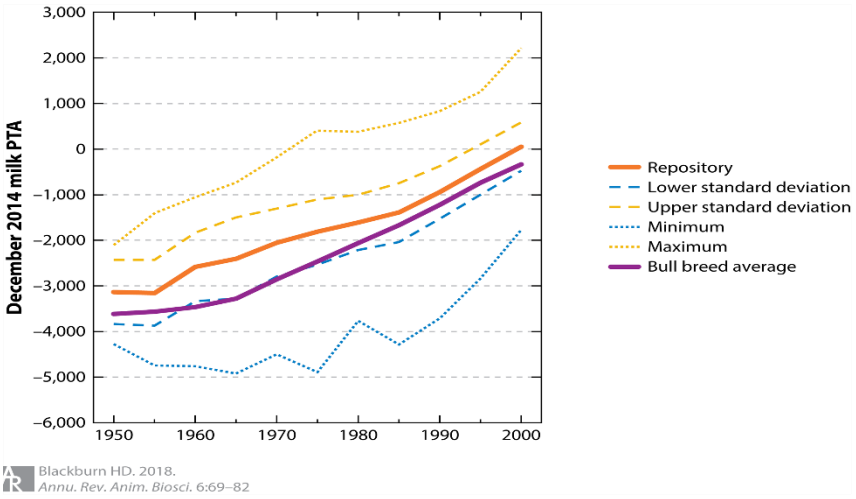
BOX 5.9

Resampling Populations to Keep the Collection Current

A common criticism of gene banks is that the collections can become dated and thus lose utility over time or make it more difficult to use due to its genetic differences with the existing *in situ* populations (Leroy et al, 2019). This issue can be addressed by executing periodic sampling over time (Blackburn, 2018). Under a resampling protocol gene banks would decide how frequently to resample a breed dependent upon the genetic change that is occurring.

For example, Guernsey is an "at risk" breed in the United States. The gene bank has samples from a time continuum of 1948 to 2019. The following graph illustrates how resampling over time has enabled the collection to keep pace with the in-situ population for the predicted transmitting ability (PTA) for milk. The graph also illustrates the diversity collected by comparing individuals from the gene bank to the annualized breed mean and two standard deviations for milk production; there are several years where animals in the collection exceed the in-situ population by more than two standard deviations. Additionally, the graph shows that while the breed is increasing for PTA for milk but at a pace that has not exceeded the PTA for bulls collected earlier in time, suggesting that animals collected may have greater longevity for competitive use than previously thought. The second figure is for Holstein which has a faster rate of change for milk PTA, but shows that resampling has kept pace with the in-situ population change.





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SECTION 6

Collection and cryopreservation of germplasm and tissues

Collection and cryopreservation of germplasm and tissues

6.1 INTRODUCTION

Long-term cryoconservation of germ cells and embryos, or germplasm, is an important tool in the management of animal genetic diversity. Animal germplasm gene banking, however, is challenging since the cryopreserved germplasm must recover, after freezing and thawing, all its specific functional capacity to generate live and healthy offspring. Cryopreservation is not a biological process *per se*, therefore, most cells and tissues require specific procedures that depend on the type of germplasm and animal species. In addition to the freezing-thawing processes themselves, the upstream methods of sampling and fertility treatments and the downstream methods of utilization all hold their own challenges.

This section reviews the basic principles of cryopreservation and then presents an overview of the procedures involved in germplasm collection, cryopreservation and use for mammalian, avian and apian genetic resources for food and agriculture. Precise protocols for many of these procedures are described in Annex 6.1-6.5 of this guideline.

6.2 SPECIFIC FEATURES OF LONG-TERM CRYOPRESERVATION

Cryopreservation is generally defined as the viable freezing and storage of biological material at ultra-low temperatures, preferably at that of liquid nitrogen (-196˚C), and subsequent thawing. Long-term cryopreservation of germplasm extends its applications, including transport over long distances, storage while awaiting the recipient animal (such as in quarantine), use following the death of the donor, maintenance of valuable laboratory lines, and conservation of endangered species and breeds.

The freezing-thawing processes can be carried out by classical cryopreservation methods including progressive decrease in temperature to reach freezing and progressive increase in temperature at thawing. Alternatively, vitrification consists of an extremely rapid freeze-thaw process expected to put the frozen cell in an “amorphous” (i.e. without a clear form or structure) state with very few crystal formations that facilitates the restoration of cellular function at thawing. However, the amorphous state is not stable, and the risk of secondary uncontrolled ice formation limits the universal applicability of this method.

Cryopreservation involves extensive temperature variations, intracellular ice crystal formation, and dramatic shifts in osmolality, all of which may cause thermal, mechanical and chemical stress leading to a loss of function (FAO, 2012). It is, in fact, quite remarkable that many cells can be cryopreserved successfully. The success of germplasm cryopreservation varies considerably among cell types and species, as a function of their biophysical and biochemical properties (Mazur *et al.*, 2008). Consequently, different germplasm types are subjected to specific approaches and protocols to minimize damage and conserve function after thawing.

The cell plasma membrane is very temperature-sensitive and is the primary site of damage. Phase transitions during cooling transform membrane phospholipids from a liquid crystalline phase characterized by high rotational and lateral mobility of lipids to a crystal gel phase in which lipid

mobility is restricted. Phase transitions and membrane protein restructuration may or may not be fully reversible upon thawing, thereby compromising normal functions, such as selective permeability, enzyme activity and cell-cell interactions. Differences in membrane sensitivity to cooling, freezing and rewarming are largely attributable to compositional variations, such as phospholipid fatty acid profile and cholesterol content. A high ratio of unsaturated to saturated fatty acid content in the membrane is associated with cold temperature susceptibility (White, 1993). Cholesterol, which renders biological membranes more fluid at low temperatures (the opposite of its effect at body temperature), decreases their sensitivity to the cold. The cholesterol:phospholipid (C:P) ratios of cell membranes may also reflect cold tolerance, with high C:P ratios conferring resistance to cellular cryo-injury (Mocé *et al.*, 2010). Enhancing cryo-tolerance by incorporating exogenous cholesterol into the membranes has resulted in improved goat sperm survival after thawing (Salmon *et al.*, 2016).

During freezing and thawing, the formation and melting of ice crystals cause osmotic shifts that induce dramatic effluxes and influxes of cellular water (Seime *et al.*, 2016). Upon extracellular ice formation, the solute concentration in the extracellular unfrozen medium increases, causing cells to dehydrate and the cells swell (and possibly burst) during thawing of the ice crystals. The resulting fluctuations in cell volume impose considerable ultrastructural stress to the plasma membrane, thereby compromising its selective permeability and other functions. Membrane permeating cryoprotective agents, such as glycerol or dimethylsulfoxide (DMSO), are usually included in the cryopreservation media to increase osmolality, thereby moderating the osmotic damage and increasing survival despite potential toxic effects. Non-permeating cryoprotectants, such as proteins (albumin), synthetic amide-based components (e.g. polyvinylpyrrolidon) and sugars (e.g. sucrose, raffinose) may stabilize membranes or limit osmotic changes during cryopreservation (Seime *et al.*, 2016).

Semen is by far the most common form of cryopreserved germplasm. Semen cryopreservation typically involves dilution in a protein and lipid-rich medium containing glycerol orother cryoprotecting agents, depending on the species, and quite rapid freezing, storage, and rapid thawing. Although sperm are structurally complex, they tend to be cryo-resistant relative to other materials such as oocytes and embryos due to their low cytosolic volumes and water content. Sensitivity to temperature is species-specific and appears to be a function of plasma membrane composition, C:P ratio, in particular. Successful semen cryopreservation is traditionally assessed based on sperm motility after thawing, although motile sperm may have sublethal damage that prevents fertilization. Moreover, cryopreservation-induced DNA damage to sperm can impair embryo competence, manifested as adverse gestational outcomes or post-natal developmental dysfunction (Bailey *et al.*, 2020). Nonetheless, long term cryopreservation of sperm for some species, such as cattle and goats, has formed the basis for highly successful industries leading to rapid and efficient genetic selection outcomes.

In contrast to sperm, the oocyte is large and voluminous with a high proportion of water. To minimize the formation of highly destructive ice crystals, two basic techniques dominate the field of oocyte cryopreservation: slow freezing and vitrification (Saragusty & Arav, 2011). For slow freezing, oocytes are dehydrated in the presence of cryoprotectants that replace most of the intra-oocyte water to inhibit internal ice crystal formation. Nevertheless, success rates are generally poor with slow freezing techniques. More commonly, oocytes are vitrified, resulting in a solid glass-like cytoplasm, free of ice crystals. Although vitrification results in fewer water crystals than slow cooling, it requires high concentrations of cryoprotectants, often DMSO, which themselves can be toxic to the oocyte.

The primary technique used for embryo cryopreservation is vitrification versus slow controlled freezing. Birds are an exception, since embryo cryopreservation is not feasible because of the egg structure. For several avian species, cryopreservation of the primordial germ cells (PGCs) extracted from the blood of early embryos is possible (Nandi *et al.*, 2016). Vitrification is currently the preferred strategy for long-term conservation of ovarian tissue to preserve ovarian stem cells and testicular tissue containing spermatogonial stem cells.

The controlled rate needed for the slow freezing method often requires programmable equipment to manage the cooling, whereas the tools required for vitrification are simple and relatively inexpensive. Vitrification can be carried out under field conditions. Nevertheless, it requires considerable experience to be carried out successfully (Saragusty & Arav, 2011).

6.3 SEMEN

Sperm was the first form of germplasm to be successfully cryopreserved in mammals, facilitated by the ease of semen collection and its role in accelerating genetic improvement. This methodology is now routinely employed with varying degrees of success in the cattle, swine, and equine industries. Semen cryopreservation was subsequently developed for several domestic bird species and more recently for other animals such as honey bees. In these latter species, semen cryopreservation is not utilized widely for routine breeding, but tends to be employed more often for genetic conservation programs or genomic selection breeding. Semen is the form of germplasm representing the largest number of samples in most national gene banks (Procedures in Annex 6.1). Even though only half of the genome is present in sperm, this predominance is expected to persist in the future in many domestic species because semen collection is a minimally invasive method towards germplasm conservation and use, and in agricultural species where other methods are not available or very difficult to manage.

6.3.1 Collection

In mammals, bull (Austin *et al.*, 1961), ram (Terril, 1940), buck (Leboeuf *et al.*, 2000), rabbit (Naugthon *et al.*, 2003) and stallion semen (Love, 1992) is collected with an artificial vagina, while the gloved hand method is utilized with boars (Awda and Buhr 2008). In some instances, such as when the male is unaccustomed to semen collection, more invasive methods, such as electroejaculation, are warranted. The use of polyurethane condoms inserted into the vagina or vaginal devices, may also provide a means of collecting higher quality semen during copulation, as it permits normal precoital sexual behaviour (Wulster-Radcliffe *et al.*, 2001).

In most poultry species, including chicken, turkeys, common ducks, guinea fowl, gander, emu, and ostrich, ejaculation is usually stimulated by simple dorso-abdominal massage (Burrows and Quinn, 1937). In specific species (quails, some ducks), the presentation of a female is needed. Male birds require training in these non-invasive methods. A high level of trust between the animals and humans that practice the collection is very important, since the welfare of the animal increases the likelihood of their cooperation and secures optimal ejaculate quality.

In honey bees, the current semen collection requires donor sacrifice (or culling).

6.3.1.1 Collection with artificial vagina in mammals

With this method, semen quality is similar to that obtained during natural mating. Each ejaculate is often collected into a separate vagina to ensure samples are not mixed and that the lubricant must be non-spermicidal. The temperature and pressure of the artificial vagina may be adjusted for each male that is regularly collected to optimize performance, to be comfortable with the animals and ensure sperm viability. Furthermore, to avoid injury to the animal, the person collecting the semen should guide the animal's penis via the prepuce into the artificial vagina and never force the vagina over the penis. In small ruminants, semen is collected 2-3 times per week in the presence of an oestrus ewe or doe. During the breeding season, repeated semen collections, two within 5 min, can be acquired. Bulls are typically collected 2-3 times per week, with 2 or 3 ejaculates per collection day, if necessary.

6.3.1.2 Gloved hand technique for boars

The gloved hand method is commonly used to collect semen from boars. Latex gloves have detrimental effects on sperm quality; therefore, polyurethane is recommended. The boar is first allowed multiple attempts to mount a dummy. The protruding penis is then firmly yet gently grasped so that the glans penis ridges are between the collector's fingers. The initial fractions of the ejaculate are usually discarded, after which the sperm-rich portion should be collected into a 37°C insulated container covered with two layers of sterile gauze to filter the gel fraction (Woelders, 1991). The ejaculation on average requires 5 to 6 minutes but may last up to 30 minutes depending on the boar. Although the gloved hand technique works well, commercial systems of automated semen collection are increasingly popular in boar studs.

6.3.1.3 Electroejaculation in mammals

Electroejaculation is frequently used to collect semen outside of the breeding season, or from males not trained to ejaculate into an artificial vagina. In ruminants, semen collected using electroejaculation may show lower sperm concentration than that collected using an artificial vagina due to stimulation of the accessory sex glands resulting in a relative increase in production of seminal plasma. If performed properly, however, the ejaculate will contain the same number of sperm. Electroejaculation is an easy and effective technique, but it can be stressful and painful. With some males it may be necessary to overcome these stresses with analgesics, anaesthetics, or sedatives prior to initiation of the technique (Orihuela *et al.*, 2009a,b, Santiago-Moreno *et al.*, 2011). Proper technique with electroejaculation, which includes sanitation of the sheath and penis, appropriate preparation and use of the probe for stimulation, and correct animal handling ensures the highest quality samples, but these methods vary depending on the species (see Evans and Maxwell, 1987, for ram and buck techniques) and may even vary by male.

The use of electroejaculation to collect boar semen is generally considered to be unsatisfactory and unnecessary, since manual collection works well. Nonetheless, electroejaculation in pigs may be useful in special instances (Fischman et. al, 2003).

The transrectal ultrasonic-guided massage of the accessory sex glands (TUMASG) is an alternative technique to electroejaculation and this requires fewer electrical stimuli or even no pulse, and there is, therefore, potential mitigation of animal welfare concerns (Abril-Sánchez *et al.*, 2019). Ultrasound examination of the accessory sex glands is performed using real-time transrectal ultrasonography to encourage ejaculation, however, if the animal does not ejaculate within approximately 15 minutes, a single electrical stimulus (lasting 5 seconds) may be provided using an electroejaculator with intermittent breaks for TUMASG. The ampulla of the ductus deferens is monitored by ultrasonography to assess when it is empty, thus avoiding unnecessary electrical stimuli. The use of TUMASG usually does not affect ejaculate characteristics compared to electroejaculation.

6.3.1.4 Postmortem epididymal sperm collection in mammals

Epididymal spermatozoa can be extracted from a valuable male for a brief period (within 8 hours in goats and ewes) after death by washing the excised testes in a Krebs-Ringer solution or other physiologic saline and then making cuts with a surgical scalpel in the cauda epididymis. Blood cells may have negative effects on sperm quality, so nicking the vasculature should be avoided. The retrograde flushing method avoids contamination and allows a larger number of spermatozoa to be recovered. The caudae epididymides are cleaned and the vas deferens cannulated with a 22 to 25 gauge butterfly needle fitted with a flexible tube connected to a syringe filled with extender. The flexible tube is pre-filled with medium before any pressure is applied. The medium is then introduced into the cauda epididymis by using manual pressure from the syringe. When the majority is filled, a small, single cut is made in the terminal part of the cauda epididymis. The fluid that emerges from the cut tubules is collected in a Petri dish.

6.3.1.5 Collection with dorso-abdominal massage in poultry

To ensure the best outcomes, semen collection should be practiced on adult males at the peak of the reproductive season. Immature males or males in the decreasing phase of the reproduction cycle should be avoided. The corresponding ages depend on the species, breed, climate, and breeding system. For example, in temperate climates, the optimal age to collect semen for cryopreservation is between 30 and 40 weeks for adult roosters under constant photoperiod, while European ganders must have semen collected, if possible, in the springs of their second and third year. The procedure is best performed with two people and begins with one person gently stroking the dorsal part of the mid-abdomen up to the tail. If the male is well stimulated, it raises its tail and exposes the cloacum containing the two genital papillae (left and right). Following that, the operator may then exert a small pressure on the ventral part of the abdomen to push the semen that is still in the deferent duct to the outside of the genital papillae. Apart from some duck species, the males of most poultry species have no penis, so the semen is collected when it leaves the genital papillae, if possible, with a soft tube connected to a reservoir. The operation is delicate since the semen must not be contaminated by other material (faeces, urates, etc.) or secretions (e.g. transparent fluid) from the cloacum, or by blood.

6.3.1.6 Semen collection in honey bees

Semen collection is currently done by gathering drones that are returning from a mating excursion. While it is possible to obtain drones from inside the hive, targeting those returning from mating flights ensures a greater proportion of mature males. The semen is most commonly collected from the eversion of the endophalis, which is a terminal act for the individual male, as it is during natural mating. The induced ejaculation procedure is done by applying pressure to the thoracic-abdominal segments, causing a partial eversion prior to the full eversion. In mature males, this results in the extrusion of sub-microliter amounts of off-white coloured semen floating on the bright white accessory gland secretions (mucus).

The eversion method requires care to maintain sanitary conditions. Drones tend to defecate during the eversion process and bacterial contamination of the semen can dramatically reduce sperm viability and cryosurvival, and also cause problems for the inseminated queen. Detailed instructions and equipment can be found in the review article by Cobey *et al.*, (2013). The equipment used to collect and to inseminate honey bees consists of a threaded syringe connected to a pulled glass capillary tip. A saline solution containing antibiotics is usually introduced to the sample during the collection process at a ratio of about 1:10 (1 part saline to 10 parts semen) (Hopkins *et al.*, 2017). The introduction of antibiotics is essential for the stability of semen and success of the insemination to follow.

6.3.2 Treatment and cryopreservation

Sperm from different animal species show highly variable resistance to storage *in vitro*. For example, whereas it is possible to store most mammalian semen outside the body at room temperature for hours without specific treatment before cryopreservation, this is impossible in poultry. The fertilizing ability of avian sperm is lost within the first 20 to 45 minutes (depending on the species) after semen collection if not treated properly.

The ability of sperm to keep their fertilizing ability after cryopreservation also depends on the species. An overarching factor for all species is that the longer the sperm remains in the female tract during natural mating, the greater the requirement for sperm integrity during cryopreservation (for birds, see Blesbois 2011, 2018). Species for which sperm must be stored for weeks to months in the female reproductive tract after insemination include birds with sperm storage tubules and honey bees with spermatheca, thus present greater challenges for semen cryopreservation.

The procedures for semen cryopreservation are therefore outlined according to species in Annex 6.1. Regardless of species, all procedures follow the general principles of cell cryopreservation involving extending sperm in protective diluents before freezing, usually containing internal and external cryoprotective agents (CPAs). The freezing and thawing curves are adapted to the species and to the availability of a specific programmable freezer in the field.

6.3.2.1 In mammals

In many species (bulls, rams, bucks, and boars) semen can be collected at remote locations and frozen on-site or diluted with either a shipping diluent or cryopreservation diluent, cooled and transported to a laboratory for freezing. This enables consistent processing and cryopreservation of samples and more uniform post-thaw results. The critical component to transporting samples in this manner is to ensure that the semen samples are handled appropriately using methodologies that are optimized for the species of interest (e.g. Purdy *et al.*, 2010a,b, see Annex 6.1 for additional details). Moreover, it is critical to ensure when cryopreserving samples that the appropriate medium and method are utilized for a species to likewise ensure the best possible post-thaw quality and fertility.

BOX 6.1

A specific interaction between seminal secretions and freezing diluent components in the goat

The removal of seminal plasma is an important consideration in goat sperm cryopreservation because the enzymes from the bulbourethral gland interfere with certain additives in extenders (e.g. egg yolk) and can hydrolyze the sperm membrane phospholipids, reducing or eliminating the chances of efficient preservation. This is not required for all bucks since, due to genetic differences, some of them do not produce those enzymes. Seminal plasma may be removed by diluting with a solution such as Krebs-Ringer phosphate glucose followed by centrifugation. The supernatant is then discarded, and the pellet of sperm resuspended in an extender. The seminal plasma removal by this classical method preserves acrosome integrity and sperm motility after freezing-thawing. Density gradient centrifugation (Santiago-Moreno et al., 2017), single layer centrifugation (Jiménez-Rabadán et al., 2012), dextran/swim-up (García-López et al., 1996) and semen filtration with Sephadex (Galarza et al., 2018) are all suitable methods to isolate spermatozoa in small ruminants and to remove unwanted components such as abnormal, moribund and dead sperm, leukocytes, epithelial cells and debris.

Glycerol is the main penetrating cryoprotectant agent used for freezing mammalian sperm. Egg yolk and milk-based extenders have been classically used to protect sperm from the detrimental effects of cooling and freezing. There are concerns, however, that pathogens can be introduced through egg yolk. Soy lecithin-based extenders may be an alternative (Layek *et al.*, 2016), however, the fertility outcomes may be not be equivalent.

The high concentration of polyunsaturated fatty acids in sperm membranes render them particularly vulnerable to oxidative damage associated with the freeze-thaw process. Antioxidants may counteract the detrimental effect of reactive oxygen species and improve the quality of frozen–thawed sperm. Among them, reduced glutathione, glutathione peroxidase, superoxide dismutase and catalase, protect against the formation of lipid peroxidation in frozen–thawed sperm (Câmara*et al.*, 2011). Resveratrol, vitamin E, L-carnitine have also been shown to help maintain sperm motility, viability and to reduce DNA fragmentation (Bahmyari *et al.*, 2020). Increases in fertility attributed to the use of these antioxidants, however, are inconsistent.

6.3.2.2 In birds

Successful semen cryopreservation protocols are available for some poultry species. Although glycerol seems to be the best cryoprotectant for poultry sperm, it must be removed at thawing to avoid a specific contraceptive effect.

In the chicken, a glycerol-based method of sperm cryopreservation gives the highest success after cryopreservation when applied to breeds showing variable levels of fertility capacity (Annex 6.1). This procedure involves the removal of glycerol by serial dilutions, and centrifugation at thawing. To simplify the semen freeze-thaw process, many experiments have used other CPAs, mainly DMSO, Methyl Acetamide, Dimethyl Acetamide, or Dimethyl formamide (DMF). These other cryoprotectants have yielded satisfactory results when applied to roosters with high fertility (see Annex 6.1). They have been less efficient, however, for roosters showing variable levels of fertility (Thelie *et al.*, 2019). The semen freezing conditions for poultry are still debated, but plastic straws have been adopted by most cryobanks, due to their ease of use and for identifying straws compared to the pellet method. Straws are usually employed with slow and moderate freezing rates, and pellets with high freezing rates (Tselutin *et al.*, 1999).

In the guinea fowl, a method using DMF and packaging in straws was standardized (Seigneurin *et al.*, 2013, Annex 6.1), however, freezing as pellet is also encouraging (Varadi *et al.*, 2013). In geese, the use of straws or cryovials were both reported to be efficient (Tai *et al.*, 2001; Lukaszewicz 2002, Varadi *et al.*, 2019).

The cryopreservation of turkey semen has been a historical challenge that is still not fully resolved. The difficulty to obtain standardized semen cryopreservation protocols for toms is certainly related to the very long storage of sperm in the female tract (up to 3 months). Promising results, however, have been recently obtained in turkeys using DMSO as membrane permeable CPA and Ficoll as non-permeant CPA (Ioro *et al.*, 2020).

6.3.2.3 In honey bees

A semen cryopreservation protocol for bees was first published by Harbo (1979), who recommended the use of DMSO as a cryoprotectant. Initially the cryopreservation mixture was 60 percent semen,

10 percent DMSO and 30 percent saline (0.85 percent sodium chloride solution). Since then, various methodologies have been tested and used with modifications to the diluent composition and additives such as 10 percent egg yolk (Hopkins *et al.*, 2012). Promising results using dialysis to introduce DMSO prior to freezing and subsequently remove DMSO after semen thawing have been reported (Wegener *et al.*, 2014). Regardless of the diluent, semen cryopreservation has always been performed using either glass capillaries (Harbo, 1979; Hopkins *et al.*, 2017) or straws (e.g. Cassou straws: Rajamohan *et al.*, 2019). While the small volumes of semen produced from honey bees seem to be well suited to vitrification, to-date, all successfully reported cryopreservation techniques utilize slow/programmable freezing rates at about 3°C/minute.

6.3.3 Thawing and insemination

Thawing semen is relatively simple, with differences in thaw rates depending on the previous freezing rates, the size of straw or device (e.g. cryotube) used, and the penetrating cryoprotectant. Thawing may be followed by the removal of the cryoprotectants to better restore the sperm fertilizing capacity but that, like the insemination method with cryopreserved sperm, depends greatly on the species.

6.3.3.1 In mammals

In mammals, the method of semen thawing varies according to the method of freezing, the cryopreservation medium, and the cryoprotectant. Sealed semen straws are usually plunged directly in a water bath at 37˚C for 30 seconds. However, different thawing rates are applied to ram semen frozen by directly plunging semen drops on two different cold supports: block of dry ice to form small pellets then submerged into liquid nitrogen, or directly into liquid nitrogen to form spherical pellets). The thawing intensity differs between the two pelleting methods: thawing in dry glass tubes placed in a 37°C water bath for 2 minutes for the first, ultra-rapid thawing rate by using, for example, a 60°C hotplate (2–5 seconds) for the second.

Trans-cervical artificial insemination using cryopreserved sperm is commonly used in cattle, pigs, horses, sheep and goats. With cattle, pigs, and horses, the insemination pipette easily passes into (pigs) or through the cervix (cattle and horses). In the sheep, the ewe cervix is a significant barrier to artificial insemination, therefore, laparoscopic intrauterine insemination is often utilized to bypass the anatomical challenges (Eppleston and Maxwell 1995). The percentage of motile spermatozoa and the quality of motility should be evaluated prior to insemination to ensure that each ewe receives 20-25 x 10^6 motile spermatozoa (one straw, half the dose) in each uterine horn. The dose of frozen sperm for laparoscopic insemination is lower than required via either the vagina (400 million live spermatozoa) or trans-cervically (100–200 million live spermatozoa). Using frozen semen deposited intra-uterine via laparoscopy has yielded high pregnancy rates (60–80 percent), as have vaginal and trans-cervical methods (Purdy *et al.*, 2020). Success seems to depend on the interaction of many factors (including ewe breed, ewe age, estrous synchronization treatment, number of sperm inseminated, and sperm quality (Donovan *et al.*, 2001; Olafsson, 1980; Paulenz *et al.*, 2007; Purdy *et al.*, 2020), thus increasing confidence in non-surgical artificial insemination as a viable option for sheep.

6.3.3.2 In birds

The method of semen thawing differs primarily according to the cryoprotectant used. Although the standard removal of glycerol at thawing is made by serial dilutions and centrifugation, specific density gradients have also been assessed (Long and Kulkarni, 2004; Purdy *et al.*, 2009). Up to now, there has been no simple strategy. The other cryoprotectants are usually not removed from semen prior to insemination.

The frozen-thawed semen is inseminated intravaginally (3-4 cm deep) in receptive females by everting the cloaca via gentle massage to expose the vaginal opening. Insemination must take place a minimum of 3 hours before or after laying to minimize semen expulsion due to the lay peristalsis. The duration of time between semen thawing and insemination should be quite short (30-45 min) to optimize sperm quality. Artificial insemination may alternatively be intra-uterine (IU), intramagnal (IM) or intraperitoneal (IP). The advantage of IU, IM and IP insemination is that the contraceptive effect of glycerol disappears. The downside is that these methods are invasive and bypass the biological filter of abnormal sperm, therefore problems of lay may arise, thereby limiting the use of these techniques (Long and Kulkarni, 2004).

6.3.3.3 In honey bees

In general, bee semen is cryopreserved in glass capillaries or in 0.25 ml plastic semen straws. The straws are thawed in warm water at 37 to 40°C, while glass capillaries are thawed by hand or warm air to avoid fracturing the glass (e.g. Hopkins *et al.*, 2012 using a hair dryer). Following thawing, and depending on the diluent used, the semen may be used immediately for insemination or reconstituted in DMSO-free diluent and stored, or transported (Rajamohan *et al.*, 2019). The use of 0.25 ml cryostraws for cryopreservation of bee semen works seamlessly with common instrumental insemination methods. The tubing that connects the threaded syringe to the pulled capillary tube used for semen collection allows for the straw to connect directly into the system and perform the insemination with modifications to the device.

6.3.4 Quality evaluation

Evaluating the semen of the donor before storage in the cryobank is important (Annex 6.2). To predict the quantity of samples available from an ejaculate and thus the number of ejaculates needed for cryopreservation. When possible, reevaluation of the semen samples prior to insemination will help to select the best samples to be used, since high variability may exist among ejaculates.

All the species in the scope of this guideline undergo internal fertilization. This means that their sperm contain an acrosome rich in enzymes (to penetrate the oocyte), a highly condensed nucleus (with the genetic material to be transferred to progeny), mitochondria (to maintain high metabolic activities and motility), centrioles and a flagellum to ensure the movement of the sperm through the female tract. In all species, basic semen quality analysis procedures are available (Annex 6.2). These include counting the sperm density, and different quality tests, mainly motility/mobility and viability/membrane integrity criteria. When possible, an evaluation of the integrity of the acrosomes and of the DNA is warranted.

6.3.5 Ethical issues

The ethical issues associated with semen collection and insemination depend on the species, the procedures used, and the views of the citizens of the country in which these activities are practiced.

6.3.5.1 Mammalian species

Collection of ejaculated semen and vaginal/trans-cervical insemination are not particularly invasive. In contrast, electroejaculation may have associated welfare concerns, as may laparoscopic insemination. Sedation may alleviate some issues related to animal well-being, although ethical considerations remain.

BOX 6.2
New tools for semen quality evaluation

The "omics" revolution of the last decade has led to the emergence of promising preliminary tools or evaluating the intrinsic capacity of perm to fertilize. One approach is the proteomic analysis of semen, either the seminal plasma and/or the sperm fraction (Labas et al., 2015; Soler et al., 2016; Druart & De Graaf, 2018). The intact cell matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (ICM-MS) analysis of sperm produces profiles of proteins and peptides in the range of 1000 to 30000 Daltons. This range is pertinent since it permits the observation of major native proteins and peptides that are key actors in reproduction, as well as peptides obtained from protein degradation. The sperm ICM-MS profile produces an "ID card" (Figure 6.1) specific to each male. This ID card has been shown to represent the intrinsic fertilizing capacity in the chicken and is now under development in other farm species.

In the chicken, this ID card has been shown to represent the intrinsic male capacity much better than any other semen quality test and is now under development in other farm species.

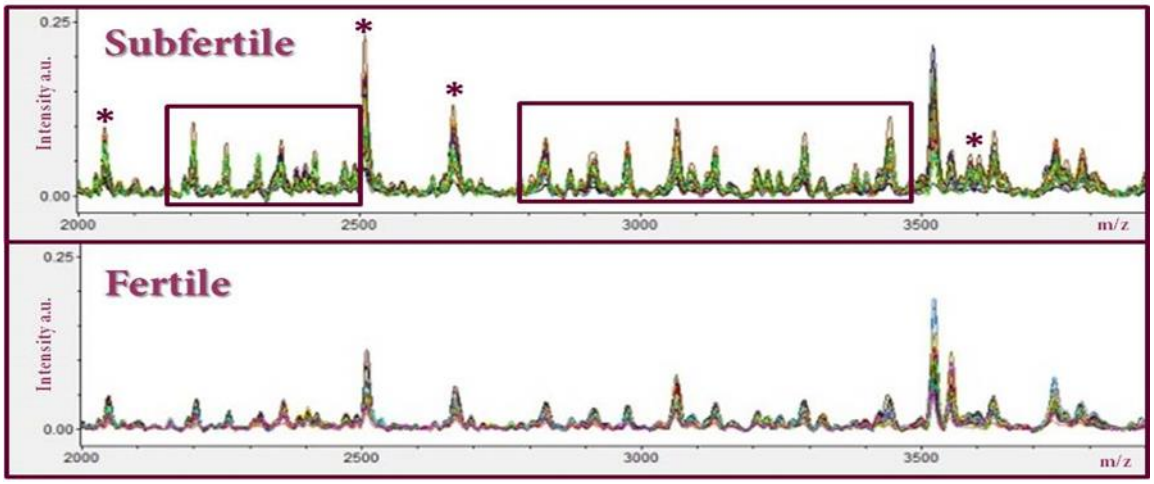

FIGURE 6.1
Difference in ICM-MS profiles between subfertile and highly fertile chicken males (Soler *et al.*, 2016)

The upper profile is from a subfertile male (40 percent fertility), the bottom profile is from a highly fertile male (90 percent fertility). The number of peaks is lower in the semen of the fertile than the subfertile male indicating a higher diversity of peptides of degradation in the semen of the subfertile male. The "*" indicates the peaks that are higher in the subfertile males. The rectangles show that the subfertile animals present a higher number of peaks than the highly fertile males.

Another new approach is the study of the RNA population present in sperm. Mature spermatozoa contains noncoding RNA, some of which are involved in the regulation of many physiological pathways, including the control of sperm motility. Deep sequencing of sperm microRNA (miRNA) has revealed differences between high and low motility sperm populations separated by use of a Percoll gradient (Capra *et al.*, 2017; Capra *et al.*, 2019). Researchers now hypothesize that bulls with moderate to high fertility (or low fertility) can be identified by differences in semen miRNA expression.

6.3.5.2 Poultry species
The methods of semen collection and artificial insemination are noninvasive, provided that insemination is intravaginal, one of the principal reasons for prioritizing this method.

6.3.5.3 Honey bees
The semen extraction process in honey bee drones is sacrificial. However, the males also perish naturally during copulation with a queen. Up to 200 drones are sacrificed to obtain 40-60 µl of semen. The technique does not disrupt the whole hive(s) and has no known consequence on the population of bees. Instrumental insemination is invasive and can only be performed in narcotized virgin queen bees. The loss of queens due to instrumental insemination is usually dependent on the expertise of the human technician. Therefore, when done properly the processes of semen collection and insemination in honey bees may be considered to have little to no ethical concerns.

6.4 EMBRYOS AND OOCYTES

Embryo cryopreservation allows the storage of the whole genome of a diploid animal and the efficient diffusion of genotypes of interest, as well as the rapid reconstitution of a herd or breed. It has been successfully developed in many mammalian farm species. In contrast, embryo cryopreservation is much more difficult for oviparian species due to species-specific egg structures and vitellus storage. However, it is now experimentally possible in honey bee for example. Since the early embryo is primarily developed from the fertilization of the oocyte, new developments in oocyte cryopreservation are also under experimentation in mammals –paving the way for conserving the female genome in cryobanks.

6.4.1 Potential use of mammalian embryos
Since the birth of normal offspring from cryoconserved mouse embryos was first reported in 1972 (Whittingham *et al.*, 1972), similar successes have been achieved in at least 33 mammalian species (see table and refences in Annex 6.3), including for major livestock species (see procedures in Annex 6.3). In those species for which collection and transfer techniques are available and operational, embryo banking is a very good option for conserving genetic diversity and offers the fastest way to restore an original breeding population, including both nuclear and mitochondrial genetic information. Embryo technology, however, is usually more costly and requires greater technical capacity than gene banking with semen. In many species, it is also much more invasive than the semen methodology.

While the birth of live offspring from frozen–thawed embryos has been reported in most common livestock species, it is technically difficult, and the expected success rates vary from species to species. The greatest success has been achieved in cattle, a species in which cryopreservation of embryos has become routine. Both slow-freezing and vitrification protocols are effective (van Wagtendonk-de Leeuw *et al.*, 1997; Arav, 2014). The success of cryopreservation is dependent on the stage of the embryo, with blastocyst yielding the best outcomes in most species.

Due to high commercial value of dairy genetics, bovine embryos cryopreservation has been the target of much research in the last decades, leading to highly efficient standard procedures with around 60 percent of pregnancies to term after transfer of frozen thawed embryos to recipients (Ferré *et al.* 2020), even after direct transfer of the content of the thawed straws (Dochi 2019). Procedures for cryopreservation of buffalo embryos have largely been developed by adapting techniques used in cattle, but fewer resources have been spent on development and refinement and as a result success

rates are generally much lower. The potential for cryopreserving sheep and goat embryos is similar to that in cattle (e.g. Fogarty *et al.*, 2000; Rodriguez Dorta *et al.*, 2007). Cryopreservation of horse embryos is less efficient (Ulrich and Nowshari, 2002). However, the emergence of vitrification techniques are encouraging (Moussa *et al.* 2005; Hinrichs 2010; Squires, 2016). Of all the major livestock species, cryopreservation of pig embryos has long been the most problematic, because pig embryos are extremely sensitive to chilling and have high lipid content. Ultrarapid vitrification in thin plastic straws (open pulled straws) provided efficient results for pig embryos at the morula or blastocyst stages (Berthelot *et al.* 2000; 2001), even after non-surgical transfer (Cuello *et al.* 2005).

Also, species differ with respect to the difficulty of collecting and transferring embryos and whether surgical or non-surgical collection procedures are preferred. Table 6.1 compares surgical and non-surgical collection for major livestock species. Table 6.2 compares transfer procedures. The information presented in the two tables should be considered when planning a gene banking program, both in choosing the type of germplasm to store and in deciding upon the quantity needed. In addition to the five major mammalian species included in the tables, other livestock species for which live offspring have been obtained from cryopreserved embryos include the dromedary (Nowshari *et al.*, 2005) and rabbit (Naik *et al.*, 2005). Pregnancies have been reported in the llama (Aller *et al.*, 2002) and red deer (Soler *et al.*, 2007) (Locatelli *et al.* 2005), as well as many other domestic, model and wild species.

TABLE 6.1

Non-surgical versus surgical embryo collection in livestock species

Embryo collection type	**Cattle**	**Sheep**	**Goats**	**Pigs**	**Horses**
Non-surgical					
Difficulty[a]	1	5	3	3	1
Percent of treated females with ≥1 embryo per collection	85	>50	>70	<35	80
Transferable embryos percollection (n)	4-8	0-8	3-8	0-5	≤1
Collections per year (n)[b]	3-6	3-6	3-6	2-4	4-6
Recommended for use?	Yes	No	Yes	No	Yes
Surgical					
Difficulty[a]	5	2	2	1	4
Percent of treated females with ≥1 embryo per collection	85	>70	>70	95	<80
Transferable embryos per collection (n)	4-8	3-8	3-8	10-25	≤1
Collections per year (n)[b]	3	1-2	1-2	2	3
Post-surgical adhesions	+++	++++	++++	++++	+
Recommended for use?	No	Yes	No	Yes	No[c]

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

[a] Difficulty of the procedure, with 1 being the easiest and 5 being the most difficult to perform.

[b] Post-surgical adhesions generally dictate the number of surgical collections per female during her lifetime. The number of surgeries per female may be designated by governmental regulations and/or an institutional review board.

[c] Frozen–thawed equine embryos >300 μm in diameter rarely produce a pregnancy following transfer.

TABLE 6.2
Non-surgical versus surgical embryo transfer in livestock species

Transfer type	Cattle	Sheep	Goats	Pigs	Horses
Non-surgical					
Success rate (%)[a]	50–80	10-15	10-35	5-10	55-80
Success rate frozen (%)	50-65	<10	<10	<10	10-20
Difficulty[b]	1	5	3	3	1
Recommended for use?	Yes	No	No	No	Yes
Surgical					
Success rate (%)[a]	55-80	50-65	50-65	60-85	60-80
Success rate frozen (%)	50-65	40-65	40-65	25-60	10-20
Difficulty[b]	3	3	3	2	2
Recommended for use?	No	Yes	Yes	Yes	No

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

Production, collection, processing and freezing of embryos are usually more demanding than the equivalent procedures for semen, and a greater level of training and experience is required. The following subsections address major issues in the cryoconservation of embryos. FAO has previously produced manuals on ET in several species including cattle (FAO, 1991a), buffalo (FAO, 1991b) and sheep and goats (FAO, 1993). In addition, commercial manuals are available for purchase.

6.4.2 Embryo collection in mammals

6.4.2.1 Recommendations before starting embryo collection

To maximize efficiency, the collection, processing and storage of embryos should be carried out by trained professionals. Many countries have specific regulations on who can perform embryo collection. Technicians will need to undergo special training in sanitation and specific techniques (see Section 7).

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

The embryo-collection team (usually two or three technical people) needs to have access either to well-maintained, clean and sanitary permanent facilities, or to a mobile laboratory, where embryos can be collected, evaluated, processed and packed. The processing laboratory needs to be clean and equipped with an appropriate working space, electricity, temperature-controlled incubator(s), microscope(s) and other technical equipment and supplies. Small equipment must be sterilized

between collections and single-use disposable materials are recommended for sanitation purposes, when possible.

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

The potential health risk can be large if the recommended procedures regarding collection and handling are not followed precisely. It is important to review the International Embryo Technology Society (IETS) recommendations for the sanitary handling of *in vivo*-produced embryos before beginning embryo collection (see Stringfellow, 1998) as well as for the serial washing of the recovered embryos (ten successive washings including two trypsin treatments). This treatment removes potential pathogens from the embryos (Pellerin et al. 2018) and samples of the washing media should be kept for pathogen screening.

Abundant results from worldwide research on the risks of disease transmission via embryos are available for cattle. Recently, the IETS published a chapter regarding these risks in small ruminants (Souza-Fabjan and Menchaca, 2020). Less information is available for pigs and is almost non-existent for other species. Any embryo collection should be preceded by an extensive clinical examination of the donor animal for the presence of diseases. Its herd or flock mates should also be checked, as should the general environment in which the animals are kept. The clinical examination may eliminate a potential donor from consideration or indicate that a treatment needs to be applied. The results of the examination may also influence the precise protocol applied for superovulation and recovery, as good outcomes can only be expected from perfectly healthy animals.

6.4.2.2 Superovulation of donors

Ideally, to maximize the success in the reconstitution of a given breed, each female embryo donor will produce at least one male and one female offspring. To reach this goal, each female will obviously need to produce multiple embryos. To increase the number of embryos per collection, donor females are administered various gonadotropin-like hormonal agents to stimulate the ovaries to produce multiple ova for fertilization and embryos for collection. Early reports in cattle, sheep, goats and pigs described the use of pregnant mare serum gonadotropin (often abbreviated PMSG) at various dose levels to superovulate donor females (Elsden *et al.*, 1978; Saumande *et al.* 1978). This agent (now termed equine chorionic gonadotropin or eCG) is still the agent of choice in pigs. However, eCG has a long half-life, and often over-stimulates the ovaries of donor cattle. Therefore, it is no longer the agent of choice for cattle in North America, although it is still used in countries where other gonadotropic agents are not commercially available. In small ruminants, it is still widely used, whereas it may be associated with the premature regression of corpora lutea, a common phenomenon in superovulated does and ewes (Pintado *et al.*, 1998). The production of eCG, however, raises ethical questions of animal welfare and replacement solutions should be preferred.

Today, follicle stimulating hormone (FSH) has become the preferred agent for superovulating donor cattle and small ruminants. FSH has a much shorter half-life in circulation and is, therefore, usually administered by twice-daily injections for three to five days (see Monniaux *et al.*, 1983; Armstrong,

1993; Mapletoft *et al.*, 2002; Figueira *et al.*, 2020). However, success using once-daily injections in cattle has also been reported (e.g. Looney *et al.*, 1981; Bo *et al.*, 1994). *Bos indicus* cattle appear to be more sensitive than *Bos taurus* cattle to FSH. Various modifications to techniques for superovulating *Bos indicus* cattle have been developed and are now in use (see Baruselli *et al.*, 2006, 2008; Bo *et al.*, 2008; Bo and Mapletoft, 2014).

Information on various superovulation procedures for cattle and buffalo is presented in FAO training manuals (FAO, 1991a,b). Some of the more commonly used superovulation schemes for cattle donors today are presented in Annex 6.5 of these guidelines. Current recommendations for optimum fertilization and successful embryo transfer (ET) are that one or two inseminations per donor cow with one or two units of good-quality semen per insemination are needed (see Schiewe *et al.*, 1987).

It is important to select the appropriate number of embryo-donor females to match each sire in the breeding schedule and to optimize rates of genetic variability in the cryoconservation program (e.g. Woolliams, 1989). Table 6.1 presents the number of transferable embryos that can be obtained after a single superovulation treatment and embryo recovery, as well as estimates the number of embryos obtainable from one donor female for one year. The figures vary considerably from animal to animal, because some females simply do not respond well to stimulatory agents. In addition, they may develop physiological conditions that make it difficult to retrieve the embryos. Thus, although 25 donor females and 25 donor males are the recommended minimum, a larger number of candidate females may be needed, because of the likely failure to obtain embryos from some donors.

The expected rates of success in both collection and transfer must be considered when determining the number of embryos to collect and store. Experienced embryo transfer professionals can achieve cattle embryo recovery rates greater than 75 percent, with four to eight good-quality bovine embryos per donor per collection. Using good-quality embryos for transfer, 65 to 80 percent pregnancy rates can now be expected in well-managed cattle operations. Expected pregnancy rates from embryo transfer in a variety of livestock species are presented in Table 6.2.

6.4.2.3 Stage of embryo development

Embryos develop through various morphological stages after *in vivo* fertilization. As the embryos divide, the number of cells (blastomeres) per embryo increases as they migrate through the reproductive tract of the female. It is important to know when the embryos can be expected to be in the uterus of the superovulated female, so that the embryos can be obtained from the uterine horns through non-surgical recovery. In bovine, the embryos reach the uterine cavity at the late morula, early blastocyst stage at Day 5 to 7 post ovulation.

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

6.4.2.4 Non-surgical embryo collection

Livestock embryos are collected from donor females by flushing the reproductive tract using a physiological flushing medium. The most often-used flushing medium for cattle is phosphate buffered saline (PBS), which can be obtained by mixing commercially available dry packets with water or purchased as a ready-prepared solution. Various other media are also commercially available. In some

species (e.g. cattle, horses and buffaloes) harvesting donor embryos is most often done using a non-surgical standing method, but in other species (e.g. pigs) a surgical approach is usually required (see review by Betteridge, 1977). In goats, with little training, it is possible to efficiently collect embryos by the non-surgical route and, thus, most of the commercial embryo recovery in some countries is non-surgically performed in goats (e.g. Brazil - Fonseca *et al.*, 2013; Fonseca *et al.*, 2019).

In the field, commercial embryo transfer companies collect cattle embryos using a simple, non-invasive non-surgical procedure. Non-surgical embryo collection and transfer pose minor risk to the cow, and reduce the time needed for harvesting embryos. The drawback of non-surgical embryo collection is that embryo recovery rates may be a little lower if collection is done by a less-experienced technician. Flushing of both uterine horns in cattle usually recovers 50 to 90 percent of available ova/embryos, depending on the experience of the technician. The potential number of embryos available for collection can be determined through palpation or ultrasonic examination of the number of corpora lutea present on the ovaries of the donor animal. However, rectal palpation of donors with a large number of ovulations yields a rather imprecise estimate. It is therefore recommended that, if possible, ultrasonography be used to evaluate the ovaries of the donor prior to the embryo collection procedure.

For flushing, a 3-way-catheter (in, out, and balloon inflation ways) is passed through the cervix into the uterine horn. A balloon on the catheter is then inflated to avoid leaking, and 750 ml of flushing medium is allowed to flow into the horn. Manual manipulation is then used to recover the embryos and the medium from the horn. When one horn has been flushed, the cuff is deflated, and the catheter is removed. It is then placed into the contralateral horn and the same flushing procedure is repeated. This approach uses about 1 500 ml of medium per donor animal.

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

Successful production of live offspring following embryo transfer in horses was first reported in the early 1970s in the United Kingdom and Japan. The non-surgical embryo collection and transfer procedures used today in the mare are easier to perform than those used in the cow. The basic non-surgical collection and transfer procedures used in the mare were reported by in the United States of America (Imel *et al.*, 1981). Several modifications have subsequently been introduced to improve the procedure (see Wilcher and Allen, 2004; Squires, 2019). The latter-stage horse embryo is large enough to be seen even without a microscope.

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

Superovulation is not efficient in mares and consequently not used. Usually, less than one embryo is produced from a donor mare per cycle for potential embryo collection. To obtain early-stage embryos (single ovulated, <300 μm in diameter) for cryopreservation would require more than 130 mare cycles to harvest 100 embryos.

6.4.2.5 Surgical embryo collection

Today, surgical embryo collection in pigs and sheep is usually performed at commercial embryo transfer units. Likewise, goats are still subjected to surgical embryo collection in most countries. Information on surgical procedures available for sheep and goats is provided in FAO (1993). In addition, see Kraemer (1989) or Menchaca and Hunton (2020). Over the years, research reports have described various non-surgical approaches to embryo collection and transfer in these species (see reviews by Candappa and Bartlewski, 2011; Fonseca *et al.*, 2019). Recent studies demonstrated that the recovery rate and the overall efficiency of non-surgical embryo collection is high and similar to surgical collection, with the advantage of inducing less stress to animals (Santos et al., 2020). Recent reports indicate that non-surgically derived embryos lead to acceptable pregnancy rates in goats (Fonseca et al., 2018) and sheep (Figueira et al., 2020), similar to those achieved using the standard surgical approaches.

Although the embryo collection in sheep may be laparoscopically (i.e., puncture with trocars and abdominal visualization by an endoscope in the female abdominal cavity inflated with air) conducted, it is routinely performed worldwide by laparotomy (i.e., paramedian incision of the white line followed by exteriorization of the uterine horns in females placed in dorsal recumbency). A detailed procedure was described by Menchaca and Hunton (2020). These authors highlight embryo collection may be performed 5.5 to 6.5 days after breeding/insemination and it is often performed earlier in sheep than in goats, due to their slightly faster embryo development. After recovery, embryos can then be identified, processed, and cryopreserved. Embryo collection in pigs is also usually done surgically. Given that pigs naturally produce multiple offspring, superovulation is usually not practiced, as embryo viability may be reduced. The collection is performed five to eight days after insemination, via laparotomy at a mid-ventral position. The animal is maintained under general anaesthesia while the procedure is performed. At this point, embryos will be in the blastocyst stage. Collection can be done earlier, at the four- to eight-cell stage, but in this case the embryos will require further culture before transfer and additional manipulation (e.g. delipidation).

6.4.3 Conventional mammalian embryo freezing

Embryos are usually frozen when they are at the morula or blastocyst stage, which is reached by five to nine days after fertilization, depending on the species. After collection, embryos are placed into a hypertonic solution containing cryoprotective permeating agent, such as glycerol or ethylene glycol and possibly some non-permeating ones, such as sucrose (see Leibo, 1992). These agents contribute to dehydrating the embryo before and during the cooling process, to avoid the formation of intracellular ice crystals during freezing or thawing. Today, most livestock embryos are frozen in sealed 0.25 mL plastic straws, similar to those used for freezing bull semen.

Because embryos are a collection of many interacting and voluminous individual cells, the freezing protocols for embryos are generally more sophisticated. For more details on the procedures for cryopreserving livestock embryos, see Annex 6.3. Cellular properties, such as cytoskeleton features or lipid droplets abundance, vary between species and between the stages of embryonic development. Thus, to minimize damages to the embryo and optimize survival rates, it is important to ensure that the cryopreservation procedure accounts for the particular characteristics of the targeted species (Rall *et al.*, 2000) (see Section 3 for basic principles of cryopreservation). The most often-used embryo freezing method is slow freezing, which is based upon reversible dehydration of the cells that prevents the most damaging effects of intracellular ice crystallization. Most technicians who use the slow-

freezing technique use an automated embryo-freezing machine that can be adapted to work under field conditions.

After the embryo and cryoprotectant are placed in the plastic straw and cooled down rapidly (1 °C per min) to negative temperature (-5 to -7 °C), a critical step in the freezing process is “seeding”, which is the act of purposefully inducing ice-crystal formation in the cryoprotectant solution surrounding the embryo, avoiding surfusion maintaining embryos in a liquid environment at negative temperature. After embryos are cooled to approximately -35 °C, the straws are plunged into liquid nitrogen for storage at –196 ºC. A summary of the methods and their applications is presented in Leibo (1992) and Rall (1992).

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

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

The high lipid content of porcine embryos makes them very sensitive to traditional slow freezing. However, *in vivo* produced porcine embryos at the expanded or hatched blastocyst stage can survive slow freezing procedures to some extent even without lipid removal (Fujino *et al.*, 2007). Field reports indicate that *Bos indicus* cattle embryos do not survive the freezing process as well as *Bos taurus* embryos (see Ballard *et al.*, 2007; Looney *et al.*, 2008), while *Bos taurus* embryos may have higher lipid content (Visintin *et al.* 2002).

6.4.4 Vitrification of mammalian embryos

As explained in Sections 3 and 6.2, vitrification is a process that uses the rapid increase in the viscosity of high viscosity solutions during rapid cooling to obtain a glassy solid phase without ice crystals formation, both inside and outside the cells (see Rall and Fahy, 1985; Rall, 1992). Vitrification involves the use of a high-concentration mixture of cryoprotective agents. Embryos placed briefly into increasing concentrations of vitrification solutions are then plunged directly into liquid nitrogen, saving valuable time, and eliminating the need to purchase an embryo freezing machine (Vajta & Kuwayama, 2006; Vajta & Nagy, 2006). For further details on vitrification procedures, see Vajta *et al.* (2005) and Vajta & Kuwayama (2006). Although vitrification is a quick procedure and does not require special equipment, it can be technically more demanding and typically yields pregnancy rates that are 10 to 15 percent lower than those obtained using slow freezing until the technicians have gained enough experience to master the technique.

Success rates with vitrification in cattle (Seidel and Walker, 2006) and small ruminants (Guignot *et al.* 2006; Baril *et al.* 2001) are now approaching the rates achieved with conventional embryo freezing. Commercial kits for vitrification of livestock embryos are now available. As vitrification methodologies improve, there are some indications that the approach may have some advantages over standard slow-freezing procedures in cattle (Vajta *et al.*, 1997; Visintin *et al.*, 2002), mainly for in vitro-derived embryos. The future for vitrification technology appears promising, especially for embryos that have lower viability following conventional cryopreservation, such as pig embryos and those produced via in vitro fertilization. Porcine embryos can be efficiently cryopreserved by

vitrification at the blastocyst and morula stages (Cuello *et al.*, 2004; Maehara *et al.*, 2012) and also at the pronuclear (zygote) stage (Somfai *et al.*, 2009). Several different devices have been tested for the vitrification of in vitro produced porcine blastocysts and were determined to be equally effective even without lipid removal or blastocoel collapse (Bartolac *et al.*, 2015). To prevent cross-contamination of samples in liquid nitrogen, closed vitrification systems and defined media have been applied with good results (Misumi *et al.*, 2013; Mito *et al.*, 2015). It is important to highlight that, in goats, in vivo-derived embryos vitrified at stage of morula frequently have not resulted in pregnancy, compared to the good rates obtained from vitrified-warmed blastocysts (Gibbons *et al.*, 2011). In sheep, vitrification by the Cryotop method (ultra-rapid vitrification of minimum volume microdrops on a cold surface) led to higher embryo survival and number of lambs born compared to the slow freezing, both with in in vivo and in vitro-derived embryos (dos Santos Neto *et al.*, 2017).

Good success has been reported using vitrification to cryopreserve horse embryos, particularly after mechanical collapsing of the blastocoele (Moussa *et al.* 2005; Hinrichs 2010).

6.4.5 Embryo sexing and genotyping in mammals

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

One simple approach to sex determination is to bisect the embryo and identify the sex of one of the halves by polymerase chain reaction (PCR) of genes in sex chromosomes. Once the sex is established, the remaining half of the embryo can be transferred to a recipient female (e.g. Nakagawa *et al.*, 1985; Herr & Reed, 1991).

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

At present, research efforts are directed towards minimally invasive embryo biopsy approaches for harvesting cells to be used in identifying potential genetic abnormalities and diseases prior to transferring the embryo. Cells from embryonic biopsy are currently used by selection companies to identify genetic traits of the embryo by using high throughput single nucleotide polymorphism (SNP) mapping and genomic selection technology. The potential for using genomic information to select the appropriate embryo to transfer provides significant benefits to commercial breeders and accelerates the genetic gain on production traits. For general cryopreservation programs, these technologies may also be useful for selecting animals or embryos with the aim of maximizing the amount of genetic variability conserved in the gene bank (de Cara *et al.*, 2011).

Embryo genotyping may also be of interest to improve animal health, for example by decreasing the scrapie sensitivity genotypes in small ruminants (Guignot *et al.* 2009; Guignot *et al.* 2011).

6.4.6 Embryo cryopreservation in honey bees

The ability to cryopreserve insect embryos in most of the species that have been assessed depends on the selection of a precise developmental stage (Rajamohan *et al.*, 2015). Insects, and invertebrates in general, often develop extremely fast to prevent predation and changes in abiotic conditions. In some species, such as the house fly, the eggs hatch in less than 6-8 hours at 37°C. In the case of honey bees, *Apis mellifera*, the eggs that are laid in well-guarded hives develop at a uniform temperature of 33-34°C and 50-70 percent humidity. Under these conditions, the bee embryos hatch in 72-76 hours (Nelson, 1915; Laidlaw, 1979). This fast development creates a limited window for intervention which makes it imperative to collect small number of uniformly developing embryos to assess their cryopreservability. Insect cryopreservation is also unlike embryo preservation in bovine, ovine, caprine, or porcine systems where the pre-morula stage embryos are often cryopreserved. The appropriate developmental stage for most insects is in their late organogenesis stage. By the time all the collected embryos reach the required developmental stage, they have diversified into developmental stages that are staggered by 0-6 hours, as is the case in honey bees. Another complexity that arises due to late-stage embryo cryopreservation is that they are not amenable to slow or conventional freezing techniques but require assisted dehydration and subsequent vitrification.

6.4.6.1 Collection of embryos

Two common embryo collection methods are of particular interest in bee germplasm cryoconservation. In the first method, Collins (2004) used a queen and drone excluding mesh to trap the queen on an empty frame with an extruded wax foundation. When this structure was placed inside the hive of the queen to be collected, it restricted the egg laying of the queen to the particular frame but allowed the workers to traverse the mesh to maintain the foundation, eggs and service the queen. The queen stayed on the frame for about 4 hours before being released and the frame was removed to the laboratory for studies on the embryos. In the second method, Rajamohan *et al.* (2020) captured the queen in the hive and placed her in a Scalvini™ cage, which is an approximately 8 x 10 cm polypropylene cage (described in Annex 6.3) with a snap cap to allow the queen into the cage. The queen was trapped for 1 hour before being released back into the hive. Because the willingness of the queen to lay eggs was much higher in the Collins technique, the Scalvini cage method often has a failure rate of 50 percent or more. This approach can be improved by leaving empty Scalvini cages in the hive overnight and trapping the queen the next day. To improve the probability of capturing overall success, the use of multiple hives, queens and Scalvini cages is recommended.

The removal of the eggs to study them using low powered transmitted light microscopy allows for a clear view of the development but is risky due to extreme fragility of the eggs. Once again there are two techniques which may be useful for this purpose. In the first approach, Collins (2004) used a Taber forceps to grab the eggs which are usually laid upright in the hexagonal cells of the hive, ~1 egg per cell. The distal end of the egg is attached to the wax foundation, while the proximal end is free standing. The spacing in the Taber forceps allows grabbing the eggs in the middle without crushing the embryo. For the second approach, Evans *et al.* (2010) used an unconventional technique of tapping the frames, which dislodged the eggs from their adhesive base and also allowed them to land on their heavier adhesive distal end on the collecting surface. With four taps, nearly 90 percent of eggs were dislodged from one side of the frame. They reported that >90 percent of the embryos collected by this method were intact and 86 percent hatched into larvae, whereas manual removal of the eggs resulted in only 31 percent success. Hence, the “tap down” protocol for egg collection is recommended for both embryonic evaluation and cryopreservation. Scalvini cages with eggs are to be tapped gently 2 to 3 times over a 9 cm sterile polystyrene petri plate. At the same time, a piece of filter paper soaked in distilled water is placed in the lid of the petri plate. The plate containing the eggs is gently inverted

onto its lid and closed. The covered plate is then placed in an incubator at 34°C and 60 percent humidity and no lights until the embryos are required for assessment and cryopreservation.

6.4.6.2 Cryopreservation

As is the case with nearly all insect species, the technology to cryopreserve honey bee embryos is still in a very early stage of development, primarily due to its technical difficulty and need for highly precise embryo staging. The current technique designed for the honey bee embryos allows for 1 to 3 embryos to be cryopreserved at the same time. The basic method for insect embryo cryopreservation is used across species due to the shared structural and developmental features (Leopold a& Rinehart, 2010). Based on the structure of an insect embryo, the basic protocol has the following features:

1. stage selection;
2. dechorionation;
3. permeabilization;
4. cryoprotectant loading and dehydration;
5. vitrification;
6. thawing;
7. rehydration and detoxification; and
8. embryo culture.

2.

In honey bees, the specific developmental stage must fulfill all the criteria laid out in Leopold and Rinehart (2010) and Rajamohan *et al.*, (2015). These criteria determine the suitability of a developmental stage for cryopreservation. In bee embryos this occurs approximately at 66-68 hours of development. The embryos are dechorionated in most insects for ~15 minutes using a 25 percent sodium hypochlorite However, in the case of honey bees, the dechorionation procedures that last more than 25 seconds cause structural instability as the chorion in the bees is not as robust as in most insects. In dipterans, the permeabilization process removes the wax layer protecting the embryo from dehydration. The permeabilization process is often a two-step process of (i) surface dehydration using 2-propanol and (ii) wax dissolution using 6 or 7 carbon alkanes that lasts less than 45 seconds. In the case of *Apis mellifera*, the process is also two staged but restricted to 5 and 15 seconds, each. In dipteran and lepidopteran embryos, the cryoprotectant loading is again a two-step treatment with increasing concentrations of ethane diol from 10 to ~40 percent supplemented with disaccharides, polyethylene glycol and fetal bovine serum. The osmotic limitations of the honey bee embryos means that the loading and dehydration steps are combined in a single-step ethane diol treatment at 4°C to reduce toxicity and osmotic effects. While in the case of most dipteran embryos, techniques have been developed to mass-cryopreserve 150 to 5000 embryos in one session, only 1-3 honey bee embryos can be cryopreserved per session. Embryos are frozen on a single hair/filament brush (Fig. 8 Annex 6.3) or a thin strip of polyvinyl pyrrolidone coated polycarbonate membrane (Millipore, USA).

Vitrification is usually done is two steps involving a rapid transfer to liquid nitrogen vapor followed by quench freezing in liquid nitrogen. The vapor treatment is well calibrated between -120°C and -135°C. In large embryonic structures, this protects the embryos from fracture damages (Rajamohan & Leopold, 2007). Thereafter the embryos are plunged into liquid nitrogen and captured in a histological tissue cassette for storage. To thaw, the embryos are brought back into the vapor phase and then plunged into a cell culture medium such as Schneiders or Grace's medium containing 0.5M (~18 percent) trehalose dihydrate. The embryos are fished and floated on fresh medium with no trehalose after 2 minutes for dipterans and <30 seconds in the case of honey bees due to their fragility. The hatching is observed, assessed and estimated after 24 hours.

6.4.6.3 Use

Honey bee embryo cryopreservation represents one of the major advancements in the pollinator conservation strategy. A hatched diploid female larva of the bee could be easily reared into a queen and this allows for the easy founding of a bee colony. Cryopreserved haploid male embryos (Note: diploid males have also been reported – Harbo and Bolten, 1981) upon hatching could serve as a source of germplasm of a specific genotype. Semen from a single male bee has been used to inseminate queens for very specific genetic manipulation studies (Richard *et al.*, 2007).

6.4.6.4 Tools for quality evaluation

In the case of honey bee embryo conservation, the currently used quality assessment is to ensure defect-free development of the thawed embryos. The proportion of embryos that hatch and the proportion of the hatched larvae that exhibit movements indicative of feeding and respiration should be recorded. Hatched larvae can be further assessed by tracking their progress in an *in vitro* environment or after grafting into a hive. If a queen bee is being derived from the cryopreserved embryos, the "queenliness" of the bee can be quantified (Slater *et al.*, 2020). The in-hive acceptability and fecundity of a queen bee with degraded queenliness has yet to be ascertained, however. If a drone is being reared, spermatozoa count and morphometrics should be assessed. Some or many of the quality assessments cited in Leopold *et al.* (2010) for cryopreserved dipterans are also appropriate for honey bees.

6.4.6.5 Ethical issues

At present, no major ethical concerns are noted or have been raised, despite the trapping of the queen to enable egg collection. Repeated trapping could lead to injury to the queen and possible rejection of the queen, thus jeopardizing the hive itself unless a replacement queen is available to ensure the viability of the hive.

6.4.7 Oocyte cryopreservation in mammals

Oocyte cryopreservation is an approach for gene banking of female genotypes in mammals. Furthermore, cryopreservation of oocytes could theoretically enable their flexible use in time and space for other technologies such as in vitro production of embryos with different males or the production of transgenic or genome-edited animals. Different procedures are currently available to freeze oocytes (Annex 6.3). However, these methods remain quite experimental allowing only low success rates of embryo production from frozen thawed oocytes in most species, except humans. Indeed, several factors make oocyte freezing challenging, such as the size of these cells, their lipid contents, the structure of their cytoskeleton, the stage of meiosis (mature vs. immature), their necessary interactions with surrounding somatic cells. Several reviews are currently available on this topic (Díez *et al.*, 2012; Mullen and Fahy, 2012; Khalili *et al.*, 2017), here we will illustrate the challenges and research perspectives of mammalian oocyte freezing with the example of pig species.

First, due to their high lipid content, porcine oocytes are very sensitive to low temperatures (Didion *et al.*, 1990) thus they do not survive traditional equilibrium freezing. Porcine oocytes can survive vitrification and although protocols have been reported with various results, an internationally accepted standard protocol has been lacking. Also, vitrification compromises the developmental potential of oocytes. For these reasons, in most laboratories, porcine oocyte vitrification is still at the experimental level and the current protocols require further improvements. Furthermore, the utilization of vitrified oocytes requires assisted reproduction techniques and the transfer of oocytes or

subsequently developing embryos into recipient females (Kikuchi *et al.*, 2016). In pigs, these technologies are not well established compared to other farm animals and humans.

6.4.7.1 Collection and cryopreservation of porcine oocyte

Immature porcine oocytes can be collected from slaughtered animals either by the aspiration or slicing of antral follicles. In indigenous pigs, slicing of antral follicles is recommended for oocyte collection since it results in higher oocyte numbers than aspiration (Somfai *et al.*, 2019). Alternatively, oocytes can be collected from live animals as well by endoscopic ovum pick up (OPU) (Brüssow *et al.*, 1997) and ultrasound-guided OPU (Yoshioka *et al.*, 2020). However, this approach has not been reported in indigenous pig breeds.

Porcine oocytes can be vitrified either at the immature (germinal vesicle) stage right after collection or at the mature (metaphase-II) stage after *in vitro* maturation (reviewed by Somfai *et al.*, 2012). Although matured oocytes can survive vitrification by the popular Cryotop method at high rates, the procedure induces apoptosis which compromises subsequent development (Vallorani *et al.*, 2012). Nevertheless, high embryo development rates were reported by others when matured oocytes were vitrified by the Cryotop method and later activated parthenogenetically (Ogawa *et al.*, 2010).

Vitrification of porcine oocytes at the immature stage seems to be advantageous since oocytes vitrified at the immature stage retain/regain a higher competence for embryo development than those vitrified at the mature stage (Egerszegi *et al.*, 2013). Furthermore, embryos obtained from vitrified immature oocytes showed a high competence to develop to live piglets (Somfai *et al.*, 2014). The combination of permeating cryoprotectant agents (pCPA), the equilibration protocol and the warming temperature greatly affect the efficacy of immature oocyte vitrification in pigs (Somfai and Kikuchi, 2021). High survival and *in vitro* embryo production rates were achieved when immature oocytes were vitrified in the combination of 17.5 percent ethylene glycol and 17.5 percent propylene glycol, after equilibration in low dose (4 percent) of pCPA (Somfai and Kikuchi, 2021). Using this CPA treatment protocol, vitrification with the Cryotop device or by the microdrop method were equally efficient (Appeltant *et al.*, 2018). Equilibration in 15 percent of total pCPA (either ethylene glycol + DMSO or ethylene glycol + propylene glycol), which is generally used for embryo vitrification appears to be harmful for oocytes (Somfai *et al.*, 2015). Warming of vitrified oocytes must be performed at 42˚C to ensure high survival rates (Somfai *et al.*, 2014). Cumulus cells should not be removed from oocytes before vitrification since they contribute to the maintenance of membrane integrity during vitrification and warming (Nguyen *et al.*, 2021). Vitrification at the immature stage by the above-mentioned approach does not trigger apoptosis in oocytes and resultant embryos (Somfai *et al.*, 2020).

6.4.7.2 Use and prospects

In vitro embryo production (IVEP) technology applying either in vitro fertilization or intracytoplasmic sperm injection (ICSI) has key importance to generate embryos from vitrified oocytes (Kikuchi *et al.*, 2016). Then, to produce piglets, in vitro produced embryos can be surgically transferred into recipients at the blastocyst stage (Somfai *et al.*, 2014). Another approach for the utilization of vitrified porcine oocytes (i.e. piglet production) is their surgical transfer at the MII stage into the oviduct of recipient females followed by artificial insemination (Gajda *et al.*, 2015).

6.5 GONADAL TISSUES

In mammals, the most widely used methods of *in vitro* gene banking are sperm and embryo cryopreservation, but these methods are not always available, such as due to health or technological problems of donors. In avian species, egg and embryo cryopreservation are not possible due to the high amount of vitellus and the special biological and physicochemical challenges associated with the avian egg. For these situations, freezing and transplantation of gonadal tissue may provide a suitable alternative for cryoconservation of the whole genome.

Donor gonads are obtained via surgical removal or from euthanized animals and then preserved via slow freezing or vitrification to enable long term storage in liquid nitrogen. In poultry, vitrification is the most effective method for gonad preservation. It is also often used in domestic mammalian species. After thawing, the grafts can be placed into the same species (allograft) near to the anatomical site (orthotopic) or in other parts of the body (e.g. under the skin, heterotopic) depending on the type of tissue and species. In birds, ovarian tissues are generally transplanted orthotopically and testicular tissues are transplanted heterotopically subcutaneously. Use of donors and recipients of different species (xenograft) is often practiced in mammals (e.g. pig, sheep and goat). In this case the organs may be transplanted under the skin of nude, immunodeficient mice and then donor gametes can be extracted from the xenografts.

Effective gonadal cryopreservation and transplantation methods have been successfully used to produce progeny from multiple species (e.g. pig, sheep, goat, rabbit, chicken, Japanese quail, honey bee, Kaneko *et al.,* 2013; Devi & Goel, 2016; Song & Silversides, 2007c; Rajamohan *et al.,* 2019; Liptoi *et al.*, 2020; Liu *et al.*, 2010, respectively) making it an effective means of preserving animals of high genetic value. Gonadal tissue cryopreservation is utilized routinely in genetic conservation programs with poultry (procedure in Annex 6.4) in many countries but is not commonly used in mammals.

6.5.1 Tissue collection

6.5.1.1 Mammalian species

In mammals, the gonads are usually harvested after slaughter (e.g. pig, goat, equine) or obtained surgically via laparotomy (e.g. ewe, rabbit). Organs from adult or prepubertal animals and embryonic organs can be used depending on the species (Kaneko *et al.,* 2013; Pimentel *et al.*, 2020; Gastal *et al.,* 2017). The gonads should be immediately rinsed in 70% ethanol and then saline solution (0.9 % NaCl) supplemented with antibiotics (penicillin/streptomycin). The cortex is cut into approximately 1-1.5 mm^3 pieces in most cases and vitrified.

6.5.1.2 Poultry species

Only the left ovary develops in most female birds. In newly hatched hens, the ovary is a triangular yellowish-white organ, about 5-6 mm long, and 1.5-2 mm wide, located in the left part of the abdomen, near the abdominal aorta and vena cava. The ovary is usually enclosed by air sacs, the left kidney, and the mesentery colon. Its texture is soft and fragile. In the male, the two symmetric testes are located below the cranial division of the kidney. The testes are attached to the dorsal abdominal wall by the mesorchium. Their form is oblong, their structure is compact, and they are covered by the serous membrane and connective tissues.

Immediately after hatching, primary oocytes are located marginally in the ovary and are in a developmentally dormant state (Song & Silversides, 2007a). Chicken ovaries can be vitrified at 24 hours post-hatch, although successful vitrification of ovarian tissue from week-old Japanese quail and 3-days-old turkey has also been published (Liu *et al.*, 2010; Liu *et al.*, 2017), indicating this window may be longer than 24 h or may depend on the species.

Following removal, donor gonads are usually cut into several (2-4) pieces and 1 to 2 pieces are typically grafted into the recipient animal. Excised gonads must be free of extraneous tissue (kidney, adrenal gland, connective tissue) prior to transplantation to ensure proper placement, minimize scarring or improper adhesion, and provide the best possible opportunity for proper function (Buda *et al.*, 2019).

The organs should be removed from euthanized chicks using sterile (optimal) or clean (acceptable) techniques. Care should be taken to ensure that no longer than 30 minutes elapses between the removal of the gonads and vitrification. Gonads may be stored DPBS-FBS solution on ice while awaiting vitrification (Annex 6.4). BOX 6.3 describes the system developed by staff of the national gene bank in the United States of America for cryoconservation of gonadal tissue in chickens.

6.5.1.3 Honey bees

Methods for the collection and preservation of seminal vesicles, and more recently testis, have been developed. Chilled and surface sterilized drones are dissected to obtain the testicular and seminal vesicle tissue (Hayashi & Satoh, 2019), which is excised and cut into 3 to 4 pieces. This tissue contains primarily immature sperm and should be collected after day 3 the drones hatch from the pupal cell (Machensen, 1955). Drone bees are collected from healthy hives in the mid-season of activity when drone production is high in the hive.

6.5.2 Cryopreservation and thawing

6.5.2.1 Mammalian species

In mammals both slow freezing and vitrification methods are used, although the latter is more common (reviewed by Devi and Goels, 2016). Likewise, the cryoprotective agents vary by species.

6.5.2.2 Poultry species

Vitrification is used for the preservation of poultry gonads and this method is well suited for small tissue pieces (Wang *et al.*, 2008; Váradi, 2016). Use of the technique means that the tissues are immersed directly into liquid nitrogen immediately following treatment with vitrification solutions at room temperature. The ultra-rapid cooling prevents the formation of ice crystals and maintains cellular integrity. Human acupuncture needles can be used to facilitate tissue handling (Wang *et al.*, 2008) of 3 to 5 organs per needle. Once on the needles, the organs are placed into successive vitrification solutions with increasing cryoprotectant (DMSO and ethylene glycol) concentrations. The needles containing the gonads are then placed then into open, labelled 1 mL volume cryovials in liquid nitrogen, where they are closed with forceps before long-term storage in nitrogen tanks (Annex 6.4).

Cells can risk damage during warming due to the osmotic changes and recrystallization (Morris *et al.*, 2012; Papatheodorou *et al.*, 2013). Consequently, the appropriate warming of donor organs is critical to achieve a successful transplantation. Prior to grafting, needles containing organs are placed in three different thawing media at 38.5°C that include decreasing sucrose concentrations (1 to 0.25 M, Annex 6.4) to remove the penetrating cryoprotectants. Thawing must be performed as quickly as possible to

preserve the viability of the cells. The quantity of the solutions must be no less than 3 mL so that the relatively large needle and organs will not cool the media prevent proper warming. Keeping the solutions at a stable temperature can be ensured by using a fixed temperature heating plate. Organs may be stored prior to transplantation in DPBS with 20 percent FBS for up to 1 hour at 0°C (Liptoi *et al.,* 2020; Barna *et al.,* 2020) (Annex 6.5).

BOX 6.3
USDA NAGP method of gonad preservation

Bulk preservation of 1-day old chick gonads is accomplished with a staff of four people. One person (P1) is assigned to sacrifice and "breast" the chicks once they are unresponsive. The second person (P2) performs the removal of the gonads and places the organs on a clean/sterile gauze pad or an absorbent underpad with a moisture barrier. The third person (P3) places the gonads on the acupuncture needles, and the fourth person (P4) performs vitrification.

To accomplish this, the media (Basal solution (BS), Vitrification Solution (V1) and Vitrification Solution 2 (V2)) are prepared as described in Annex 6.5 and aliquoted into separate 2.0 ml microcentrifuge tubes (1.75 ml per tube). This ensures that when a needle with gonads is placed into the tube it is completely submerged in the solution. Multiple tubes for each medium should be prepared. Cryotubes should be labeled with an identification number that corresponds to the chicks, sex, line, breed, etc. and the top of the cryotube should be punctured (approximately 3 mm hole) to allow equilibration of liquid nitrogen and minimize the chance for vials to explode upon removal from storage.

Large quantities of chicks can be processed if the following methods are employed. P1 sacrifices and breasts the chicks (removes the skin of the bird by placing their left thumb in the clavicle of the chick, grasps both wings with the right hand and pulls to separate the skin from the carcass) in groups of 10 to 20 ensuring that the intestines remain over the gonads. P2 removes the gonads as described in Annex 6.5 and places them on the clean surface in front of P3. The gonads should be separated by testes and ovaries, placed accordingly on the needles, and placed in Basal solution on ice by P3. It is critical that once the gonads are excised that they are only minimally exposed to air or are kept moist with BS. Once a reasonable number of needles are prepared (< 12) a group of gonads (ovaries or testes) are passed to P4. P4 moves the needles to tubes containing V1 after blotting the tissue free of BS with a clean cloth. These transfers are performed at 5 seconds intervals and repeated with V2. After incubation in V2 the samples are blotted to remove excess amounts of the solution and then placed in the prepared cryotubes submerged in liquid nitrogen for vitrification. The cryotubes are then capped and placed in storage. Processing samples using this teamwork enables preservation of 100 (males) to 120 (females) chicks per hour.

6.5.2.3 Honey bees

Samples of seminal vesicles are frozen using a defined medium containing cryoprotectants and then stored in cryovials (Rajamohan *et al.,* 2019). Removal of DMSO by gentle centrifugation of the samples following thawing is necessary prior to use (Annex 6.4).

6.5.3 Use of the frozen-thawed gonadal tissues

6.5.3.1 Mammalian species

In mammals frozen-thawed ovarian tissues can be grafted orthotopically (e.g. ewe, rabbit). Immunodeficient mice can be the host of both ovarian and testicular tissues via xenografting (e.g. pig, goat). It is possible to activate the preantral follicles in the ovaries of fetuses by optimizing germplasm utilization (e.g. goat) and gain sperm from immature testicular tissues (e.g. pig) (Kaneko *et al.*, 2013; Pimentel *et al.*, 2020).

6.5.3.2 Poultry species

The transplantation surgery is frequently a significant challenge for cryoconservation with gonadal tissues. Ovariectomy with newly hatched recipient chicks is difficult because the ovary rests on the adrenal gland and the aorta, which makes damaging the vasculature a very real concern and may result in uncontrolled bleeding (Song & Silversides, 2006; Buda *et al.*, 2019). Removal of the ovary can be done using fine forceps, iris scissors, or forceps designed for eye surgeries (Liu *et al.*, 2013b), but this often results in an incomplete removal of the recipient's ovary. In this case, the recipient hen will be a chimera and will produce a mix of donor and recipient derived progeny, because a complete ovary can develop from a single tissue piece from either the donor or recipient. The ovariectomy should be done precisely starting from the cranial part of the ovary and by applying bipolar electrocautery (Liptoi *et al.*, 2020). In males, the technique is the same as in females, and similar precautions should be exercised (Song & Silversides, 2006, 2007a, 2007b; Liu, 2013; Liptoi *et al.*, 2013). In this case, removal of the testes can be performed with fine scissors and forceps (Annex 6.5).

Potential recipient chicks should be sexed after hatching so that they can be matched with a donor of the same sex and this will minimize the time between thawing and grafting. Anesthesia of recipient chicks is induced by intramuscular administration of xylazine and ketamine and maintained with isoflurane gas during the operation. The intervention can be carried out on a table sanitized with 70% ethanol and equipped with a heating pad to maintain the body temperature of the chick. A head lamp or other lighting may be necessary because of the small size of the incision (2-3 cm). During the procedure the yolk sac is carefully removed through this incision using the technician's fingers, the gastrointestinal tract is pushed aside, the genital organs can be accessed, followed by the ablation (Song & Silversides, 2006, 2007a, 2007b; Liu, 2013; Liptoi *et al.*, 2013; Barna *et al.*, 2020) (Annex 6.5).

The prepared donor ovarian tissue is grafted as close as possible to the anatomical location (Liptoi *et al.*, 2020; Barna *et al.*, 2020). Testicular tissue can be grafted subcutaneously through a small cut in the skin of a castrated chick (Liu, 2013). The chicks should be kept in a heated room, under infrared heat lamps during the whole pre-, peri-, and postoperative care (Annex 6.5).

Although the transplanted organs can adhere and develop without any treatment, the use of immunosuppressive therapy after surgery may increase the acceptance and function of donor gonads. Steroid injection just after intervention can prevent acute immune response and edema. Mycophenolic acid can be used for supporting later gametogenesis, because due to the inhibition of B and T cells, it facilitates the implantation of donor tissue (Song & Silversides, 2006; Song *et al.*, 2012; Liptoi *et al.*, 2013; Barna *et al.*, 2020) (Annex 6.5).

Not all genotypes are suitable recipients among the domestic chicken breeds (Liptoi *et al.*, 2013, 2020). When done properly, the acceptance rate of transplanted gonadal tissues is 70 to 80% and the

proportion of donor-derived progeny from them averages 33 to 50 percent. However, in some cases the acceptance of grafted donor organs is low or zero (Song & Silversides, 2006; Liptoi *et al.*, 2013). Selection based on the genetic distances of suitable breed pairs (with using closely related breeds being preferable) seems to be a useful method for creating appropriate donor/recipient combinations (Bodzsar *et al.*, 2012; Liptoi *et al.*, 2020). However, determination of the optimal genetic/physiological background requires further research to facilitate the selection of proper recipients for any donor genotype. Rectifying this situation will enable more-successful preservation and utilization of indigenous or endangered chicken breeds as well as commercial lines that become obsolete as a result of genetic selection (Liptoi *et al.*, 2020).

6.5.3.3 Honey bees

Cryopreservation of seminal vesicle and testicular tissue has the advantage of making it feasible to derive gametes from drones that are not yet ready to mate or from whom the gametes could not be obtained by induced ejaculation. The technique may also be applicable to other hymenopterans and pollinators (Campion *et al.*, 2021).

6.5.4 Tools for quality evaluation

6.5.4.1 In vitro test in mammalian species

Cellular integrity and quality of frozen-thawed ovarian tissue has been explored using tissue culture and the terminal deoxynucleotidyl transferase nick end labeling TUNEL assay (Gastal *et al.*, 2017). Similarly, histological analysis with hematoxylin – eosin staining is used to compare the status of the gonads in different maturation phases (Kaneko *et al.*, 2013; Pimentel *et al.*, 2020).

6.5.4.2 In vitro tests in poultry species

The tools for quality evaluation of frozen-thawed gonadal tissues are limited in poultry. Histological examination of the fresh and thawed gonads of newly hatched chicks stained with hematoxylin-eosin can be used, but this technique did not identify structural differences between fresh and vitrificated gonads (Varadi, 2016). The viability of frozen-thawed gonadal tissue can be evaluated via tissue culture. Very simply, if a fibroblast explant grows, it means that the tissue is viable. However, with this method, it cannot be certain whether the explant remains functional or retains its ability to produce gametes. Liu et al. (2010) applied the trypan blue assay for estimation of cell viability in Japanese quail. Tissue pieces were stained with trypan blue to microscopically identify the dead cells (blue) and the living cells (unstained).

The DNA integrity of fresh and frozen-thawed ovarian tissue in turkey using the TUNEL assay combined with hematoxylin and eosin staining has also been investigated by Liu *et al.* (2017). They concluded that apoptosis was most frequent in the fresh tissue of one-day old birds presumably due to intrinsic selective mechanisms. However, it is uncertain whether this method truly evaluates the functionality of cryopreserved ovarian tissue because it is difficult to establish if the cell death is caused by the freezing method or other biological factors.

Due to the small size of the poultry gonads, *in vitro* examination of a portion of them significantly reduces the amount of tissue that can be grafted. Still, it is important to investigate the quality of frozen-thawed organs to test applied or developed methods. Checking 1 to 2 pieces from each series of frozen organs is sufficient to test efficacy. There may be individual differences, but previous studies have shown that the primary limiting factor is finding the appropriate recipient rather than the damage caused by vitrification.

It is recommended that organs be examined under a stereomicroscope immediately prior to transplantation. Any foreign tissue should be removed with scissors and sharp tweezers and can be further cut if necessary. Preparing the organ in this way shortens the duration of surgery and facilitates grafting.

6.5.4.3 In vivo tests poultry species

In poultry, the true efficacy of gonadal cryopreservation is proven by progeny production (Liu et al., 2017). That technique demonstrates whether the tissue pieces survive the freezing-thawing procedures, if the tissue is properly transplanted into recipients, and if the gonads have attached, developed, and finally function. As noted earlier, previous investigations demonstrated that the success of transplantation is strongly dependent on the appropriate recipient/donor pairs (Liptoi et al., 2013, 2020). When the match is appropriate, the rate of acceptance of the frozen-thawed donor tissues of mixed breeds is similar to those achieved when tissues are transplanted between birds of the same breed. Prior to full experimentation or breed reconstitution, it is advisable to perform test transplantations and to sacrifice a few individuals at the eighth week of life and perform histological examinations to determine if the breed combination supports organ grafting and is suitable for the production of gametes.

Donor-derived progeny can potentially be identified according to the feather color. However, if the recipient has a similar color and if their ablation was incomplete, it is not an accurate method. Molecular genetic markers can always be used to confirm the lineage of the progeny (Liptoi *et al.*, 2020).

6.5.4.4 In vitro test in honey bees

The *in vitro* tools to study cellular viability include the live-dead assay using Sybr-14 for viable cells and counterstaining with propidium iodide to identify cells with a damaged plasma membrane. In addition, the TUNEL assay has been used to ascertain the presence or absence of DNA nicks (Wegener *et al.*, 2014).

6.5.5 Ethical issues

Gonadal tissue transfer of poultry and mammalian species is an invasive intervention. Although it is approved for use in many countries, including Canada, Hungary, United Kingdom and USA, some countries still require a strict authorization process. Consequently, before applying the procedure, it may be necessary to acquire country-specific permits from the relevant authorities. Furthermore, in the case of honey bee, although the extraction of tissue does involve sacrificing drones, in many countries it does not require animal care authorization because the benefits of the procedure include preserving and sustaining pollinator populations.

6.6 DIPLOID GERM CELLS AND SOMATIC CELLS

Diploid germ cells are present in the early developmental stages before the initiation of gametogenesis and meiotic reduction. These cells are valuable components within germplasm collections as they are mitotically active and can be increased in number *in vitro*, or complete gametogenesis when re-introduced into the gonads of host animals. Some of the progeny from the host animal will contain the genome of the donor germ cells. The use of embryonic germ cells was developed first in fish and later in the chicken through the study of primordial germ cells, which are easily recognizable and may be

extracted at specific early stages of embryonic development. They now constitute a key tool for gene banking for chickens (Procedures in Annex 6.5).

Procedures for other diploid germ cells are also under development. The use of mammalian spermatogonial germ cells from the testes are now emerging for future applications in livestock conservation. Somatic diploid cells could be directly reprogrammed into functional gametes and their use can be expected in the future for breeding rare and valuable individuals from livestock species. The use of somatic nuclei for cloning is also a method that is sometimes employed in mammals.

6.6.1 Chicken primordial germ cells

Primordial germ cells (PGC) are diploid stem cells in the embryo that are the precursors of male and female gametes. In some species, PGCs are not yet sexually determined towards becoming male or female gametes and in many vertebrate species will form sperm or eggs when transplanted into surrogate host animals of the same species. PGCs are very few in the embryo, so they need to be propagated *in vitro* to increase their number before biobanking. In mammalian species, the PGCs can only be propagated *in vitro* for short periods. However, PGCs can be propagated *in vitro* indefinitely for chicken breeds (van de Lavoir *et al.*, 2006). In bird species, PGCs can be easily collected from the embryonic blood at the time of their migration toward the gonads or from other embryonic tissues; the laid egg blastoderm or the pre-meiotic stage embryonic gonad. Isolated PGCs can then be increased in number through *in vitro* culture before biobanking. After thawing and re-amplification *in vitro*, PGCs must be transplanted into a surrogate host embryo where they will develop into functional gametes and produce offspring (van de Lavoir *et al.*, 2006, Whyte *et al.*, 2015). Using this process alone or in combination with frozen semen would restore the male and female genotypes of individual breeds in a single generation. In this context, the cryopreservation of amplified *in vitro* PGCs is of great interest for poultry species as a conservation strategy complementary to sperm-based biobanking techniques.

6.6.1.1 PGC collection and cryopreservation in the chicken

Avian PGC collection and culture is described in Annex 6.5. These cells can be easily isolated from early avian embryos when they migrate through the circulatory system during their normal life cycle. They can also be isolated from the embryonic gonad after completing their migration to this organ and are suitable for culture. The *in vitro* propagation of chicken PGCs, however, is technically complex, uses specialized cell culture reagents, and requires laboratory cell culture facilities. The cryopreservation of cultured PGCs is relatively simple and comparable to protocols for cell lines that utilize cryopreservation medium containing DMSO.

6.6.1.2 PGC use in re-establishing chicken breeds

PGCs must be reintroduced into surrogate hosts of the correct sex where they complete normal development and gametogenesis (Annex 6.5). Injection of PGCs into surrogate avian host embryos is technically demanding as the cells must be injected into the embryonic blood circulatory system of a laid egg through a small window made in the shell. The egg must then be incubated until hatch and the chick raised to sexual maturity for mating.

6.6.1.3 Tools for quality evaluation

It is important to evaluate the quality of the PGC lines that are stored. PGC lines are defined as PGCs cultured from a single embryo (genotype) to increase cell numbers and frozen in multiple samples in the gene bank. Validation of cell lines by *in vitro* and *in vivo* tests should be performed for each cryoconserved breed.

***In vitro* characterization of PGC lines.** The established cell lines should be tested to define the essential characteristics and ensure that the cell populations are truly homogenous and have the specific characteristics of this cell type. The potential tests are listed below.

- Sex determination: For sex determination of PGCs lines, embryonic tissue samples are usually collected during the PGC isolation procedure. The sex of PGC lines is important for the efficiency of reintegration into host embryos and gene bank storage. DNA sequencing of the host embryo can also be used for later use in regenerating genetic diversity.
- Germ cell and stem cell-specific immunohistochemistry of PGC lines (Lázár *et al.*, 2018): Germ cell-specific staining of cells shows that the cells retain their ability to develop into gametes during the long-term storage. Two commercially available germ cell-specific antibodies are DAZL and SSEA-1, a stem cell-specific antibody widely used in PGC studies.
- Gene expression of germ cell and stem cell specific markers of PGC lines (Lázár *et al.*, 2020, Poultry Science, under review): The most important and widely used germ cell-specific marker genes are *DDX4* and *DAZL*, whereas *POUV* and *NANOG* are good examples for stem cell-specific genes used in the PGC validation process.

Mycoplasma contamination. Mycoplasma can be vertically transmitted via the egg. PGCs have been shown to not carry mycoplasma from infected hens but cell lines can become contaminated. A PCR test will validate the sanitary state of the cells (Onuma and Kuwana, 2011).

***In vivo* validation of PGC lines.** The cultured PGCs need to be characterized *in vivo* to investigate the cell function and their ability to migrate into the gonads. The principle of the method is to label the cell line samples with a fluorescent dye (PKH26 Red Fluorescent Cell Linker) and then inject them into the recipient embryo. The injected embryos are dissected on Day 6 of embryonic development and the recipient's gonads are examined to see if the injected cells are incorporated. However, the true test for function is progeny production from the surrogate hosts.

Ethical issues. The collection of avian PGCs is invasive for the donor egg and embryo that are sacrificed. However, this procedure is carried out at the early embryonic stages of eggs, so it is of no or little ethical concern to most countries. The introduction of donor avian PGCs into host animals is made by injection into host embryos. In avian embryos, this has a minor impact on embryo development. The impact on the welfare of the hatched host is also minimal; the host will behave - breed and lay eggs as are normal for the host breed.

6.6.2 Spermatogonial stem cells

Male germ cells remain mitotically active in the testes of the adult livestock animal. The spermatogonial stem cells (SSC) of the adult testes, continually self-renew and differentiate into functional spermatozoa. SSCs can be directly purified from the testes and cryopreserved or propagated *in vitro* to increase their number before cryopreservation. SSCs can subsequently be transplanted into recipient host animals and achieve the successful repopulation of the host testes where they differentiate into functional spermatozoa. The semen produced can be subsequently used for insemination or natural mating. The use and development of permissive surrogate host animals for allogenic germ cell transplantation is described below. In theory, SSC cryopreservation and transplantation should be applicable to all livestock species and the culture of SSCs is under development for several mammalian species (Pramod, 2014; Oatley, 2016; Zhang, 2017) However, the culture of SSCs is difficult and, similar to PGCs, the cultured cells must retain their ability to repopulate the gonad of a host animal. In the future, it may be possible to differentiate SSCs directly

into functional gametes *in vitro*, which would eliminate the requirement for the use of surrogate host animals.

The SSCs can be isolated from animals postmortem or during veterinary castration. The SSCs need to be injected into the mammalian testes to compete their development into functional spermatozoa. The injection procedure is technically demanding, but is not generally considered an invasive procedure, because the testes lie external to the body cavity in mammals.

6.6.3 Surrogate host animals carrying transplanted reproductive cells

6.6.3.1 Preparation of surrogate hosts

Cryobanked diploid germ cells must be transplanted into host animals, i.e. ”surrogate” hosts, in which they divide and complete their maturation into functional gametes. Transplanted germ cells must compete with the animal’s own germ cells and elimination of the endogenous germ cells will ensure that all offspring are derived from the cryopreserved material. Chemotherapeutic reagents and irradiation can be used to eliminate the endogenous germ cells, but these compounds can have a detrimental effect on the animal’s health and welfare. Thanks to new biotechnological advances, genome editing can be used to create mutations in genes responsible for germ cell survival and development. Livestock animals containing these edited genomes are completely healthy yet are functionally sterile. Transplantation of the gene banked diploid germ cell material into these hosts restores the fertility of the animal and the genetic contribution to offspring from the host animals will arise solely from the donor material. Livestock sterile hosts have been developed for SSC transplantations for pig, cattle, and goat species (Ciccarelli, 2020). Both male and female sterile surrogate hosts have been made for chicken and were shown to produce offspring from cryopreserved transplanted diploid germ cells (Woodcock, 2019; Ballanatyne, 2020). Use of sterile interspecific hybrids as recipients may also be an option. These approaches have been used successfully in fish species and are also being developed in domestic fowl (Yoshikawa *et al.*, 2018; Molnár *et al.*, 2019).

6.6.3.2 Ethical issues

The ethical issues described above governing embryo and animal transplantation will still be relevant as the procedures develop. For most countries, new regulatory approval and legislation regarding offspring produced from genome edited host animals will be required before these techniques can be used in biobanking programs, but restrictions for surrogate animals may be less strict if they are not introduced into the human food chain.

6.6.4 Preservation of diploid cells for cloning or the *in vitro* production of gametes

6.6.4.1 Collection and preservation of material

Cryopreserving tissues for isolation and culture of fibroblasts and other somatic cell types in gene banks is a simple protective option for preserving rare breeds of livestock and other animal species. The cryopreserved cells can be used for somatic cell nuclear transfer (SCNT) or ”cloning”. Cloning of livestock species is feasible for cattle, sheep, horses, pigs, goats (Wilmut, 1997; Cibelli, 1998; Baguisi, 1999; Polejaeva, 2000; Galli, 2003). The animals produced from the cloning procedure may be considered a type of hybrid, as they will contain the somatic nuclear genome from the fibroblast cell and the mitochondria from the recipient host oocyte used in the cloning process. This is a valuable option to regenerate a few rare animals with superior genetics to return to *in situ* breeding populations. Somatic cells can be easily frozen and stored, making this procedure relatively inexpensive in terms of

cryopreservation. In contrast, the process of cloning is a technically demanding and expensive procedure, and the progeny may show some negative side effects due to epigenetic dysfunctions.

Important advances have been made in the ability to ”reprogramme” somatic cells into pluripotent stem cells. The expression of a set of transcription factors in the somatic cell can cause it to acquire stem cell-like properties and are known as induced pluripotent stem cells (iPSC;Takahashi, 2006). The pluripotent cells can then be guided to differentiate into different somatic cells and tissues. An aspiration of this research is to be able to differentiate iPSC directly into sperm or oocytes that can be used for *in vitro* assisted fertilization, followed by ET into surrogate host females. This technology is in its infancy, but great progress has already been made using small animal model species (Hayashi, 2011; Hayashi 2012). In the coming decade this approach should be applicable to livestock species (Su *et al.*, 2020). The ability to form functional oocytes *in vitro* could be coupled with frozen semen currently in gene banks to create viable embryos for transfer into surrogate females.

6.6.4.2 Ethical issues

Ear punch samples can be taken for cryopreservation of somatic cells during normal livestock ear tagging. This procedure will increase the stress to the animal and must follow local veterinary guidelines and regulations. Tissue samples can also be isolated during slaughter or from recently post-mortem animals. Fibroblasts can be easily cultured from both sources and used for iPSC generation or for SCNT.

6.7 GENERAL CONCLUSIONS AND RECOMMENDATIONS

Over the past few decades, the scientific community has developed a number of methods of germplasm conservation that have permitted the building of collections, and their efficient use to reintroduce genetic variation into populations *in vivo*. The recent European project “Innovative Management of Animal Genetic Resources (acronym IMAGE) (2016-2020) greatly improved animal gene bank management through an exhaustive review of the strategies presently developed, methodological improvements, and suggestions of new directions to be developed. Semen is and will probably stay the main germ cell type cryopreserved. Embryo collections are common in many domestic mammals and in development in others. In oviparous species, where the embryo cryopreservation yet to be established, the storage and use of gonadal tissues and PGCs are growing. In the future, the application of these biotechnologies will be expanded, and integrated into the ethical evolution of breeding. In particular, the reprogramming of somatic cells into germ cells represents an exciting challenge for the future of germ plasm cryopreservation.

Finally, in most domestic species, given the present state of the art of reproductive cryobanking, we recommend the constitution of large collections of cryopreserved semen (often not invasive) accompanied by a targeted collection of diploid germplasm type, notable embryos in mammals, PGCs or gonadal tissues in birds, and embryos or gonadal tissues in honey bees.

In all cases, the safety and sanitary status of the resources must be controlled to avoid any potential disease contamination of any future progeny. The different countries regulation must be respected. The welfare of the animals must also be a priority of the teams that manage the sampling and the use of the reproductive collections.

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SECTION 7

Sanitary issues and recommendations

Sanitary issues and recommendations

7.1 INTRODUCTION

Germinal products, i.e. germplasm samples such as semen, oocytes and embryos, may represent a source of risk for the spread of animal diseases, including zoonoses. They are collected or produced from a limited number of donors but may be used widely in the general animal population. Therefore, if not handled properly or not classified with the correct health status, they may be a source of disease for a large number of animals. Such cases have caused substantial economic losses in the past (Eaglesome and Garcia, 1997) and high sanitary standards are thus very important for animal gene banking.

To prevent the risk of the spread of disease, regulations at international, regional or national levels provide that germinal products be collected, produced, processed and stored at specialized facilities and be subject to special animal health and hygiene regimes. At the same time, for animals to be admitted into those facilities and be classified as donors of germinal products, they are required to comply with appropriate animal health standards than those applicable to the general population, including test and quarantine prior to entry to the establishment. Other common elements of these regulations are requirements for storage of the germinal products and the traceability rules for their movement, which include marking the straws or other storage vessels and the obligation that each exchange of the material is accompanied by an animal health certificate.

Regulation of international exchange of germinal products from mammals is laid down in section 4 of the Terrestrial Animal Health Code (OIE, 2019) of the World Organization for Animal Health (OIE). The Terrestrial Code provides standards that should be used by the veterinary authorities to set up measures providing for early detection, reporting and control of pathogenic agents, including zoonotic infections, and preventing their spread via international trade of animals and animal products, while avoiding unjustified sanitary trade barriers. Based on these standards, countries may reach bilateral agreements to allow international trade of germinal products.

At the regional level, some organizations, like the European Union, have established their own regulations on movements of germinal products within and between their member states. Nevertheless, these regional regulations have used the Terrestrial Code as a reference and therefore include very similar requirements.

At the national level countries can regulate the movement of germinal products, but requirements from the Terrestrial Code or regional regulations are often used as a reference. Thus, in many countries, OIE recommendations regulate the sanitary aspects of collection, processing, storage and transport of germinal products, both at multi-national and national levels.

The OIE regulations were developed to ensure the highest levels of biosafety in order to avoid the spread of animal diseases in the trade of germinal products from mainstream breeds. If these recommendations are to be met, a large investment must be made in each facility dealing with germinal products, which will presumably be compensated by the benefits obtained in the trade of safe germinal products. Although their activities are not commercial, whenever possible animal gene banks should consider following the OIE regulations, being the best international standard available. In

addition, OIE regulations are mandatory if the gene bank is interested in distributing germplasm to other countries in the future, although the final requirements for international exchange are agreed bilaterally.

On the other hand, the requirements imposed by regulations at national, regional and international level can be a serious burden for the collection of germinal products, especially in the case of locally adapted breeds. These breeds are often raised in less intensive production systems with fewer biosecurity measures and less stringent animal health programmes. As a consequence, the farms often do not meet the OIE requirements for sending donors to centres and the donors do not pass the necessary tests. In addition, the quarantine period, testing and requirements for facilities may lead to high costs for collecting a small number of samples of little real commercial value.

At-risk breeds are often found in only a few locations, leaving little opportunity for selection of donor farms based on sanitary conditions. Disease outbreaks may also present an urgent need to collect germplasm from animals in the affected area as well as a health and sanitation risk for the gene bank.

BOX 7.1
Sanitary measures for gene banks – Experiences from China

The Chinese government has identified the conservation and sustainable utilization for animal genetic resources (AnGRs) as a priority. However, like many other countries, in China biodiversity, and thus AnGR, are still facing challenges:

1. Drastic climate change and human activities, like natural hazards and urbanization, have rapidly deteriorated the habitat of many animals, including endangered livestock breeds.
2. To meet the huge national demand for food, researchers and breeders have focused on high-productivity breeds and large-scale breeding, leading to, amongst others, rapid loss genetic diversity and local genes.
3. Several animal disease outbreaks since 2000 have underlined the need for conservation of local breeds, a need which currently is far beyond the existing conservation capacity.

To protect biodiversity, China established the national gene bank for livestock and poultry in 1992. Up to December 2019, the gene bank had conserved 670,000 samples of germplasm material and contributed to the conservation of 249 local breeds, of which 39 are endangered. However, gene banking in China is still in its infancy and there is an ongoing need for development.

A recent example is the outbreak of African swine fever (ASF) in 2018. Among the more than 1.1 million culled pigs were many local breeds with good genetic traits, which was cause for concern. The Chinese government activated, besides the national emergency plan, a series of policies related to gene bank development in order to sustain the conservation and development of the pig industry. This serious incident highlighted the importance of gene bank preservation, as well as the need for adequate sanitary recommendations to respond to different epidemics in different regions.

The following recommendations regarding sanitary status were issued for, but not limited to,

gene banking in China:

- Sanitary status is the basis for conservation work in animal gene banks and provides guidance in case of epidemics.
- Sanitary status may differ at home and abroad. Attention should be paid to sanitary protocols according to the epidemic situation in other countries when collaborating on germplasm material collection.
- Domestic collection and storage of germplasm must follow OIE sanitary standards. Cryopreservation should involve quarantine and sampling of donors, pathogen and quality testing of samples, production and freezing of genetic material, regular testing after storage and prior to future utilization. Only donors that meet OIE sanitary standards and are free of infectious diseases can be used as the object for germplasm collection to avoid the transmission of pathogens across the gene bank.
- Laboratories that prepare and store genetic materials need to meet the criteria of ISO standard 14644-1 (ISO, 2015). In addition, large gas-phase liquid nitrogen (LN) storage tanks should be used to avoid contact between the sample and LN, cross-infection of the sample, and safety risks of personnel and samples caused by the explosion of the cryotubes from LN tanks.
- Laboratories should strengthen their management and use of good laboratory practices.
- Operators should receive training at regular intervals on relevant standard operations and regulations concerning gene bank operations, including sanitary aspects.
- On-farm biosecurity should be strengthened. Bio-safe farms should be used and animal disease testing reports for diseases prohibited by OIE should be checked when choosing donor animals.

Source: Dr. Zhao Xueming

To allow collection from local breeds or of relevant material in case of an outbreak, it is highly recommended to establish specific derogations in the national animal health laws to regulate the gene banking for these specific situations, especially the collection out of an authorized centre (Box 7.2). Collaboration between the institutions in charge of animal health and management of AnGR is vital to balance the need for avoiding the spread of diseases with the need of conservation (see Box 7.2).

BOX 7.2

A tailor-made regulation for the collection of reproductive material destined for gene banks in Spain

Spain has a large livestock heritage consisting of 189 breeds, of which 165 are native and 140 are classified as endangered. The activities for the conservation of livestock breeds date back to the last century and are currently organized in a national plan of action. *Ex situ* conservation has been carried out on a large number of breeds. Nevertheless, the requirements of the EU animal health legislation were a burden for collecting material from local breeds, mainly because of their feral behaviour, free range breeding and practical obstacles to fulfilling conditions of the regulation.

In the last regulatory update (Royal Decree 841/2011) in 2011, EU regulations were maintained as a reference for the collection and storage of germinal products in Spain.

However, an exception was included to allow the collection of reproductive material intended to be stored in gene banks without complying with these regulations (mostly to collect semen from farms), provided that these activities did not pose a threat to public or animal health.

To apply this exception, the following procedures were developed to regulate the collection of reproductive material in the field for the different livestock species:

- Collection must take place on farms that are classified as officially disease-free under EU rules on intra-Community trade. This condition is waived only in the case of animals that are genetically highly relevant for the conservation of the breed.
- The donor will be subject to the same serological tests as those identified in the EU's intra-Community trade regulations, and in addition, etiological tests will be carried out to detect pathogens in the semen.
- In the case of serial collections over several days, the donor must remain isolated and samples must be taken at the beginning and end of the collection period.
- The material must be stored separately while awaiting the analytical results.
- If any of the tests are positive, the collected material must be destroyed, except in extreme cases where its storage is justified by a genetic expert. In these cases, strict requirement for storage and s use are to be established.
- Collections may only be carried out by specifically authorized centres under official control.

The development of these procedures has been coordinated between the animal health units and those responsible for the conservation of livestock genetic resources. At present, 17 centres throughout Spain can carry out the collections in the field, which has increased the conservation of material from endangered local breeds in gene banks.

Source: Dr. Fernando Tejerina Ampudia

Each country will need to balance its breed conservation strategy with compliance with national and international health regulations. Decisions should be based, in part, on the types of diseases that are present and how contagious, virulent and damaging to animal production they are. Animals that have a highly contagious and possibly fatal disease, such as foot-and-mouth disease or African swine fever, should not be sampled except in the most dire of circumstances (i.e. if no non-infected animals exist). Furthermore, the intended use of the germinal products collected should be taken into consideration, with strict animal health requirements if the material is intended for use in field populations and more lenient requirements in case the material is only used for long term conservation in a gene bank core collection.

In all cases, as much information as possible about the health status of the farm of origin and the donor animals must be collected and stored, to identify and mitigate potential risks.
In this context, Commission Delegated Regulation (EU) 2020/686 concerning the approval of germinal product establishments and the traceability and animal health requirements for movements of germinal products within the Union is of great importance. For first time, the European regulations allow the movement of germinal products between gene banks in different countries, on the basis of bilateral agreements and without having to fulfil the animal health requirements for the rest of the centres (see Box 7.3).

BOX 7.3

New possibilities for gene banks in the European Animal Health Regulation

In December 2019, the EU approved Commission Delegated Regulation (EU) 2020/686, which supplemented Regulation (EU) 2016/429 of the European Parliament and of the Council (EU, 2020). These regulations regard the approval of facilities for germinal products and the traceability and animal health requirements for movements of animal germplasm within the EU.

The new delegated regulation (article 2.10) defines gene banks as "a repository of animal genetic material for *ex situ* conservation and sustainable use of genetic resources of kept terrestrial animals, held by a host institution authorised or recognised by the competent authority to fulfil these tasks".

More importantly, the new regulation recognizes specific procedures for the movement of germinal products between gene banks in different member states. The requirements for such movements are defined in article 45 and demand firstly that the semen must come from an endangered breed and, secondly, that a bilateral agreement is signed between the two member states involved. The conditions of the agreement concern the use of the germinal products (*ex situ* conservation and sustainable use of AnGR) as well as information about the health status of the material, with specific attention to foot-and-mouth disease and Rinderpest virus.

Recognition of the specific needs of gene banks in the European animal health regulation was of utmost importance to avoid unjustified burdens to gene banking. This regulation provides an opportunity for competent authorities of member states to develop derogations for cross-border exchange of germinal products by national gene banks.

Other novel measures in the new regulation, alleviating some previous impediments and thereby facilitating the work of gene banks, are:

- Germplasm samples (semen, embryos, oocytes) of one species with the same sanitary status may be transported in the same tank;
- Samples of sheep and goats with same sanitary status may be transported in the same tank; and
- Samples with different sanitary status and/or of different species must be stored in separate tanks, but may be stored in the same room, provided the tanks are clearly marked and no cooling agent can pass from one tank to the other.

The EU regulations and recommendations have focused on mammals, however, and mainly the large livestock species (i.e. cattle, sheep, goat, pig and horses). International recommendations for poultry and other species (e.g. rabbits or camelids) are lacking. In such situations, national derogations must be put in place to collect germinal products from these species.

Finally, in addition to the animal health regulations, gene bank managers must be aware of and comply with other relevant rules and regulations. These rules may address issues such as animal welfare, environment and animal identification, as well as the most recent regulations derived from the Nagoya Protocol on Access and Benefit-sharing of the Convention on Biological Diversity (CBD, 2011). Section 9 addresses these topics in more detail.

7.2 COLLECTION

Ideally, donor animals should be free from OIE listed diseases relevant for the species (OIE, 2019), even if the disease is present in the country. The country collecting germplasm for a gene bank may decide to use approved collection centres. For animals with acceptable sanitary status this approach is feasible and the best way to build a genetic archive from these samples. The collected material may then be stored, traded and used freely on a national and international level. This approach guarantees a maximum of biosafety but has some disadvantages. First, the approach is cost intensive as well as time consuming probably adding years to collection development. Moreover, many local breeds will not be able to meet the sanitary requirements for approved collection centres and many countries do not have approved centres for all species of farm animals.

For countries that are not concerned with international exchange of germplasm, options for collecting material outside of approved centres are (i) to use collection centres with lower sanitary standards; (ii) to collect material on farm; or (iii) to use slaughterhouse material. The use of the germplasm collected from these sources may or may not require national derogations for gene bank material. If a derogation is needed, it should state the exceptions from the national regulation and the conditions for collection, testing, storage and use of the material for the country.

Utilizing collection centres with lower sanitary requirements may be a satisfactory solution. The sanitary status of each donor should be documented and quarantine measures are recommended. The following recommendations (most of them derived from the OIE standard) may be used to establish derogations for collection centres at national level:

1. The collection centre should only house donors or other animals that comply with the same animal health and biosecurity requirements. Distinct sets of equipment and instruments should be maintained for each group of animals with different sanitary status. The instruments and equipment must be employed exclusively in the collection centres.
2. As animals enter the collection facility, they should be maintained in isolation buildings or pens for a specified period, during which they can be tested for various diseases, placed on selected rations and trained for collection. Ideally, personnel that handle quarantined animals should not be involved in the care or collection of animals that have already passed the isolation phase.
3. Once animals have passed the health tests they should be moved into the main facility, where they will be kept while their germplasm is collected. At this stage in the collection process, the main health concerns relate to the potential for introducing a disease from outside the facility. Such risks can be minimized by requiring animal handlers to follow specific sanitation protocols and by keeping rodents and wild birds out of the facility. The risk of contamination from bedding can be addressed by cleaning animals prior to collection. Even though the animals in the collection facility will have passed through quarantine, equipment for germplasm collection such as artificial vaginas and collection tubes should be kept clean and changed for each animal. The centre must be divided in a clean and dirty areas, without cross contamination between them. In addition, the entry of unauthorized persons must be prevented, and authorized visitors must comply with the animal health and biosecurity requirements.

To develop specific derogations at the national level for the collection of germplasm on-farm, different aspects must be considered (see also Box 7.4):

1. Collection on-farm is an option, and its utilization is justified by the genetic value of the donors and the impracticability of collection at designated centres.
2. Field collection of germplasm requires a mobile collection team with adequate equipment for collecting samples, and preferably for processing and short-term storage
3. The sanitary status of the farm/herd/donor should be documented as completely as possible. As no quarantine for the donors is possible, testing should be done before the samples are added to the collection. Backup samples of non-germplasm material like blood or tissue should also be collected and sent to determine the health status of the animal for specific diseases. The sample can also be stored for future testing.
4. During field collection, collectors must respect the sanitation of each collection site, and minimize the risk of spreading diseases. Shipping boxes and other supplies used at one collection site should never be used at other sites. The boxes and supplies may be reused after they have been sanitized, but only at the same collection site. If frozen materials arrive at the gene bank, the liquid nitrogen tank or dry shipper should be sanitized with a 10 percent bleach solution after it has been warmed to room temperature. Non-disposable equipment, such as electro-ejaculators, must be sanitized and rinsed between animals.
5. If personnel travel from site to site, very specific sanitation practices should be implemented. The undercarriage and tires of vehicles should be washed, preferably with a disinfectant, after each site. Likewise, the boots of the personnel should be disinfected, or covered with disposable covers that are discarded after leaving each site. Clothing should also be laundered or changed between collection sites or disposable suits should be worn. Polyvinyl or nitrile gloves should be worn and changed between handling different animals.
6. Conditions in the field may mean that maintaining animals in quarantine for any length of time is impractical. One approach to address the risk of collecting germplasm from infected animals in the field is to draw blood and semen samples from the donor animals tested for the relevant disease(s). Obviously, the germinal products must not be used until the laboratory results arrive at the gene bank.

For species where the necessary biotechnical methods are already in place, germplasm may be obtained from slaughtered animals. Maintaining hygienic conditions at the slaughterhouse is possible and should be maintained at the same level as during on farm collection. Routines like washing the surface of the isolated gonads decrease the risk of spreading diseases. Backup samples should also be collected and stored from every donor. For gene banking purposes, an adequate number of samples can usually be obtained by using epididymal semen or oocytes from isolated ovaries. When collecting ovaries or gonadic tissue (i.e. for avians), the regulations on animal by-products of each country and/or region must be consulted and fulfilled. In the European Union these types of samples are within the scope of the regulation on animal by-products and gene banks must follow these regulations, although they may seem strict for gene banking activities.

7.3 TESTING

Samples should be free from OIE listed diseases**.** In **s**ituations where samples cannot be considered free of these diseases, the trade-off between the risk of spreading a disease and conservation of biodiversity should be assessed on a case-by-case basis. Not all OIE listed diseases are transferable by germplasm. Use of AI and/or ET may in fact disrupt infection chains present in natural mating conditions and help to control the spread of venereal diseases and some zoonoses. However, some infectious agents tolerate the processing and freezing procedures.

BOX 7.4

Sanitary practices for collection, processing, storage and use of AnGR germplasm and non-germplasm material in Uganda

Uganda, like the rest of Africa, manages livestock in endemic situations for some OIE-listed diseases. Control through vaccination is only carried out for some of diseases following outbreak situations. Uganda is mostly collecting and storing semen for artificial insemination (AI) from bulls, boars, and a few bucks. Collection is done by the government, research institutions as well as some private organizations and individuals.

One of the challenges is that biosecurity is often limited at the field collection sites. However, a minimum level of biosecurity may be attained. At the on-station laboratories, regular testing is performed for common endemic diseases such as brucellosis, East Coast Fever, trichomoniasis, campylobacter, vibriosis, mycobacterial diseases and campylobacteriosis. Further biosecurity measures include restricted access to the animal houses of the bull stud, use of foot baths prior to entry into the bull stud and semen collection area, rooms where the staff change their clothing prior and after working with the animals, as well as an access window between the bull stud and the laboratory for passing collected semen.

In the laboratory, the staff work with appropriate protective clothing and other equipment and observe specific hygienic measures to avoid cross-contamination.

Field collection of germplasm is not common in Uganda. Collection is done by a multi-disciplinary team that includes a veterinarian, laboratory technicians, animal scientists and sometimes social scientists. Limited financial resources impact biosafety and biosecurity, procedures are optimised to ensure collection of quality material. Protocols for collection and handling of the different samples are strictly adhered to, even under field conditions.

Field collection of non-germplasm material like blood and other tissues has been initiated for cattle, goats, chickens and pigs. When semen is collected outside AI stations, blood samples for testing for health status are collected. Samples are only collected from animals that are visibly healthy and ascertained by a veterinarian. Semen for gene banking is stored in a restricted area that is only accessible to the trained staff.

Uganda is still building its capacity for appropriate and sanitary gene banking. Disease resistance genes are important for livestock managed in Africa and should be researched upon and promoted for long term survivability and biodiversity. Cryoconservation supports this research and helps ensure that these valuable genes are not lost.

Source: Dr. Sheila Butungi

Therefore, testing for diseases should be carried out before and after collection of samples and, in the case of older material in storage, before use of the material. Backup storage of blood and/or tissue samples as well as germplasm for further testing is important in this respect. In case of oocytes or embryos, samples of the washing agents should be stored as well. Test samples may be stored in normal freezers apart from germplasm. In case of diseases transferable by semen, samples can be split at collection and part of the undiluted sample can be used for PCR testing.

When collecting on-farm or at the slaughterhouse, testing the donors before collection is not always possible. In that case, samples should not be added to the collection immediately; they should be quarantined in separated storage until the test results are available.

7.4 PROCESSING

To be allowed for international trade, germinal products must be processed in authorised laboratories, following the OIE regulations. The laboratory need not be on the same premises as the collection site, so several collection sites of the same hygienic standard may share one laboratory. For example, in Europe, sexing of bull semen takes place in only a few specialized laboratories. All AI centres selling sexed semen from their bulls make use of the services of these laboratories. This practice is safe because the AI centres and the sexing laboratories all share the same hygienic standardsbased on EU regulation 2020/686.

The semen laboratory should be physically separated from the semen collection facilities and include separate areas for cleaning and preparation of collection tools such as artificial vaginas, semen evaluation and processing, semen pre-storage and storage. Entry to the laboratory should be restricted to authorised personnel. The laboratory should be constructed with materials that permit effective cleaning and disinfection and the laboratory should be regularly cleaned. Work surfaces for semen evaluation and processing should be cleaned and disinfected after use.

Protocols for biosafety need to be in place to maintain minimal sanitation standards and prevent any cross-contamination between samples. All instruments that come into contact with the semen in the processing must be cleaned and either disinfected or sterilised prior to use, except for new single-use instruments. Any biological product originating from animals used in the processing of semen, including diluents, additives or extenders, should be obtained from sources that present no animal health risk or are treated prior to use so that such risk is prevented.

For collection outside of collection centres, a mobile laboratory is a good alternative. All steps of processing germinal products are performed in a controlled environment. Disinfection routines after each processing step prevent the spreading of infectious agents between charges. When leaving a farm with possibly infected donors the outside of the mobile lab should also be disinfected with special attention to the tyres and bottom of the vehicle.

Processing may also take place at the site of collection, if suitable premises and the necessary equipment are available. Seminal plasma may carry infectious agents. By centrifugation of the sample and re-suspension of the sperm cells a dilution process takes place. Use of media with added antibiotics, which is compulsory in some countries and the EU, prevents the multiplication of bacteria in the processed sample. The discarded seminal plasma can be stored separately for testing at a later time, if desired.

Embryos and oocytes are to be washed in sterile media several times during the preparation for the freezing process to eliminate most of the potentially infectious agents present on the membrane of the cells, according to the recommendations from the International Embryo Transfer Society (IETS) Manual (IETS, 2009). The processing of embryos or oocytes should be done in a laminar flow facility or other suitable facilities where all technical operations associated with specific sterile are conducted. In addition, it is recommended (IETS, 2009) that the embryo collection team routinely submit to a certified laboratory samples of non-viable embryos or oocytes, flushing fluids or washing fluids for bacterial and viral testing. Tissue samples for the conservation of somatic cells can also be washed before packing.

All samples should be packed in sterile vessels and the vessels sealed to avoid direct contact between the content of the vessels and the freezing agent. Disinfection of the surfaces of the sealed vessels and the use of new cooling and freezing agents for each charge prevents contamination of the freezing

machine and the transport and storage facilities. The processing of the germinal products includes the labelling of the vessels, which is essential for traceability and ensuring the health conditions of the germinal products in the future. Following the International Committee for Animal Recording (ICAR) guidelines for bovine species (ICAR, 2017), the vessels should be marked with the following (in case of embryos and oocytes with a unique reference number to set a cross reference with a paper or accessible electronic files):

- The date of the collection or production of the germinal products, in case of embryos, the date of freezing;
- The individual identification of the donor animals;
- The identification of the collection or production establishment (optional); and
- The breed of the donor (especially for semen);

Other information may be added, such as species, batch or a bar code. The hygienic status of the sample should be documented clearly in the processing protocol and on the vessels themselves as part of the labelling. To attain a high biosafety level, the staff of the collection centres and the laboratories must receive adequate training in disinfection and hygiene techniques to prevent the spread of diseases.

7.5 STORAGE

As outlined in the previous guidelines (FAO, 2012) the sanitary status of samples is a crucial factor either due to risk of cross contamination in the storage facility or risk of infection during subsequent utilization. The classic storage method for samples is in liquid nitrogen (LN) tanks. The samples are stored submerged in the liquid phase at -196°C. The technology is well-established and tanks are available in many sizes and for a multitude of storage vessels. With regard to sanitation, the method has the disadvantage that infectious agents may pass from infected samples into the LN resulting in cross contamination of other samples (Bielanski, 2012). With both technologies, the possibility of pathogens from the environment contaminating the LN or the LN vapour exists. The pathogens may survive in the liquid phase or in the vapour and may contaminate the surroundings after thawing or when dumping used LN. Only new LN should be used when filling tanks and no LN should be allowed to pass from one tank to another. If tank equipment like canisters is transferred from one tank to another they should be thoroughly cleaned and disinfected.

Samples of different sanitary standards should be stored in different tanks. Sanitary status “overrules” species, i.e. samples from different species but similar sanitary status may be stored together. Each tank must carry a label clearly stating the sanitary status of the content. Frozen material from potentially infected donors should be kept in separate tanks and not be allowed to enter the main collection with higher hygienic status.

Storage premises require high biosecurity. They should be fenced and precautions against entry by vermin should be taken. Storage rooms should be easy to clean and should not connect directly to other facilities like stablesor rooms for cleaning collection equipment. A separate room for visitors or people fetching breeding material from the gene bank should be provided to minimize the risk of contamination of the storage rooms. Only trained staff should access the storage rooms and a record of their entry should be kept. Information about the location of each vessel and its sanitary status must be

recorded and stored in a data base (see Section 8) as part of good management practice and to facilitate proper utilization of the material in the future.

7.6 PERSPECTIVES

International recommendations (OIE) and bi-lateral and potentially national regulations on trade in AnGR products have often been an obstacle for the establishment of germplasm banks of endangered breeds (Oldenbroek, 1999). The requirements in these regulations focus on a high health status of genetic material from mainstream breeds. The trade in germinal products from these breeds generates sufficient profit to enable implementation of costly disease prevention measures. For many local breeds, the resources required by the above regulations are not available. As a consequence, the *ex situ* conservation of AnGR for endangered local breeds has often been slowed, delayed or made impossible.

However, an increasing number of countries are choosing to set up tailor-made regulations (Box 7.2) for gene bank activities, so that collection and storage of reproductive material in gene banks is allowed to take place in a manner that ensures adequate protection of animal and public health. The development of new techniques for detecting pathogens in samples themselves, such as PCR, can be a great advantage in moving from a health qualification of the animal to a health qualification of each sample.

In addition, new techniques allow the re-testing of old storage material with modern and more sensitive/specific methods compared to at the time of collection, or even assessing the sanitary status of reproductive material stored without testing (see Box 7.5). In both cases, new testing techniques will substantially improve the information level on the sanitary status of the material in gene bank.

The new sanitary regulations concerning endangered transboundary breeds and how to preserve them via bilateral agreements, as shown in the example to the new European animal health law, offer new perspectives for utilization of cryopreserved AnGR. This implies that coordination of the conservation programs of endangered transboundary breeds, which until now has faced many obstacles (see Box 7.6) can be greatly improved. An example of this is the case of the Turopolej pig, which has a collection of semen in the Austrian gene bank, which was collected according to national regulations but cannot be used in transboundary collaboration with neighbouring countries until the new EU regulation enters into force in 2021.

Tailor-made regulations for *ex situ* conservation activities can allow material with different sanitary status to be stored in the same collection and even in the same storage room if separate tanks are maintained. This underlines the need for accurate recording of all necessary information, including sanitary status, in suitable database systems that also allow safe exchange and utilization of the material. International databases such as EUGENA (https://www.eugena-erfp.net/en/) could be a suitable platform to facilitate bilateral agreements between countries. Section 8 deals with data management in more detail.
Finally, the development of new conservation technologies based on the collection of gonadal tissues will require an adaptation of the regulations on animal by-products in Europe, to allow exceptions where the tissues collected are intended for the establishment of germplasm banks. This issue may be pertinent in other countries and regions.

BOX 7.5

Sanitary measures for collection, storage and gene banking in Vietnam – challenges and initiatives

In Vietnam, more than 70 local breeds are endangered. In 1990 the Vietnamese government initiated a conservation program for animal genetic resources, managed by the National Institute of Animal Science (NIAS). From 1990 to 2015, collection was done primarily by storing semen for artificial insemination (AI) from bulls and boars.

A more recent initiative is the Vietnamese - Japanese project "Establishment of a cryo-bank system for Vietnamese native pig resources and a sustainable production system to conserve biodiversity", running from 2015 to 2020. In this project, semen and embryos from 6 pig breeds were selected, resulting in a total of 7,121 straws and 216 embryos stored at NIAS.

In addition, the FAO project “Cryoconservation of local Vietnamese pig breeds as protection against loss from African Swine Fever” was carried out in 2020. In this project, the ear tissues of 100 local pigs from 5 breeds were collected and conserved at NIAS.

To date, a proper legal and regulatory framework governing the collection, use and conservation as well as sanitary issues regarding gene bank material has not been put in place.

In Vietnam, several OIE-listed diseases are endemic and control through vaccination is only carried out for some of diseases following outbreak situations. Typically, testing for diseases has not been done before or after collection of samples for gene banks, which means that the sanitary status of these samples/ animals is not known. Storage of samples at the gene bank is done either grouped according to breed or time of collection.

In addition, national technicians have not all received professional training in good sanitary measures during genetic material collection and training courses about semen and tissue collection according to OIE standards have not been organized.

The Vietnamese situation underlines the need to establish a legal framework for the national gene bank including the management of sanitary issues. This should include recording the sanitary status of the farm/herd/donor as well as performing tests for relevant endemic diseases before and after collection of samples. Regarding storage, the recommendation will be that germplasm samples of one species with the same sanitary status should be stored in one tank. Samples with different sanitary status and/or of from different species must be stored in separate tanks. Finally, regular training courses on sanitary issues relevant for collection, storage and conservation of genetic material should be provided.

Source: Dr. Ngo Thi Kim Cuc

BOX 7.6

Recommendations for sanitary measures at the West-African Regional Animal Gene bank in Burkina Faso

Framework for the establishment of the West African regional animal gene bank. To put in place an effective framework for the management of African animal genetic resources (AnGR) and to face the threat of loss of their diversity, the Inter-African Bureau for Animal Resources (AU-IBAR) developed the project on animal genetic resources titled "Strengthening the Capacity of African Countries to Conservation and Sustainable Utilization of African Animal Genetic Resources", which was subsequently funded by the EU. The project aimed at strengthening the capacity of countries and regional economic communities to use and sustainably conserve African AnGR through the institutionalization of political,

legal and technical instruments essential for the judicious use of AnGRs at a national and regional level.
The project established hosts for sub-regional gene banks throughout Africa and Centre International de Recherche-Développement de l'Elevage en zone Subhumide (CIRDES), a sub-regional institution, was selected to host the gene bank by the 15 Economic Community of West African States (ECOWAS) countries. The official launch of the gene bank took place in July 2019 in Bobo-Dioulasso (Burkina Faso) and was the opportunity for stakeholders to visit the bank and understand the role of the different facilities provided by the project.
Challenges at the West African regional animal gene bank. The conservation and sustainable use of AnGR at the regional gene bank set up at CIRDES requires the existence of legal and regulatory frameworks to govern the collection, use and exchange of genetic materials between ECOWAS countries as well as sanitary issues. However, this legal framework is currently under development. This means that the gene bank is not yet operational to receive genetic materials from the ECOWAS countries but semen has been collected from West-African transboundary cattle breeds available at the experimental farm of CIRDES. The collected breeds included Zébu Peulh Soudanais, N'Dama, Baoulé, Borgou and Somba.
National technicians have been trained in observing good sanitary practice during genetic material collection to avoid any contamination during and after the collection process. Training on semen collection and conservation was organized for participants from the 15 ECOWAS countries in 2019 at CIRDES, but sanitary issues were not emphasized. Follow-up training on sanitary recommendations will thus be a high priority once the legal framework is established and the sub-regional gene bank becomes operational.
Source: Dr. Isidore Houaga

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SECTION 8

Databases and documentation

Databases and documentation

8.1 INTRODUCTION

As noted in Section 1, for many gene banks, the stored resources consist not only of the biological material, but also of all the associated data. Data administration systems are of critical importance to gene banks, not only for day-to-day management of the collections, but also for allowing potential users to access up-to-date information on the stored material. Improving the availability of information will help to increase the utilization of the gene bank collections. Basic information about gene bank collections should be easily accessible to potential users via the internet, without the need to independently gather additional information from outside the gene bank information system. Approaches that are currently used vary from spreadsheet software to more advanced computer and software systems specifically designed for regular gene bank management and stakeholder utilization (Zomerdijk *et al.*, 2020). Use of a data management system based on spreadsheets is discouraged (except as a provisional initial step for small gene banks with minimal budgets) and such a system will have practically no value for seamless sharing of information with gene bank users. In the context of long-term management and utilization of the gene bank's collection, the information about the material is as important as the samples stored within the gene bank.

The previous guidelines on cryoconservation (FAO, 2012) introduced the essential components of a gene-bank information system, including the data to be captured for each donor animal and for each stored sample. Information technologies have developed rapidly since 2012. While the basic information required for data management and knowledge associated with gene banks remains nearly the same, new types of information about genetic resources have become more readily available or easy to obtain, including genomic and geographic data. In addition, as gene banks gain importance to support the management of *in vivo* populations and for research, the demand for information by stakeholders and the scientific community is likely to increase, prompting the need to establish a data system that provides online access to information that fully characterizes the stored material.

This section reviews the information of critical importance for internal gene bank management and discusses the new types of information available for improved management of gene bank collections. It then addresses issues about placing of gene bank data in the public domain and finally provides examples of several information systems in use for gene banking and animal genetic resources.

8.2 CRITICAL ISSUES FOR MANAGEMENT OF GENE BANK DATA

Due to the special nature of the biological data, several challenges may be encountered when establishing or revising a scheme for data management for gene banks. General issues regarding management of bio- and gene bank data can be identified and are listed below. While databases and information systems will usually be constructed by professional software developers, it is critical for gene bank managers to drive the process of building the system and be full partners in the development and deployment of the software. They must understand the issues of importance for the software developer and be able to communicate with the developer regarding to the needs of both the gene bank and its users.

8.2.1 Data management plan

The first step in developing an information system will be drawing up of a data management plan (DMP). The DMP describes the data to be collected and managed and defines the main rules for data collection and entry, storage and sharing, while taking into account the relevant rules and regulations on data protection. The DMP shall designed considering both present and future needs, and be regularly updated as novels kind of information become available, new data flows are undertaken, or the legal background changes.

The DMP should include a specification of "metadata". Metadata are the set of complementary information that describes the sample of material in the gene bank, as well as information that defines the data. The metadata therefore facilitates comprehension and utilization of the data by other users. As an example, for a sample of material in a gene bank, the metadata are the data fields necessary to characterize the sample (such as (i) type of material, (ii) date of collection, and (iii) animal identification number, etc.), as well as the rules that specify details about of the type of data in each field (such as (i) text, (ii) numeric, in YYYY-MM-DD format, and (iii) alphanumeric). Data systems for gene banks will have different levels of metadata, such as for individual samples or for different donor animals. These concepts will be developed more fully later in this section.

8.2.2 Database technological solutions

Database management systems (DBMS) are software packages designed to manage and optimize the storage and retrieval of data from databases. A DBMS provides a systematic approach to manage databases via a user interface. The various options for DBMS vary widely. Different types of software may be necessary to achieve the final desired system. Software options may include free open-access applications, commercial software, or a combination of the two.

The decision about the most appropriate DBMS is very important. Development of a functional DBMS implies a substantial investment in time and money, even if free software can be used. The criteria for selecting a DBMS for gene bank data should include (i) the expected size of the gene bank; (ii) the input mode and data flow that is in place or will be adopted; (iii) the need to export data or subsets of it; and (iv) the needs for linking to external public and private databases.

8.2.3 FAIR guiding principle

Good data management should adhere to the FAIR principle, stating that scientific data should be made Findable, Accessible, Interoperable, and Re-usable (Wilkinson *et al.*, 2016). Box 8.1 Describes the characteristics of the FAIR guiding principles in more detail.

BOX 8.1
The FAIR guiding principle

The following are the main characteristics of a data management system to ensure that it adheres to the FAIR guiding principles:
To be Findable:

- F1. (meta)data are assigned a globally unique and persistent identifier
- F2. data are described with rich metadata
- F3. metadata clearly and explicitly include the identifier of the data it describes
- F4. (meta)data are registered or indexed in a searchable resource

To be Accessible:

- A1. (meta)data are retrievable by their identifier using a standardized communications protocol
  - A1.a the protocol is open, free, and universally implementable
  - A1.b the protocol allows for an authentication and authorization procedure, where necessary
- A2. metadata are accessible, even when the data are no longer available

To be Interoperable:

- I1.1 (meta)data use a formal, accessible, shared, and broadly applicable language for knowledge representation.
- I2.1 (meta)data use vocabularies that follow FAIR principles
- I3.1 (meta)data include qualified references to other (meta)data

To be Reusable:

- R1.1 (meta)data are richly described with a plurality of accurate and relevant attributes
- R2.1 (meta)data are released with a clear and accessible data usage license
- R3.1 (meta)data are associated with detailed provenance

8.2.4 Data security and protection

Data security is an essential aspect of any database implementation. Sensitive data and any personal information a gene bank holds must be stored securely to adhere to national regulations and to protect it from loss or theft.

Gene bank managers must consider both the physical risk of destruction of data (such as from equipment failure, loss of electricity, fire, or theft) and the risks from hacking or from unintentional corruption of data through human error. Any system must address the issue of keeping the data secure, regardless of whether the data are available to the public or kept entirely in-house.

Risk management and prevention may include steps like establishing computer and software redundancy, and development of continuity of operation plans if the main computing system fails. Data redundancy or backup systems should be physically separated from the primary system. A third backup system may even be prudent, potentially using cloud storage. Secondary power sources such as batteries or generators may be considered to prevent a loss of service due to electrical blackouts. Staff using the system must be properly trained to operate the hardware and software, which will help prevent human error. With regard to cyber-security, a firewall should be used to prevent hacking and access to sensitive data should be limited to people with an approved level of clearance.

Data collection should start with a definition of the level of data privacy and property rights at each step of data management: (i) maintenance of property rights on collection and storage at the gene bank level; (ii) permissions for data transfer to other external storage archives; and (iii) rights to publication or other types of intellectual property. Sensitive data in animal gene banks are usually related to ownership rights and personal information such as the breeder's home or business address and other contact information. However, commercially sensitive data may also be associated with sample and animal data, especially when genomic information is stored, and should be accounted for in a data protection statement.

Different countries and regions may have regulations regarding data sensitivity that must be followed if data are shared. In Europe for example, the General Data Protection Regulation (GDPR) came into force in May 2018, with the aim of providing a high level of protection to individuals' personal data. To avoid placing considerable constraints on scientific research, the GDPR also provides for a two-level framework to enable derogations when scientific research is concerned (Stauton *et al.*, 2019, van der Burg *et al.*, 2020).

8.2.5 Definition of data type

A formal definition of the data and their properties (often referred to as "attributes" of data), which indicates to the computer compiler how the programmer/provider intends to use the data, must be specified for sample and animal metadata. This document should state the expected format for each data type. This concept can be shown with a few examples:

1. *Numeric*: a number, with units specified. The database developer must indicate if units are given with or without abbreviations. The use of terms defined in the "Units of measurement ontology" (https://www.ebi.ac.uk/ols/ontologies/uo) is encouraged (For example, a birth weight could have a value of 1.3 and the units specified as "kilogram").
2. *Dates*: a record of when an event occurred. The format must be described; the use of the following format is recommended, YYYY-MM-DD for dates or YYYY-MM for months.
3. *Text*: simple narrative text. The language must be indicated, as well whether the text is undergoing any check or limitation (such as number of characters).
4. *Alphanumeric:* a combination of numbers and text.

8.2.6 The metadata ruleset

The data types for all data should be compiled in a document called the metadata ruleset. This document should describe with brief sentences the specifications for all sample/animal metadata, including the range of allowable values. The following is an example ruleset for data on the storage conditions for a biological sample in general:

Sample storage (limited value) How the sample was stored. Temperatures are in degrees Celsius. "Frozen, vapor phase" refers to storing samples above liquid nitrogen in the vapor Allowed values are:

- ambient temperature
- cut slide
- frozen, -80 degrees Celsius freezer
- frozen, -20 degrees Celsius freezer
- frozen, liquid nitrogen
- frozen, vapor phase
- paraffin block
- RNAlater, frozen -20 degrees Celsius
- TRIzol, frozen
- paraffin block at ambient temperatures (+15 to +30 degrees Celsius)
- freeze dried

8.2.7 Assignment of a unique identifier and mandatory descriptors

Samples submitted to the database should be assigned a unique identifier and a minimum set of descriptors (i.e. the metadata) matching the metadata rule set, ensuring high quality and comparable data across the diverse collections in different storage formats and languages.

8.2.8 Other data-related issues

8.2.8.1 Geographical data

A geographic information system (GIS) tool should be used for identifying and storing the geographical origin of the samples. This information becomes particularly relevant when assessing the origin of breeds or when studying the genotype by environment interaction or assessing the adaptation ability.

8.2.8.2 Ontologies in animal gene banks

An ontology is a standardized vocabulary that describes objects and the relationships between them in a formal way. Ontology allow sharing of information among the people and software agents (software that facilitates data exchange). Trait ontology is useful so that researchers and stakeholders may communicate with each other more consistently and effectively. Development and definition of ontologies specific to gene and bio banking is an area of critical need for the future to facilitate standardization of associated data systems. An example of an ontology that is currently of common use for livestock is ATOL: The Multi-species Livestock Trait Ontology (Golik *et al.*, 2012).

8.2.8.4 Genomic data

In recent years, several national and international projects (e.g. Bovine HAPMAP Consortium *et al.*, 2009; Stella *et al.*, 2018) have characterized genetic diversity using genotyping and sequencing of thousands of individuals from local, commercial and experimental populations. Much of these data are already in the public domain. Thus, genomic data represent a fundamental feature of samples hosted in gene banks and should be stored and, when possible, made available to stakeholders in commonly-used formats.

For storing and exchanging genotype data files, the so-called “PLINK” format has become a universally recognized standard recommended for gene bank databases (Purcell *et al.*, 2007). The PLINK files are either a bundle of plain text files or of binary files. Various applications are provided by PLINK to manage the data files and to perform statistical analyses (Harvard University, 2017).

Genome sequencing data are highly informative but present significant computing and bioinformatics challenges in terms of data storage capacity, computation time in management, and internet connectivity and bandwidth for transfer of information. The most used format is FASTQ, which is a text-based format for storing both biological sequence information, such as for DNA, and their corresponding quality scores (Cock *et al.*, 2009).

The massive size of genome data files - genome sequence information of for a single animal can contain billions of data points – means that management of genomic data in a local database may prove very inefficient, due to storage and computing capacity limitations. Moreover, more and more frequently, a policy of open data is applied by funding agencies and peer reviewed scientific journals which requires genomic data to be deposited onto public archives and made available to the scientific community. In this context, when gene banks have genomic data that they wish to make public, it is advisable for them to upload the data onto public archives administered by entities such as the European Bioinformatics Institute (EBI) and DRYAD (Dryad, 2021) and link their sample records to these archives, rather than storing the data locally.

8.2.8.5 Non-standardized data

Digital documents, photos and videos can provide valuable information about a gene bank sample and its donor animal or breed. This information should be included in databases where feasible. In general, these types of "data" do not have a standardized type. The metadata should clearly indicate the type of object (e.g. digital file, photo, video) and type of file (e.g. docx, PDF, jpg, tiff, mp4).

8.3 MANAGEMENT OF DATA FOR INTERNAL USE

The management of data for internal use is mainly concerned with sample retrieval, essential description and storage of technical information for sample management. However, it also provides the basis for data sharing, for querying and analyses that support decision making and optimization. Furthermore, the database should record the information necessary to trace a sample of material from the time of its entry into the gene bank until it exits the bank for utilization, as well as the conditions of its exit and subsequent utilization.

Tables 8.1 and 8.2 lists the information (metadata) regarding donor animals and stored samples, respectively, that should be considered required or recommended for gene banks, irrespective of their size and other characteristics.

Table 8.1 **Donor animal information: required and recommended additional database fields**

Data field	Data type[a]	Necessity	Examples	Data sensitivity
Animal ID				
Repository ID[b]	A/N	required		Low
Official national ID[b]	A/N	recommended		Medium
Breed association ID[b]	A/N	recommended		Medium
Producer ID	A/N	recommended	Round Oak Rag Apple Elevation	Medium
Markings	A/N	recommended	e.g. tattoo number, description	Medium
Animal demographics				
Birth date	A/N	recommended		Medium
Sex	A/N	required		Low
Provider				
Owner name[c]	T	required		High
Breeder name[c]	T	recommended		High
Geographic location	A/N	required	e.g. mailing address or geographical coordinates	High
Taxonomy				
Species	T	required	Latin name preferred	Low

Breed	T	required		Low
Population	T	required		Low
Production environment				
Natural[d]	T	recommended	e.g. semi-arid, arid, subtropical, tropical	Low
Production system	T	recommended	e.g. extensive, mixed crop-livestock, intensive	Low
Phenotype				
Body measurements	N	recommended	e.g. weights and measurements at various ages	Medium
Visual identifiers	A/N, D	recommended	e.g. coat colour, markings, digital photographs	Medium
Production traits	N	recommended	e.g. milk yield, fleece weight, litter size	Medium
Genotype				
Pedigree	A/N, D	recommended	3 generations if possible	Medium
Genetic test results	A/N	recommended	e.g. genetic defects	High
Genetic markers	A/N	recommended	SNP, sequence, microsatellite, chip used	High
Breeding values	N	recommended	e.g. for production traits	Medium
Breed information				
Census data	N	recommended	breed averages	Low
Phenotypic descriptors	A/N	recommended	known genetic attributes	Low
Genotypic descriptors	A/N	recommended	production systems where the breed is prevalent	Low
Production systems	A/N	recommended	breed averages	Low

[a] T = text, A/N = alpha numeric, N = numeric, D = digital file

[b] May all be the same number

[c] May be the same

[d] Can be obtained from geographic information systems if geographic coordinates are recorded

Table 8.2. **Sample information: recommended minimum database fields (to be associated with animal identification)**

Trait	**Data type**[a]
Collection	
Date	Date
Location	A/N
Sample type	
Material	T
Sample quality (examples)	
Semen	
- Temperature at arrival in lab	N
- pH at arrival in lab	N
- Pre-freeze motility	N
- Post-thaw motility	N
Embryo	
Grade before freezing	A/N
Stage of development	A/N
Quality after freezing	A/N
Straw Information	
ID	A/N/ or bar code
Freeze date	N (can be multiple)
Species	A/N
Breed	A/N
Number of straws/containers per animal	
Storage information	
Storage conditions	A/N
Kind of straw, ampule or pellet	A/N
Tank	N
Placement in tank	N
Collection method	
Semen	A/N
Embryo	A/N
Oocyte	A/N
Somatic cells	A/N
Freezing protocol used[b]	
Semen	A/N
Embryo	A/N
Oocyte	A/N
Somatic cells	A/N

Sample ownership	
Semen	A/N
Embryo	A/N
Oocyte	A/N
Somatic cells	A/N
Sample sanitary status	
Semen	A/N
Embryo’	A/N
Oocytes	A/N
Somatic cells	A/N
Sample distribution	
User (person or organization)	A/N
Contact information	A/N
Date of release	Date
Terms of utilization (e.g. copy of material transfer agreement)	D

[a] T = text, A/N = alpha numeric, N = numeric, D = digital file

[b] Thawing instructions may be included as additional information.

Tables 8.1 and 8.2 are not exhaustive. The following are examples of complementary information and data types that may be relevant for the gene bank samples:

- specimen collection protocols (digital files; A/N): stand-alone files or URL links to the protocol used to collect this sample;
- description (Text): brief descriptions of any special features of samples not fully captured by the basic data of the sample (Table 8.1);
- photos or videos of the raw or processed sample (digital): microscopic images to indicate quality of the sample;
- staff initials (Text): the initials of the laboratory technician or contact for the sample; multiple persons can be provided;
- documents (digital files): word processing or PDF files of important documents, such as protocols applied to process the sample, material transfer agreements and other contracts;
- organization URL (A/N): the website(s) of organizations associated with the sample; and
- publication DOI (A/N): Publication(s) associated with the sample, supplied as the digital object identifier (DOI) which can unambiguously point to the publication.

In addition to information directly related to stored gene bank samples, a gene bank may find it valuable to store other types of complementary information in their database. For example, as described in Section 2, quality management of a gene bank involves the documentation of key processes that ensure the bank operates correctly. This documentation should be stored in the database. The database is also a logical tool to store and manage records of gene bank operations, such as monitoring of storage room temperatures and oxygen levels.

8.4 SHARING OF DATA WITH EXTERNAL STAKEHOLDERS

Gene banks must manage their collections in ways that promote their utilization by increasing access not only to the stored material but also to the potentially vast amounts of data that describe them. As discussed earlier in this section, examples of such data include genomic information, geographic "layers" of descriptors of the characteristics of the location where the donor animal was living, and data about the animal's breed. Ideally, data and tools should flow in both directions, from gene banks to users and from users to gene banks, to add value to the collections and facilitate their further use.

Few gene banks will be willing to make all of their data available to the public. Therefore, a gene bank's information system should be built to differentiate between the privately and publicly available data. Implementation of a userid-password or other system or regulate and monitor access to the different parts of the data system is strongly recommended. This system should also the rights associated with each user, such as to which areas of the system the user has access, and whether the user can change the data or simply read it.

Two main options exist for making data information systems available online: (i) in-house server; and (ii) commercial "cloud" service provider. The in-house option may provide greater control of access to the data but equipment will require maintenance and will likely need to be replaced on a regular basis as technology advances or the amount of information to be handled increases. The gene bank will also need to establish a secondary data storage unit for backing up the data and commit to following a regular (preferably daily for active gene banks) schedule for backing up their data. The in-house option may be prohibitive for a small, independent gene bank, but may be feasible if the gene bank is associated with a larger institution, such as a university or government research institute or ministry. With a commercial provider, the need for equipment purchase and maintenance will be eliminated, as will some of the need for a staff member specialized in these tasks. Nevertheless, some in-house capacity in information technology will be required in any case. Most commercial providers will include services to back up the data on a regular basis, as well as guaranteeing a certain level of data security. With a commercial provider, the gene bank risks losing some control over its data, and may lose data access if internet failures occur. In terms of costs, there is no general rule on which option is more favourable. Each gene bank will need to undertake its own cost comparison if it is considering these two options.

8.5 INTERNATIONAL INFORMATION SYSTEMS

Several international information systems are of relevance for national gene banks. Each has its own roles, some of which may be overlapping. Among these roles are international data sharing and reporting to meet international obligations. Some of the systems can also be used for managing internal sample data. Brief descriptions of these systems are given below.

8.5.1 DAD-IS and EFABIS-net

The Domestic Animal Diversity Information System (DAD-IS - http://fao.org/dad-is) is not a gene bank database, but it plays an important role for countries undertaking cryoconservation. DAD-IS, developed and maintained by FAO, is the web interface to the Global Databank for Animal Genetic Resources. The European Farm Animal Biodiversity Information System network (EFABIS-net) is the network of European national "nodes" providing unique interfaces to DAD-IS. The DAD-IS holds

information on more than 15 000 national breed populations (representing around 8 800 breeds and about 40 species) from 182 countries. The data in DAD-IS include basic information about each breed's physical appearance (including photos) and other defining characteristics, geographic range, history, uses, common management conditions, productive and reproductive performance, population size and any institutional form of management, including breed associations, *in situ* conservation programmes or gene banks. Data in DAD-IS are inserted exclusively by the National Coordinator for Management of Animal Genetic Resources of each country (National Coordinator), an official nominated by the government. Because of this formal, single point of entry approach, completeness of data for each breed varies widely among countries.

The formal approach for data entry is necessary, however, because the data in DAD-IS are official information from each country and are used for intergovernmental purposes. For instance, DAD-IS is recognized by the Convention Biological Diversity as its Clearing-House Mechanism (CBD, 2020) for monitoring of the genetic diversity of domesticated livestock. DAD-IS is also the source of data for Indicators for the UN's 2030 Agenda for Sustainable Development, commonly known as the Sustainable Development Goals (SDG). Specifically, DAD-IS is the source of data for SDG Indicators 2.5.1b and 2.5.2 which address cryoconservation and *in vivo* conservation of animal genetic, respectively.

Indicator 2.5.1b is *Number of animal genetic resources for food and agriculture secured in medium-or long-term conservation facilities.* For this indicator, only local breeds are included, and a breed is considered to be "secured" if the amounts of cryoconserved genetic material are sufficient to allow the reconstitution of the breed, should it become extinct *in vivo*. Countries are requested to insert into DAD-IS the numbers (if any) of doses of semen, embryos, oocytes and somatic cells for each of their breeds and the numbers of donor animals providing each type of material. The National Coordinator can either indicate whether these quantities are sufficient for breed reconstitution given the goals of the country and typical reproductive efficiency of stored samples or allow for DAD-IS to calculate sufficiency according to a standard algorithm. Countries lacking gene banks are also requested to explicitly insert this information to eliminate uncertainty regarding the status of their breeds.

Gene bank managers should communicate regularly with their respective National Coordinators to provide data on material stored and thus help improve the quality and quantity of gene bank data in DAD-IS. Insertion of these data is also important for countries to meet their international obligations with respect to monitoring of implementation of the SDGs. DAD-IS includes a web page where all National Coordinators and their contact information are provided: (http://www.fao.org/dad-is/national-coordinators/en/).

FAO has recently developed an application programming interface (API) that allows the exchange of data between DAD-IS and other systems. The API will facilitate the transfer of data from national databases to DAD-IS, eliminating the duplication of efforts associated with reporting in both systems. Also, DAD-IS has the capacity to upload comma separated value files that contain the variables reported in DAD-IS. National systems that can export their data as CSV files can easily share their data with DAD-IS report on the official indicators of animal genetic diversity.

8.5.2 ANIMAL Genetic Resources Information Network

All animal gene banks have the common need to manage information about their respective country's genetic resources. The Agricultural Research Science, research agency of United States Department of Agriculture developed a first version of a database to deal with gene bank information, called Animal

GRIN (Genetic Resources Information Network), in 2001. As time passed the need for continual evolution and improvement of Animal GRIN, including to facilitate international cooperation, became apparent. In 2006, the programmes for animal genetic resources for food and agriculture in the United States of America (USA), Brazil and Canada formed a consortium to develop, implement and use a common information system to accomplish effective banking of biological material as well as to monitor *in situ* conservation programmes. This represented the first time that three countries from different economic blocs have become partners to build a common database for genetic resources. This new information system is recognized today as Animal GRIN v.2 in English and "Alelo Animal" in Portuguese. It serves as a successful example of the type of collaboration encouraged by the Interlaken Declaration on Animal Genetic Resources (FAO, 2007).

Animal GRIN v.2 was developed in a customized way to meet each country's needs. For example, the USA programme (National Animal Germplasm Program, NAGP) does not include *in situ* conservation; hence, a live animal database was not part of the initial version of the database. This feature was included in version 2, however, to allow Brazil (Embrapa) to document the populations being conserved *in vivo* at their research centres. Version 2 of the system was implemented after discussions with numerous stakeholders including both traditional and emerging industries dealing with not only traditional livestock, but also aquaculture and honey bee species. The type of information included consists of a strong taxonomic structure, animal identification, pedigrees, germplasm/tissue storage, phenotypic information, descriptors of the production system where the animals were raised and recently, genomic (single nucleotide polymorphism chip data) and geographic coordinates.

The database can be publically viewed, providing real-time access to the database from anywhere in the world. The reports are generated via simple point and click procedures, requiring no prior knowledge of the database. AnGR management tools allow evaluation of the genetic diversity stored in the gene bank and assessment of needs before addition to the collection. The tools can be used to compare the different countries' collections of the same transboundary breeds and to develop tools that assist with *in-situ* conservation activities. Another important new and strategic feature implemented in 2021 is the capacity to share data with DAD-IS Information System.

The system has different URL for each country, but can be accessed from anywhere in the world:

- USA (https://agrin.ars.usda.gov/);
- Brazil (http://aleloanimal.cenargen.embrapa.br/) and
- Canada (https://www.agr.gc.ca/eng/scientific-collaboration-and-research-in-agriculture/agriculture-and-agri-food-research-centres-and-collections/animal-genetic-resources-of-canada).

8.5.3 EUGENA

The European region, through its Regional Focal Point for Animal Genetic Resources (ERFP), has established the European Genebank Network for Animal Genetic Resources (EUGENA). EUGENA is a network of member gene banks in European countries with the overall aims to support *ex situ* conservation and sustainable use of AnGR and facilitate the implementation of the Global Plan of Action and the Nagoya Protocol for Access and Benefit Sharing in Europe.

The specific objectives of EUGENA are:

- to support gene banks in ERFP member states to fulfil their individual roles and objectives;

- to improve monitoring and assessment of AnGR kept in *ex situ* collections in ERFP member states by sharing information on gene bank collections;
- to improve gene bank operations and procedures in ERFP member states by sharing information;
- to contribute to the long-term conservation and maintenance of AnGR in *ex situ* collections in ERFP member states;
- to use synergies for *ex situ* conservation and sustainable use of AnGR by joint activities of gene banks in ERFP member states;
- to increase the efficiency of *ex situ* conservation and sustainable use of the genetic diversity of transboundary breeds;
- to promote harmonization of acquisition and access terms for *ex situ* conservation and sustainable use throughout the gene banks in ERFP member states;
- to facilitate improved quality management of the gene banks in ERFP member states;
- to create an element of European research infrastructure for conservation and sustainable use of AnGR; and
- to facilitate a regional European approach for international cooperation and exchange of AnGR in the context of the implementation of the Nagoya Protocol for Access and Benefit Sharing.

In 2017, the ERFP developed the EUGENA web portal as a single-entry point to the register of EUGENA gene banks in Europe. Via this portal, information is available to the public about EUGENA, how it operates, and which countries and gene banks are participating. Users can therefore follow the activities of the network. The web page provides an overview of the collections at each of the member gene banks and contact information for each gene bank. This system allows all gene banks (including those without their own web pages) to present their collections and provide researchers, policy makers and breeders with valuable and timely information.

The EUGENA portal includes a free download section with templates of all relevant documents for gene banks wishing to join the network. All gene banks can benefit from the guidelines for the development of material acquisition agreements (MAA) and material transfer agreements (MTA) (see also Section 9), which are freely available for download from the portal.

The EUGENA portal contains a register of all member countries, the number of member gene banks in each country, the number of breeds and species represented in the collection and the total number of samples reported to the portal.

The individual page of each country in the portal is a dashboard, containing statistics of the distribution of the samples by breed, species and material type, a register of the member gene banks in the country including a map with their administrative locations, contact details of the country representative and links to other relevant websites. The dashboard provides links to the FAO country profile, to the list of breeds reported to DAD-IS by this country, and to its Access and Benefit Sharing Focal Point.

The register of the member gene banks in EUGENA contains the abbreviated name of the respective gene bank, the year when it became member of EUGENA, its internal EUGENA ID, and the total number of species, breeds and samples reported to the portal.

Many countries have multiple gene banks, so each bank also has its own dedicated page. The individual page of a given gene bank is a dashboard, containing the contact details of the gene bank, a map with the location of its storage facilities, distribution of the samples per material type, and a list of all the breeds represented in the collection. In addition, for each breed the list includes the amount of preserved material, the collection period, and the number of donors. As EUGENA uses the same breed names as DAD-IS and EFABIS-net, each breed is automatically linked to its respective data sheet in DAD-IS.

The portal has a built-in advanced search tool allowing the user to query data by combining several search criteria, including gene banks, species, breeds, material type, percentage of material with defined EU sanitary status and others. The search tool is the first stop for users searching for material from a certain breed within the EUGENA network. The tool provides export of the search results in machine-readable CSV format. The EUGENA portal is accessible on the following address: https://eugena-erfp.net.

8.5.4 IMAGE Data Portal

In recent years, a large amount of genome sequence and genotyping data have become available through publicly funded research projects and breeding programmes. In addition to the information produced by modern genomic technologies, other types of very valuable information are also available, such as existing gene bank information, GIS data and phenotypic data. Therefore, large research projects, which generate data and information on thousands of samples and individuals, need to properly organize the collection and recording of genotypic and phenotypic data in order to facilitate the submission of these data to public archives/databases. Experience shows that information is often segmented with a lack of direct connection between the different sources of information, which has hampered the full exploitation of the currently available genetic resources.

The European Union Horizon 2020 project IMAGE (Innovative Management of Animal Genetic Resources; 2021) was carried out from 2016 to 2020 with the overall aim to enhance the use of genetic collections and to upgrade animal gene bank management. One of its goals was to create ad hoc user-friendly solutions and interfaces to aggregate information from different resources and allow both simple and complex queries. More specifically, the key task was to create a web portal that would facilitate the integration and transparent use of the vast information stored within more than 60 gene banks/genetic collections spanning 20 European countries, combined with the collection of newly generated data as part of the IMAGE project (IMAGE, 2021).

The IMAGE web portal integrates data from gene banks and other collections with genomics data, GIS data, as well as other information generated in the project. The solution that was developed comprised the following:

1. a well-defined metadata rule set ensuring high quality and comparable data across the diverse collections in different storage formats and languages;
2. development of a single “Inject tool” helping gene bank managers to enhance, standardize, tag and submit data to a Common Data Pool that integrates all gene bank records from across Europe;
3. sustainability by archiving of this data within the BioSamples public archive of EBI; and
4. a bespoke data portal that integrates gene bank metadata with generated ”omics” datasets from within IMAGE and cross referencing to other gene bank and breeding database resources from across Europe, such as DAD-IS and EUGENA.

Within the data portal, a GIS tool is included to assist the user in identifying/storing the geographical origin of the samples as well as displaying individual/population genetic parameters and biological attributes through interactive maps. Querying across all types of data is also expected to facilitate targeted search to identify genetic material of interest residing somewhere in the partner gene banks and collections. Furthermore, starting from data derived from the portal, computing tools and methods have been developed to browse the diversity of sample and/or genomic data. The Diversity Browser is a stand-alone tool that computes principal component analysis (PCA; Reich *et al.*, 2008) of a reference dataset and a batch of samples of interest.

Finally, an interactive web interface to guide the use of genetic material was created. It allows selective downloading of collection and genotype information to be leveraged using a linked R software package (MoBPS – See also Section 5) that provides a flexible framework to simulate complex breeding programs and compare their economic and genetic impact.

Although the IMAGE Data Portal was developed through a project of the European Union, its use is open to gene bank managers and their data from any country.

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SECTION 9

Legal issues: Acquisition, storage and transfer of gene bank material

Legal issues: Acquisition, storage and transfer of gene bank material

9.1 INTRODUCTION

Adoption of national cryoconservation measures and establishment of "facilities for *ex situ* conservation of and research on plants, animals and micro-organisms, preferably in the country of origin of genetic resources" are obligations of the Parties to the Convention on Biological Diversity (CBD, 1992).

The need for establishment of *ex situ* gene banks was also recognized by governments adopting the Global Plan of Action for Animal Genetic Resources (Global Plan of Action; FAO, 2007). Strategic Priority 9 calls for establishment or strengthening *ex situ* conservation programmes and identifies six actions necessary to achieve this goal. One of the actions includes establishing modalities to facilitate use of genetic material stored in *ex situ* gene banks under fair and equitable arrangements for storage, access and use of animal genetic resources (FAO, 2007). Moreover, the Interlaken Declaration, through which the Global Plan of Action was adopted, recognizes the important role of private ownership in the management and conservation of AnGR and thereby the necessity for animal owner concurrence in the collection and management of germplasm for gene banking purposes (Blackburn and Boettcher, 2010).

The need to conserve genetic resources of cultivated plants and farm animals was strengthened by adopting the Strategic Plan for Biodiversity 2011-2020 (CBD, 2010a). Target 13 of the Aichi Biodiversity Targets requires that "by 2020, the genetic diversity of cultivated plants and farmed and domesticated animals and of wild relatives, including other socio-economically as well as culturally valuable species, is maintained, and strategies have been developed and implemented for minimizing genetic erosion and safeguarding their genetic diversity". Some of this diversity can be only maintained in *ex situ* collections.

Many governments, recognizing the importance of national cryoconservation programmes for animal genetic resources have committed themselves to provide the financial and other resources needed to establish and maintain gene banks. The involvement of public financial support for long term *ex situ* conservation strategies in many circumstances required development of policies and a legal basis for establishment and operation of *ex situ* facilities. Such measures could take a form of specific national legislation, the inclusion of gene bank-related regulations into existing law, or through delegation of responsibility and power over management of gene bank-related issues to relevant public or private entities.

Government involvement in cryoconservation also implies responsibility for the implementation of the Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization (Nagoya Protocol; CBD, 2010b), which is an instrument to execute the third objective of the CBD. Storing animal genetic resources in a gene bank provides a public repository of these resources that are, in general, obtained form privately owned animals. For many countries, national laws or specific regulations dealing with gene banks have not yet been fully established. Generally, legal frameworks of a wider scope are already in place, such as animal breeding, animal health and sanitary regulations, legislation dealing with protection and conservation of genetic diversity including conservation of local endangered breeds, as well as private property and

contract laws, but specific measures or regulations are needed for the establishment and management of gene banks.

Gene bank specific legislation, although directly related to the establishment and management of the gene bank and therefore very important, is often accompanied by other laws to be observed by the gene bank, for instance veterinary legislation (see also Section 7). Animal breeding laws may also have separate provisions regarding information on the individual donor of reproductive material that has to accompany a breeding animal or a sample of its germinal material. Such information may be also required by law on samples provided to (and by) the gene bank. Therefore, gene bank managers must be aware of the national laws and policies that establish operational boundaries and have clear protocols and instruments to be able to operate within such framework.

Management of gene banks includes entering various types of agreements on acquisition of germplasm or other biological material as well as procedures to make stored material available for use upon request. The rules guiding such agreements should be transparent and they should include rights and responsibilities of the gene bank, the users of the gene bank's material and, if relevant, obligations and rights of the original owners or donors of the samples. Policies and procedures for drawing up such agreements should be established. Box 9.1 provides a glossary for laws, agreements and other regulatory elements concerning genetic resources in gene banks.

BOX 9.1

A glossary regarding laws, agreements and other important terms relevant for genetic resources

ABS, Access and Benefit-sharing: Access and benefit-sharing refers to the way in which genetic resources may be accessed, and how users and providers reach agreement on the fair and equitable sharing of the benefits that might result from their utilisation.

ABS-CH, Access and Benefit-Sharing Clearing-House: a key tool for facilitating the implementation of the Nagoya protocol. It is a web-based information system to share domestic ABS related information, for instance to check if the country of origin is a party to the Nagoya Protocol and established access measures (https://absch.cbd.int).

CNA, Competent National Authority: is responsible for granting access or, as applicable, issuing written evidence that access requirements have been met and for advising on applicable procedures and requirements for obtaining prior informed consent (PIC) and entering into mutually agreed terms (MAT).

DDD, Due Diligence Declaration: is a declaration submitted to DECLARE where the user demonstrates that the access to the genetic resources and/or the traditional knowledge associated with genetic resources was done in accordance with the regulations established by the provider country.

DECLARE: a web-based tool which enables users of genetic resources to submit the due diligence declarations required by Article 7 of EU ABS Regulation.

IRCC, Internationally Recognized Certificate of Compliance: The Nagoya Protocol

establishes that domestic access permits that are made available to its ABS-CH shall constitute "internationally recognized certificates of compliance". All Parties with users in their jurisdiction must recognize such certificates as the evidence of acquisition of the genetic resource and, if relevant, an associated traditional knowledge in accordance with applicable ABS measures of the provider country.

MAA, Material Acquisition Agreement: Agreement for material intended to be stored in a gene bank for conservation, sustainable use, research and development of animal genetic resources.

MAT, Mutually Agreed Terms: Conditions agreed upon in the contract by providers and users for the access to and the utilization of genetic resources as well as the sharing of benefits in accordance with the Nagoya Protocol.

MTA, Material Transfer Agreement: a contract that accompanies the physical transfer of a genetic material from the gene bank collection to a user. The MTA stipulates the terms for the transfer, such as how the recipient will be allowed to use, make available and dispose of the material obtained.

The Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization to the Convention on Biological Diversity; Nagoya Protocol: an international agreement which aims at sharing the benefits arising from the utilization of genetic resources in a fair and equitable way. It entered into force on 12 October 2014.

PIC, Prior Informed Consent: In the context of ABS and the Nagoya Protocol, PIC refers to the administrative permit given by the competent national authority of a provider country to a user, prior to accessing genetic resources. However, the term is also used in relation to the right of indigenous and local communities to take a free and informed choice on whether they wish to give access to genetic resources or traditional knowledge associated with genetic resources. Parties to the Nagoya Protocol are obliged to include their Indigenous and Local Communities in the process of granting access to genetic resources and traditional knowledge associated with genetic resources.

9.2 LEGAL BASIS, STATUTES, DECISION MAKING BODIES AND PROCEDURES

The legal basis and processes of establishment of gene banks differ substantially among countries. Policies may be established by the gene bank management or their decision-making bodies or may be developed at a higher level, such as through national legislation. Box 9.2 describes establishment of legal frameworks for animal gene banks using examples from different countries.

Each country/gene bank needs to define clear procedures/protocols for all aspects related to processing of animal biological material and associated information including acquisition, storage, as well as transfer and use of gene bank material. The tools that are most useful in establishing internal gene bank procedures include standardized documentation such as material acquisition agreements (MAA) and and material transfer agreements (MTA).

Gene bank policies and specific regulations related to acquiring and dispersing germplasm generally follow a guiding principle that the overall objective of the gene bank is to facilitate the conservation,

sustainable use and development of AnGR to support the country's livestock sector. Therefore, the conditions for the acquisition or release of germplasm should not be unnecessarily restrictive as to limit the development and enhancement of collections or the use of the material stored in the repository.

Box 9.2
Examples of national frameworks for establishment animal gene banking

The French National Cryobank was initiated in 1999 as a project, co-funded by twelve organizations involved in the management of animal genetic resources. They included research institutes, artificial insemination (AI) centers, veterinary laboratories and a federation of domestic breeds associations. All these organizations signed the National Cryobank convention, with the French Agricultural Ministry being the main signatory and the major source of financial supporting for the bank (Duclos and Danchin-Burge, 2012).

In **the United States of America,** in 1990, Congress (USC) enacted legislation on the National Genetic Resources Program, describing establishment, purpose, administration and functions of the programme (USC, 1990). This law provided the United States Department of Agriculture (USDA) with a mandate to conserve AnGR. As a result, in 1999 a decision was taken to initiate the National Animal Germplasm Program (Blackburn, 2009). The programme has been under continual development over the years to enhance the scope of objectives and activities performed; the current edition covers the 2017-2022 period (https://www.ars.usda.gov/research/project/?accnNo=433404). The programme has to implement a number of USC law provisions, for instance the gene bank "have the right to make available on request, without charge and without regard to the country from which the request originates, the genetic material that the programme assembles". So the policy of the USC provided a clear guidance on this aspect of germplasm management.

In **Czechia,** the key provisions on National Program for the Conservation and Utilization of Genetic Resources are included in the Czech Animal Breeding Act (No. 154/2000 Col.). According to this law, the Ministry "shall include a gene bank in the National Programme on the basis of its written request, provided that the gene bank's operating rules guarantee the proper preservation and registration of genetic and biological material in accordance with the specification of the stored genetic and biological material" (CZECHIA, 2000). The state budget covers operating costs of the gene bank, provides funds for the purchase of reproductive material obtained on regular basis (mostly semen and embryos) as well as costs for its processing (transport, maintenance and insurance of animals kept in artificial insemination stations and preparation of samples). The Ministry Decree to the Breeding Act sets the minimum stock (core collection) for each breed and material from the gene bank is only made available for utilization if the quantity remains above this limit (CZECHIA, 2017).

Gene banks must contend with a range of different types of agreements concerning the acquisition and release of germplasm. Because of the long-term nature of the gene bank's mission and the interest of a variety of stakeholders, it may be useful to establish a multi-stakeholder board of interested parties (see also Section 1 or 2). This committee may or may not be the same as a National Advisory Committee on AnGR (FAO, 2009). The board's mission would be to provide advice and recommendations on the gene bank strategy and on policies for acquiring and distributing germplasm.

The board would be able to provide the gene bank with advice on how agreements between actors should be formulated.

One of the very important issues to consider is the ownership of the stored material, and the associated conditions for (future) use of the material, as donor animals are most often a private property. As suggested by Blackburn and Boettcher (2010), the gene banks may choose to either obtain ownership over the germplasm entering the collection or develop specific working arrangements to obtain material from individual breeders, companies or breed associations who will maintain rights over the material. The role and rights of owners of donor animals or their biological material can differ substantially, leading to differences in the processes of decision making in gene banks. The gene bank in the USA has a specific procedure for deciding if material can be released, as described in the previous guidelines (FAO, 2012). Other examples are provided in BOX 9.3.

BOX 9.3

Examples of varying decision-making in gene banks and in the ownership of donor animals or their biological material.

In **the French Cryobank,** the decision-making bodies are the Group Council, representing all partners, and the Cryobank Scientific Committee consisting of 13 experts in cryopreservation, population genetics, biodiversity and sanitary rules (Duclos and Danchin-Burge, 2012). This committee served exclusively for the purpose of the gene bank until 2011 but since then its role and functions have been broadened (Tixier-Boichard, 2012).

Different solutions were adopted in **China**. According to Chapter II on Protection of Genetic Resources of livestock and poultry of the Order No. 45 of December 29, 2005 by the President of China, "No gene banks of the genetic resources of livestock and poultry sponsored by the Central Government or provincial governments shall dispose of the protected genetic resources of livestock and poultry without approval by the administrative department for animal husbandry and veterinary medicine under the State Council or by such a department under the people's government at the provincial level" (Order No. 45, 2005). In this case the decision-making process stays with the national/local governments that financially support maintenance of the gene banks.

In **Czechia** the material stored in the gene bank becomes the property of the National Coordination Center and has no commercial value. The center decides on the conditions of its further use. Standard agreement procedure is used for any receipt and transfer of material (Matlova, 2012).

Similar approach was adopted in **the Netherlands** with quality management system (QMS) procedures for acquisition and use of biological material. Decisions are taken by gene bank management in consultation with relevant breed societies (Hiemstra, 2021).

In **Denmark**, the government supports collection and storage of genetic material from the native and locally adapted breeds of cattle, pigs, horses, sheep, and goats. The AnGR stored in the gene bank become property of the government. Distribution of the material is free of charge, after the approval of the National Advisory Board on AnGR (Martyniuk *et al*., 2019).

Contrarily, for the **French** National Cryobank, the governing Council decides which biological material can be put in or taken out of the gene bank. A depositor keeps ownership rights over

material but leaves the collection management to the Group Council. If a depositor wants to use his own samples, the decision process is much faster to keep the gene bank's management practical. Also, to use a sample, the depositor's agreement is mandatory, and reconstitution of the stock is obligatory (Duclos and Danchin-Burge, 2012). If commercial use is planned, a financial agreement must be made between the applicant, the material depositor and the gene bank.

The ownership issues are even more complicated in the case of establishment of regional gene banks. Five African regional multi-purpose animal genetics banks were launched in July 2019, located in Botswana, Cameroon Uganda, Tunisia, and Burkina Faso, (https://www.asareca.org/news/three-regional-animal-gene-banks-simultaneously-launched-across-africa). These banks will have to deal with various difficult issues such as access to the gene bank collection and purposes for access; the rights and conditions of approval, and potential benefit sharing (AU-IBAR, 2015). These issues are complicated by the fact that activities will be undertaken across borders and each of the banks are still in the process of resolving these issues.

9.3 THE NAGOYA PROTOCOL AND NATIONAL LEGISLATION ON ACCESS AND BENEFIT SHARING

Since 1993, when Convention on Biological Diversity came into force, gene banks managers have had to take into account also issues of access and benefit sharing. The states have the sovereign rights over their own resources and can exercise control over the genetic resources within their jurisdiction. According to Article 15 of the CBD, a prior informed consent (PIC) of the party providing genetic resources is needed for legal access (unless that party has decided otherwise) and utilisation and benefit-sharing must be done according to mutually agreed terms (MAT). Following these provisions, domestic access and benefit sharing (ABS) legislation was established in various countries, some of them also included specific ABS regulatory measures for ex situ collections.

The Nagoya Protocol also addresses a number of very important issues. For instance, Parties to the protocol are required, in the development of their ABS measures, to create conditions to promote and encourage research which contributes to the conservation and sustainable use of biological diversity, to pay due regard to cases of present or imminent emergencies that threaten or damage human, animal or plant health and to consider the importance of genetic resources for food and agriculture and their special role for food security. FAO (2019) has summarized the elements to be considered in national policies regarding ABS.

9.3.1 Implementation of the Nagoya Protocol

As of 10 March 2021, 130 countries had ratified the Nagoya Protocol. As reported in the interim national reports on the implementation of the protocol, the majority of countries (68 percent) have considered the importance of genetic resources for food and agriculture in the development and implementation of their national ABS frameworks (Garforth, 2018).

To provide an example, in the European Union (EU), the Nagoya Protocol is implemented in a harmonized way. Regulation (EU) No 511/2014 (EU, 2014) establishes compliance measures for users of genetic resources and associated traditional knowledge arising from the Nagoya Protocol. The EU ABS Regulation regulates only the implementation of the third pillar of the Nagoya Protocol within the Union and its common market. The access measures are under the sovereign rights of the member

states and benefit sharing contractual arrangements are decided bilaterally by parties establishing MAT (Martyniuk and Kozłowska, 2018).

The EU ABS regulation provides for checks on user compliance to be carried out by the member states and establishes two voluntary mechanisms facilitating compliance: best practices and registered collections. Regulation (EU) 2015/1866 lays down detailed rules for the implementation of EU ABS regulation as regards the registry of collections, monitoring of user compliance and best practices. It establishes two checkpoints: (i) the first at the stage of receiving funding for research that involves utilization of genetic resources or associated traditional knowledge; and (ii) the second at the stage of final development of a product. In 2016, the European Commission adopted a guidance document on the scope of application and core obligations of the EU ABS regulation. In 2021, the EU adopted the Guidance document on the scope of application and core obligations of Regulation (EU) No 511/2014, addressing various ABS issues, relevant for users from various economic sectors as well as for upstream users in research and gene banks (EU, 2021).

BOX 9.4

The Nagoya Protocol: key information

The Nagoya Protocol (CBD, 2010b) implements the third objective of the CBD and builds on the provisions set in Article 15 that require PIC and MAT to obtain access to genetic resources from the provider country. Thus, the Nagoya Protocol provides a strong basis for transparency and legal certainty and contributes to building trust between users and providers of genetic resources and associated traditional knowledge. It also creates incentives to conserve and sustainably use genetic resources, and therefore enhances the contribution of biodiversity to development and human well-being.

The Nagoya Protocol encompasses three major building blocks: access to genetic resources, benefit-sharing and compliance. It sets out core obligations for its Contracting Parties to take measures to for its implementation.

- The first pillar of the Nagoya Protocol is **access obligations**. Under the Nagoya Protocol, as required by Article 15 of the Convention, access to genetic resources is subject to the PIC obtained from the provider country, unless that country decides not to regulate access.
- The second pillar is **benefit-sharing obligations**. The Nagoya Protocol requires that benefits "arising from the utilization of genetic resources as well as subsequent applications and commercialization" are to be shared in a fair and equitable way with the Party providing the genetic resources. The benefits are shared according to written agreement (MAT) and they may be monetary or non-monetary.
- The third pillar of the Protocol is **compliance obligations**. Development of a national ABS legislation or regulatory measures is the obligation of the Parties to the Nagoya Protocol and a most important source of information for potential users of genetic resources from this country.

The Nagoya Protocol creates specific obligations to support compliance with the domestic legislation or regulatory requirements of the provider Party, and the contractual obligations that are reflected in the MAT. The protocol requires to monitor utilization of genetic resources through establishment of the internationally recognized certificate of compliance (IRCC) and establishment of checkpoints at the national level. The protocol established an Access and Benefit-sharing Clearing-House (ABS-CH),

an internet-based information system (https://absch.cbd.int) for contracting Parties to share domestic ABS related information, such as on their national focal points and competent national authorities, ABS legislation and administrative measures as well as permits issued at the time of access. The IRCC serves as unique evidence that the genetic resource covered by the certificate has been accessed in accordance with PIC and that MAT have been established and supports monitoring of utilization of genetic resources.

BOX 9.5

Examples of AnGR in national ABS legislative, administrative and policy measures and implementation of ABS-related measures by gene banks

A survey on national ABS legislation regarding AnGR and gene banks in Europe, conducted as a part of the IMAGE project, showed that only five countries (Croatia, Denmark, Estonia, Portugal and Albania) had specific access measures for AnGR and only three countries (Bulgaria, Denmark and Croatia) reported to have in place specific ABS regulatory measures for the management of animal gene banks (Martyniuk *et al.*, 2019). A few European countries that have introduced ABS regulations have decided to exempt AnGR used in food and agriculture from the scope of their national ABS regulations (France and Spain).

An IMAGE survey showed that only 29 percent of germplasm collections and 5 percent of genomic collections were using MAA. Material Transfer Agreements or similar tools were reported by 25 percent of germplasm collections and 37 percent of genomic collections. It was noticed that the level of awareness regarding the necessity to document the source of material entering gene banks and to document transfer of material to other banks or users is still rather limited. This situation might be related to the fact that European banks have mainly collected domestic AnGR and may have either other types of long-term agreements or were not that concerned about developing uniformed documentation of acquisition or transfer of their material. Nevertheless, such documentation is necessary to demonstrate the legal status of the genetic resources anytime they are transferred or used by the gene bank. Another issue is the limited information on ABS conditions attached to AnGR in the example MAA and MTA documents that were provided by survey respondents (Martyniuk et al., 2019).

9.4 ACQUIRING SAMPLES

Gene banks should have procedures and agreemetns in place for acquiring and distribution samples as samples are acquired from various sources which require different legal or regulatory approaches and frameworks.

9.4.1 Samples obtained from private entities

In the vast majority of instances, livestock animals have been historically considered private property under private control and only a limited part of AnGR is in the public domain. Cryopreserved germplasm from commercial breeding programmes is usually owned by breeding companies or private breeders. Therefore, acquisition and exchange depend on appropriate private property and contract law and require agreements transferring ownership from the breeders to the gene bank.

Genetic material can be accessed by soliciting the germplasm as a donation or through purchase from the owner. If a livestock owner donates samples of germplasm to the gene bank, he or she thereby waives all claims to the germplasm. When a gene bank buys a specific animal or its germplasm from the owner, the gene bank normally obtains unconditional rights to that genetic material.

Material for gene bank collection also may be acquired in cooperation with livestock owners in the context of specific activities such as national conservation programmes. In this case, the livestock owner may charge a fee for access to the animal and the germplasm collected. By doing this the owner may or may not forego further claims on the germplasm collection. Alternatively, the owner may prefer an agreement that facilitates the holding of the germplasm by the gene bank without transfer of ownership, or the livestock owner may wish to maintain ownership of the germplasm for a specified period while it is in the gene bank (also known as an embargo), after which the germplasm becomes the property of the gene bank. Such an approach can protect breeders, at least for a period, from competitors that may want to acquire the samples for the purpose of gaining an advantage. If the owners do not want to forego their rights to germplasm stored in the gene bank, managers must determine whether material stored for a long time (and made redundant by the acquisition of newer samples) should remain in the gene bank or be returned to the owner. For example, participants in the National Program for the Conservation and Utilization of Genetic Resources in the Czech Republic provide biological samples to the genomic collection of the gene bank. Upon request by the bank, they may also allow the collection of reproductive material (semen and embryos) from their animals for the gene bank under conditions of financial compensation.

9.4.2 Acquisition of gene bank material from other entities

Such situations may include occasional acquisition of materials (such as from completed research projects or due to reorganization or termination of operations of other gene banks) where all obligations and rights concerning the material are transferred to the receiving gene bank.

Gene banks may also accept to be put in trust of material under safe conditions for a defined period. The provider can retain ownership and then terms about storage costs, liability and/or accessibility to samples must be set in a negotiated agreement regarding custody. Ownership of samples then may be transferred to the gene bank after a certain period, possibly with an embargo period for use.

Alternatively, such materials may be donated without specific restrictions and access to them is then regulated according to the general criteria established by the respective gene bank. The gene bank and donor may also permit relatively free access to the samples but notify the donor each time one of their samples is requested (safekeeping with notification).

Finally, exclusive safekeeping may be applied, whereby the provider of material has exclusive access to the samples and decides whether access to a third party will be given. These models may apply for a limited period, after which the material becomes the sole property of the gene bank (de Vicente and Andersson, 2006).

9.4.3 Gene bank samples acquired from abroad

National genebanks generally focus on conservation of national genetic resources but can also deal with exchange of genetic material between countries (e.g. operating as a regional gene bank, for research purposes). Samples may be also donated by or purchased from a source in a foreign country or supplied by a national third party who obtained them from a foreign country.

A specific case involves backup storage of a complete or partial duplicate of a foreign gene bank's collection upon a bilateral agreement. Countries may also consider development of a bilateral germplasm exchange programme, as a security measure, but this presupposes a number of specific arrangements, which may substantially limit implementation of such solution (Blackburn and Boettcher, 2010).

In the first place, all veterinary and sanitary requirements set for effective management of biosecurity risks associated with the transfer (import health standards) valid in the country receiving samples must be met. There may also be situations where it is necessary to acquire samples that do not meet all current requirements (for example, they come from historical collections and their veterinary status is unknown). Exceptions may exist for such cases, but samples must usually be documented, stored, and handled separately from other gene bank materials.

In the EU, intracommunity trade or transfer of germplasm of purebred animals require also zootechnical certificates. Such certificates are based on the information received from the breed organization, AI center or embryo production team according with the respective domestic or regional health legislation. In the EU, model forms for the zootechnical certificates accompanied germinal products have been developed for the trade within the EU or for germinal products entering the EU (EC, 2017). Traceability and animal health requirements for movements within the Union of germinal products of certain kept terrestrial animals are also set down (EC, 2020).

Any samples acquired (physically accessed) from abroad after 12 October 2014 (when the Nagoya Protocol entered into force) are subject to the ABS requirements. For cross-border movement, the management of the gene bank must take certain measures to comply with the ABS principles. which must be checked and implemented in gene bank procedures. The gene bank must be able to do the following:

- prove that the samples were accessed/acquired legally from the country of origin and/or legally obtained from other sources;
- document the terms and conditions of use and eventual transfer to third parties (PIC and MAT); and
- comply with the benefit-sharing obligations associated with the samples.

Appropriate documentation declaring these facts must be acquired and retained with the material. These documents may include: PIC and MAT or IRCC, MAA or MTA, veterinary certificates and any other permits (such as for export, research, import) on a case-by-case basis. Many countries have their own ABS legislation that preceded the Nagoya Protocol and remains valid, which may require other types of permits.

9.4.4 General good practice upon receiving material

Gene banks should undertake the following measures for receiving material from another country:

1. To check if the original permit for collecting samples allows supply to third-party users and, if this is the case, to make the information on the permit available for potential users and to supply it together with any material to the potential users.
2. If the permit does not allow the transfer of material to third parties, the material cannot be made available, but it can be marked in the catalogue or gene bank database with a reference to the Competent National Authority (CNA) that issued the original permit, so that the

potential user can contact that CNA to either seek a new permit and negotiate a new MAT for access to the collection material in the country of origin.

3. It is possible that requested genetic material is present in a collection without PIC and MAT (acquired before October 12th, 2014). Then the gene bank should seek and transfer all necessary information to the requestor through submitting a due diligence declaration with a web-based tool (DECLARE).
4. To exercise due diligence, by using the ABS-CH tool the gene bank should established whether the requested material is a subject to the Nagoya Protocol requirements and whether the country of the gene bank has ratified the Nagoya Protocol. If this is the case it should be checked whether requested material in the gene bank collection has been acquired in accordance with the providing country ABS legislation, and if any additional institutional requirements are fulfilled.

9.4.5 Material Acquisition Agreements

The previous guidelines (FAO, 2012) considered the most common situation, that is obtaining material from domestic sources, but gene banks must also anticipate the acquisition of material from foreign sources, particularly for use with advanced biotechnologies and other types of research. In such situations, the Nagoya Protocol becomes relevant and it is necessary to negotiate and conclude relevant ABS agreements.

Many developing countries regulate or are considering regulating access to their native breeds. This will result in applying different standards for access to local breeds and mainstream international breeds that are available on a commercial basis (Martyniuk *et al.*, 2017). National policies play a key role in setting rules for access to material from local breeds. If material access is not directly determined by any higher-level legislation, it is appropriate for countries to develop and firmly enshrine national rules on how to apply for obtaining access to genetic resources, including rules dealing with possible rights of local communities associated with AnGR, and place them on the ABS-CH portal.

A gene bank must prove the legal status of, or legal access to, the samples anytime the material is transferred to other collections or users. The gene bank must therefore establish proper procedures to document the origin of every accession and the conditions for their use and further distribution. For that purpose, a Material Acquisition Agreement (MAA) is the most appropriate document. If stored material has been accessed from a country regulating access to own genetic resources, documentation also include PIC, MAT and IRCC, if the provider country is issuing such permits.

Before developing MAA, the gene bank should consider:

- national laws and regulations (included the ABS regulations where relevant);
- national and international veterinary and sanitary regulations;
- breeding regulations;
- internal rules of the institution hosting the gene bank;
- data protection rules; and
- ownership of the material.

Gene bank may develop their own MAA model in consultation with relevant stakeholders that provide samples to the gene bank. The model can be further amended for every individual acquisition and it is highly recommended to have it reviewed by a legal expert.

A potential set of elements in the MAA is provided in the Guidelines of Material Acquisition Agreements for gene banks developed by the European Regional Focal Point for Animal Genetic Resources (ERFP, 2019), which are provided in Annex 9.1.

9.5 ACCESS TO GENE BANK COLLECTIONS

9.5.1 Use of gene bank collections and access criteria

Gene banks may only provide access to their own collections if the material to be released is distributed in compliance with (i) the conditions under which the material was received; and (ii) the national laws of the country where the gene bank is located (Brink and van Hintum, 2020). A decision to provide germplasm from the gene bank collection will depend on the purpose, principles and criteria set by the governing body of the gene bank and based on conditions set in the MAA.

Access to genetic materials is sought by different types of users for various reasons and, therefore, different access criteria may be set for various purpose of use.

- **National conservation programmes such as support of *in vivo* conservation or breed re-establishment in case of extinction**. The access conditions will therefore depend on the provisions of the relevant national programme. As a rule, the genetic material is provided on the recommendation of the National Advisory Committee or a designated authority to the cooperating entities implementing the programme.
- **Breeders or breed associations and private breeding companies for genotypic reorientation of selection goals and strategies, creation of synthetic breeds or introgression of genes of specific interest.** Release of gene bank material for such purposes to both non-governmental and private breeding companies must not harm the competitiveness of the provider of the germplasm. When germplasm is to be used for generating live animals, the gene bank may want to consider requesting that the user redeposit germplasm from the resulting progeny.
- **Research organizations.** Some research projects could ultimately lead to new developments and possibly to commercial applications (e.g. DNA studies of the genetic background of traits). For these cases, it is recommended to set the conditions for the use of data arising from the project or their publication in the gene bank database.

BOX 9.6
Obtaining material from registered collections in the European Union

According to the EU legislation, collection holders have the obligation to seek, keep and transfer information to subsequent users of transfered materials, as proof that genetic transferred resources were accessed in accordance with the national ABS legislation or regulatory requirements (namely date/place of access, source, any rights and obligations, PIC and MAT, and possibly IRCC).

The objective of a registered collection was to help users of AnGR to fulfil their obligations. Collections included in the register apply standardised procedures for aquiring genetic resources, exchanging (with other collections) and supplying materials (to third persons/parties for their utilization) only with documentation. Such documentation provides evidence that the genetic resources were accessed (acquired) in accordance with ABS legislation and regulatory requirements and with MAT. Where possible collections also establish or use unique identifiers for samples supplied.

The CNA shall verify if a collection meets the requirements for recognition as a collection and can be included in the EC register. Users that obtain a genetic resource from a collection included in the register shall be considered to have exercised due diligence as regards the seeking of all necessary information. This should prove particularly beneficial for academic, university and non-commercial researchers and contribute to a reduction of time, administrative and compliance requirements. In the EU, such a system is enshrined in Commission Implementing Regulation (EU) 2015/1866 (EC, 2015).

9.6 TRANSFER OF MATERIAL

9.6.1 Transfer procedure

Distribution of material from gene bank collections can only be done when it complies with the conditions under which the material was acquired according to the domestic legislation of the country where the gene bank is located. As a general rule, material acquired for non-commercial purposes cannot be made available to a third party for commercial purposes.

The Nagoya Protocol and more precisely the national laws for its implemention lay down rules concerning the transfer of genetic material within the given temporal and geographical scope. Countries that are not among the Protocol Parties may also have their own ABS legislation. In addition, material collected and stored and pre-Nagoya Protocol could also be subject to national ABS legislation that was in force in the country of origin at time of collection. In many situations, documents and permits required for the transfer to a third party might not be available. In this case, a practical solution could be to only distribute the material under conditions set in the MTA.

The procedure of gene bank material transfer is initiated after the gene bank receives a written request from a potential user. The requestor should provide all the information that is needed for the decision-making process on the release of the material, in particular:

- legal entity and affiliation of the applicant;
- type and quantity of genetic material requested:
- purpose for which the material will be used: and

- possibly the import permit (if the requestor's country regulates entry of that material).

For breeding purposes, the requestor should specify also the material use objectives (such as breed conservation project, breed improvement or research), which may require additional confirmation of the respective breeders' organization. For research purposes the request should provide the project identification, its objectives and expected outputs, collaborators and sponsors.

The gene bank may decide to prepare standard application forms for different types of use. Also, the gene bank should have a standard procedure in place for assessing the application, which follows the objectives of the gene bank and allows for a reasonably objective decision about whether or not release of material is justified.

The gene bank must also make sure that it has enough material in the collection to satisfy the request. Depending on the gene bank policy, release of the requested material may not be allowed if it is also available in the commercial sector.

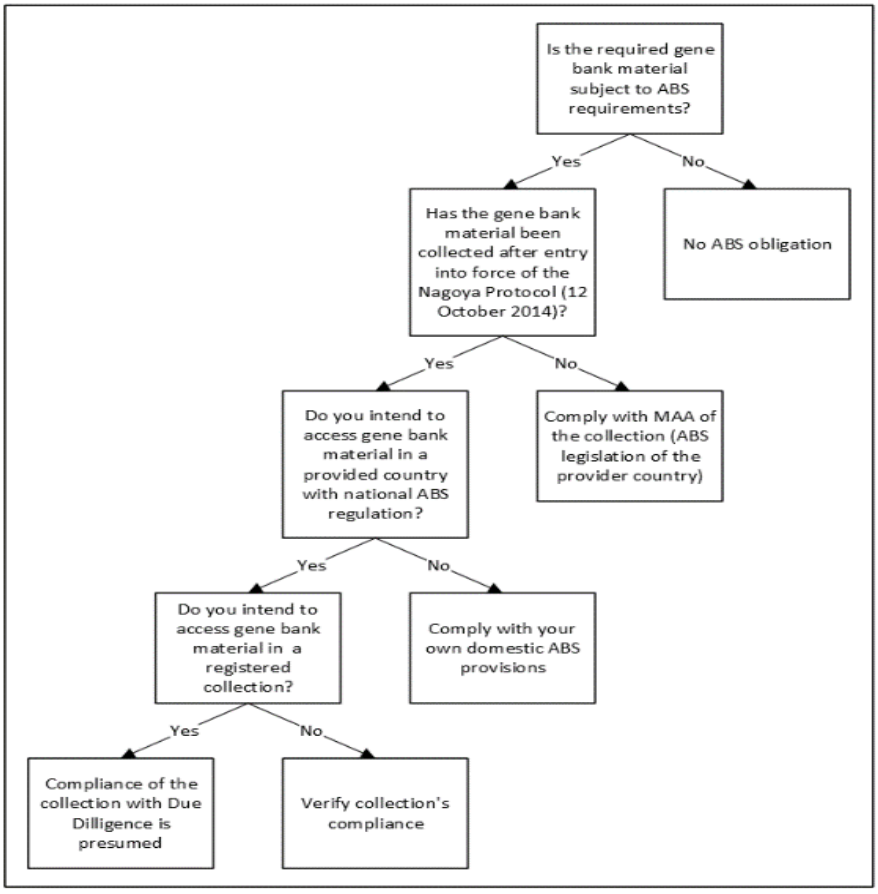

Figure 9.1
Decision tree showing ABS-related factors to consider before accessing material from a gene bank collection

9.6.2 Material Transfer Agreements

The MTAis a contractual arrangement between gene bank and users of the released material which governs their respective rights and obligations, including terms of access and benefits to be provided,

if relevant. The content of the agreement should be determined by the parties involved. Diversity in national ABS and contract laws, as well as different interests of gene banks and users may lead to a wide range of options when actual provisions are negotiated. A potential set of elements in the MTA is provided in the Guidelines of Material Acquisition Agreements for Gene banks developed by the European Regional Focal Point for Animal Genetic Resources (ERFP, 2019) and included as Annex 9.2.

The MTA underlies the physical transfer of genetic material from the gene bank collection to a user and stipulates the terms for the transfer, that is, how the user will be allowed to dispose of the material obtained:

- the extent to which replication, alteration or utilization the material is permitted;
- limitations on third party transfer or conditions and procedures that should apply if such a transfer is allowed (provided that any subsequent transfer should be subject to the same conditions that the initial transfer was subject to);
- which research and development activities the user will be able to undertake; and
- prohibition or permission to commercialize the transferred material and associated traditional knowledge, including the results of research and development;

Other specific provisions may include, for example, consent to the following:

- provision of germplasm samples from the live animals generated from the utilization of the material provided by the gene bank;
- feedback on project results - submit phenotypic and/or genotypic data resulting from the research project into a gene bank or other publicly available database after it has been published; and
- acknowledgement of the gene bank in any resulting publications.

Provisions supporting the legal certainty of the participants in the process of transfer and use of the material should also be included, such as:

- warranty and liability (agreement or waiver by the user to accept any risks associated with the health status of the material and to observe the appropriate sanitary/veterinary precautions regarding the use of the material);
- governing laws and procedures for dispute settlement; and
- treating personal data in accordance with relevant data protection laws.

Finally, ABS provisions (for material that is transferred with the Nagoya Protocol-related obligations) should also be addressed, if relevant:

- types of benefits that could arise from utilization and/or commercialization the material; and
- how the benefits would be shared.

9.6.3 Intellectual property rights

Material in gene bank collections released for research purposes may be associated with traditional knowledge of relevance for the research objective. With advances in biotechnology and the potential to use the unique properties of individual animals or their germplasm through novel methods such as genome editing (Selvam, R. 2020), the issue of intellectual property has become more pronounced not only in research, but also in animal breeding.

Research project funding agreements usually set out how the results will be published and used, including the treatment of data handling and confidentiality. A practice recommended by the World Intellectual Property Organization is to identify the background intellectual property of all parties prior to entering into a research agreement and to clarify beforehand access rights to foreground and background intellectual property to prevent any potential conflicts of interest (ALLEA, 2019). This approach may be applied also in MTA if the traditional knowledge is transferred together with the gene bank material.

9.7 IMPACTS OF ACCESS AND BENEFIT=SHARING ON GENE BANK OPERATION

The gene bank management should consider ABS legislation in the following situations:

- when a gene bank is located in a country regulating access to AnGR;
- when a gene bank would like to access AnGR from the provider country, regulating access to AnGR, to enhance its own collection; and/or
- When a gene bank is located in a country that does not have access measures for AnGR but may introduce specific provisions, like registration of users.

Gene banks, while developing their own ABS-related protocols and procedures, must balance these with relevant national legislative, administrative and policy measures. For example, gene banks need to know if sampling to enhance domestic collections requires approval from the CNA. Gene banks also need to be fully aware of their potential role, if any, in the authorization procedures if domestic or foreign users would like to obtain samples from their gene bank.

Although at present animal gene banks are mainly involved in storing material from local and international transboundary breeds already being used in their own country, that situation may change in the future. Joint research projects or activities concerning novel transboundary breeds may prompt gene banks to acquire AnGR from abroad, which will require knowledge and application of relevant ABS procedures.

Gene bank managers should become familiar with the legislative, administrative and policy measures involving ABS in potential provider countries, and understand the associated processes for access. Such information should be available on the ABS-CH, as it is an obligation of contracting parties arising from Article 14 of the Nagoya Protocol. Gene bank managers should obtain this information before submitting an access request.

The country from which genetic material will be access is an important consideration, as countries differ substantially differences in their national ABS legislation and regulatory frameworks, especially in their practical implementation. This may mean considerable differences in terms of the time needed to complete a formal request process and the costs involved.

Gene banks should also carefully consider conditions for making available material acquired and entered into the collection before 12 October 2014, the date when the Nagoya Protocol came into force. The provider country may have different time-frame related provisions in their domestic access legislation and some provider countries had their national ABS law already in force before 12 October 2014.

The ABS-related information should be a part of MAA and MTA. As was elaborated earlier in this section, every gene bank should develop its own or adapt already existing MAA and MTA, and these documents should be reviewed on a regular basis. MAA and MTA should be integrated into the workflow of the gene bank. Gene banks are encouraged to consider European Regional Focal Point guidelines while developing their own customized MAA and MTA (see Appendices 9.1 and 9.2). Although developed in Europe, much of the information provided is valid for all countries.

The major impact of ABS measures on animal gene banks is the need to maintain a detailed documentation of the source of any biological material that is introduced into the gene bank. Such information associated with samples must be stored in the gene bank database and made available for potential users to provide evidence of their legal access and compliance with the national legislation associated with the Nagoya Protocol.

There are two aspects of benefit sharing in the gene bank context. The first type of benefits arise from the fact that AnGR gene banks have been established worldwide and can provide biological material from their collections. Effective animal breeding depends on continuous research, generation of new knowledge and its successful application in selection programmes. Gene banks have a very important role in providing the opportunity to obtain specific biological material as a basis for livestock research. This benefit should be considered while developing national ABS legislative, administrative and policy measures.

The second type of benefits are executed on the bilateral level, through MAT, when a user obtains genetic material from the gene bank of a provider country. The benefit sharing provision may substantially differ if access to genetic resources was granted for research purposes only, or for research, development and potential commercialization. As stated previously, there are countries that, taking into account a number of distinctive features of AnGR, have decided to exempt livestock genetic resources from domestic ABS measures. Such an approach facilitates unhampered trade in breeding material and can help support regional and global scientific exchange of livestock genetic resources (Martyniuk *et al*., 2018).

9.8 RECOMMENDATIONS FOR GENE BANK MANAGERS

- Gene banks' managers should have a good understanding of the domestic legislation related to *ex situ* gene banks and AnGR conservation, veterinary requirements, animal breeding and ABS.
- The internal gene bank procedures and rules regarding acquisition, ownership and transfer of genetic material should be developed, implemented, regularly reviewed and updated.
- The governing/technical bodies of the gene banks should develop standard documentation for the gene bank, such as MAA and MTA, end ensure its implementation. Such documentation should be stored in the gene bank and used to prove the source of material and conditions attached to the material, if any.
- If the gene bank is collecting samples from abroad, gene bank managers should have a clear understanding of ABS legislative, administrative and policy measures of each provider country and strictly follow procedures to obtain permits. This information should be available at ABS-CH or obtained directly form ABS NFP or ABS CNA. The gene bank management must store the records of PIC and MAT (IRCC if available) associated with samples from provider countries to have proof of their origin and legal status. Such documentation may be needed during monitoring of

gene bank operation and should be made available to future users that request access to AnGR from gene bank collection.

- The gene bank must maintain a frequent and open communication with their stakeholders and raise awareness about the administrative procedures for its operation.

9.9 REFERENCES

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SECTION 10

Capacity building and training

Capacity building and training

10.1 INTRODUCTION

Knowledge and skills are critical components of smooth and effective operation of cryoconservation programmes. This section will emphasise obtaining and disseminating the knowledge required for developing a gene-banking strategy and for collecting, preserving and using germplasm collections. It also involves the bigger picture of utilizing cryoconservation as a critical component of overall management of animal genetic diversity within a country. Capacity building and training are therefore crucial elements of gene bank management. Capacity is featured prominently in the Global Plan of Action for Animal Genetic Resources (Global Plan of Action; FAO, 2007), where Strategic Priority 14 is to "strengthen national human capacity for characterization, inventory, and monitoring of trends and associated risks, for sustainable use and development, and for conservation".

Within a country, capacity building and training usually involves three domains:

- Academic and technical training in national universities and research centres;
- specialized training for gene bank staff; and
- outreach and capacity building for gene bank stakeholders.

By establishing strong systems for each of these domains and continually strengthening and updating them, a country can ensure that its cryoconservation activities contribute to long-term and comprehensive sustainability of its AnGR.

10.2 ACADEMIC AND TECHNICAL TRAINING IN NATIONAL UNIVERSITIES AND RESEARCH CENTRES

National universities and other educational and training institutions will play a major role in ensuring national capacity for gene banking. Strategic Priority 13 of the Global Plan of Action is to "establish or strengthen national educational and research facilities". Education at academic institutions such ast universities and research centres usually involves two distinct programme phases, at the undergraduate and graduate levels. Both levels are important for optimal national management of AnGR. Undergraduate programmes primarily involve one-way transfer of existing knowledge to students, whereas graduate programmes involve more two-way exchange of information, as well as generation of new knowledge through research activities.

Undergraduate level programmes are important for all gene bank stakeholders, to ensure a basic understanding of the existing knowledge for the major topics of general importance for management of AnGR. This should begin with establishing the importance of agriculture in the sustainability of humankind and the planet and the role of livestock production. Additionally, the role of livestock production at both local and global levels should be addressed. Students should be made familiar with the range of possible livestock production systems and the advantages and disadvantages of each. This discussion should include introduction of students to the agroecosystem approach to agriculture and livestock production. Courses on breeding and genetics should include management of AnGR and the need to optimize genetic improvement with maintenance of genetic variation. Students should be made aware of the Global Plan of Action for Animal Genetic Resources (FAO, 2007) and any relevant national plans and policies for AnGR. Courses on reproductive physiology should cover the utilization

of different types of germplasm and their cryopreservation. Information management is a continually more important topic for almost all academic pursuits and real-life applications and gene banking is no exception (see Section 8), so training in this field is also essential.

Graduate programmes impart knowledge on narrower topics at a relatively greater depth than undergraduate programmes. They usually aim at training people for an academic or research career, and many gene bank stakeholders will not attend graduate school. Effective graduate programmes of will enable students to become national experts on one field of study related to gene banking but must also avoid excessive specialization. Graduates will require a holistic knowledge base to fully appreciate the context in which they apply their specific skills. Academic fields of importance include population and quantitative genetics, genomics, reproductive physiology and biotechnology.

The amount of global scientific knowledge is growing at an astounding and continually increasing rate. Where feasible, cooperation and collaboration across universities and among countries to develop joint training programmes may be a solution to ensure students have access to experts across a wide range of disciplines. Accordingly, Action 3 of Strategic Priority 13 of the Global Plan of Action is to "establish or strengthen, in partnership with other countries, as appropriate, relevant research, training and extension institutions…to support efforts to characterize…and conserve animal genetic resources". This goal can be accomplished through several mechanisms, ranging from frequent informal communication between pairs of colleagues, to collaborative projects, to formal programmes for exchange of faculty and/or students. Groups of universities may even formally or informally agree to specialize on different aspects of animal genetics or management of AnGR.

Comprehensive training on animal gene banking and management of AnGR in general involves a non-traditional curriculum that often must be developed through combining components of existing classes. This can be a particular constraint for developing countries. The Global Plan of Action (FAO, 2007) highlights this need, as Strategic Priority 16 is to "strengthen international cooperation to build capacities in developing countries and countries with economies in transition…". This goal can be addressed in multiple ways, including bilateral agreements between governments and specific universities and/or research institutions, cooperation through an international intergovernmental or non-governmental organization or as a work package of an international project. As an example of the latter case, partners in the IMAGE project conducted training courses with partner institutions in Argentina, Colombia, Egypt and Morocco (Box 10.1). Although it's impossible to create an expert through a week-long course, such events can be very effective in disseminating the knowledge necessary to perform a specific task, such as undertaking a given analysis or using a specific type of software, and offer great opportunities for networking.

BOX 10.1
Training courses for non-European partners in the IMAGE project

The European IMAGE project (Innovative Management of Animal Genetic Resources - www.imageh2020.eu) included a work package on Outreach and within this topic a task on capacity building in the four non-European partner countries (i.e. Argentina, Colombia, Egypt and Morocco). As a rule, each of the training courses was one week in length, with one day consisting of a workshop for a wide range of stakeholders in AnGR and 4 or 5 days of training activities for graduate students. To expand the impact of the training courses, students from neighbouring countries were invited, either in person or online through video streaming. Lecturers included both

scientists from IMAGE partner organizations and local experts.

Consistent with Action 2 of Strategic Priority 16 of the Global Plan of Action on Animal Genetic Resources, which addressed international collaboration, the content of each event considered the particular interests of the host country. Each local organizer proposed the topics to be addressed and then worked with IMAGE partners to identify the most appropriate instructors. The topics were not limited to gene banking, but also included matters related to management of AnGR in general. The courses comprised lectures, practical sessions and group discussions with presentation of results.

The following is an example of some of the topics selected for each country:

Argentina (37 participants)

- Genetic diversity: importance, definition and measurement criteria
- Characterization of population structure by pedigree analyses and molecular markers
- Conservation of AnGR
- Introduction to the Domestic Animal Diversity Information System (including practical session)
- Community-based strategies for animal genetic improvement: animal conservation at local breeders' level
- Practical lesson: Strategy design for conservation of breeds in risk of extinction

Colombia (40 participants)

- Validation of paternity and reconstruction of pedigrees
- Molecular and genomic characterization
- Landscape genetics and genomics
- Conservation of AnGR
- Reproductive technologies for the conservation of pig breeds

Egypt (25 participants)

- Phenotypic characterization: different approaches and purposes
- Livestock conservation strategies: Development and rationalization of gene banks
- Practical computational analysis of SNP-Chip data
- Recent approaches in *In-vitro* conservation of Egyptian AnGR

Morocco (20 participants)

- Genetic diversity in livestock species: Domestication, local adaptation, diversification of livestock breeds
- Building gene bank collections and using stored material
- Advances in cryoconservation technologies
- Neutral adaptive diversity and decisions for balancing conservation and production

10.3 SPECIALIZED TRAINING FOR GENE BANK STAFF

Staff of gene banks will presumably be already among the top national experts in AnGR at the time of their hiring. However, they will need to continue to upgrade their capacity throughout their career, including both further refining their current specialized knowledge and gaining new skills. As mentioned previously, the global amount of scientific knowledge is continuously increasing at an ever-growing rate and this rule applies to the knowledge associated with operating a gene bank.

Reproductive and cryopreservation techniques change and improve. Genomic biotechnologies continue to provide more information at a lower cost per unit of information. New processing and storage equipment continue to enter the market. Computational technologies for data management become more comprehensive resulting in vast amounts of often complex data. Therefore, the organization of periodic training programs in different areas is crucial for continuous updating of the staff involved in managing AnGR. Training for gene bank staff can occur in three ways: (i) internally within the gene bank; (ii) with other gene banks; and (iii) with third-party organizations.

As explained in Section 2 of these guidelines, continuous improvement is a key feature of optimal quality management. For example, adoption of new technology and methods can improve data management, increase cryopreservation efficiency, enhance staff productivity and safety, and reduce costs. Most gene bank staff will be passionate about their work and curious about learning new theories and applying new methods. All these are reasons why gene banks need to have a capacity building programme for their staff.

Within the gene bank, employees should receive on the job training. Even the most highly skilled new employees will likely not be familiar with all the tasks required of them. Gene banks with sufficiently large numbers of employees may consider establishing a mentorship programme to encourage greater cooperation between senior and junior staff.

Individual gene banks vary widely. For example, they differ in terms of their size, years of operation, species and breeds targeted for conservation, protocols for cryopreservation and approaches for quality control and management. At the same time, they have many commonalities. Therefore, they present many opportunities for knowledge exchange. As described in Section 2, one simple opportunity for cooperation between gene banks is peer review of procedures as part of quality management. Other possibilities include short term visits of staff from one gene bank to another. When a single staff member receives training, the logical solution is for the staff member to visit the bank providing the training. For cases where multiple staff receive training, its more efficient for the beneficiary bank to host the expert from the knowledge-providing bank. Cooperation between gene banks from developing and industrialized countries may be a particularly beneficial mode of capacity building.

Finally, the capacity of gene banks can be improved through interaction with entities that are not gene banks. Universities and research centres are obvious examples. Such cooperation will allow the gene bank to benefit from new and/or improved approaches for material collection or preservation that have been developed by researchers in reproductive physiology. Genetics researchers can provide assistance and training on development and utilization of gene bank collections. In return, the researchers will have the opportunity to see their work be applied in the field. Private companies are another potential source of capacity building. For example, as part of the sale, vendors of equipment or reagents may provide follow-up to ensure that they are being used properly and efficiently.

Box 10.2 details the approach Iraq has taken during their ongoing process of establishing a gene bank.

BOX 10.2

Capacity building for gene banking in Iraq

The Ministry of Agriculture in Iraq in 2010 decided to begin the process of establishing a gene bank for AnGR. Having no previous experience in this regard, assistance from a country with experience in gene bank operation was considered necessary. An informal request for assistance was therefore distributed on the Domestic Animal Diversity Network (DAD-Net), the FAO mailing list for AnGR. Many countries responded to the request and offered their help in tackling this issue. In the end, a short-term training period was arranged with the national gene bank of the United States of America, at the National Laboratory for Genetic Resources Preservation. The experience gained was crucial for upstream preparation for establishing the national animal gene bank but was limited to a few employees and topics, whereas the sciences behind operating a full capacity gene bank are diverse. These sciences have either been absent or overlooked in the curricula at any study level in Iraq and in many developing countries. There is an urgent need to revise university curricula by including such subjects.

Technology transfer was another major need in Iraq and this was addressed by collaboration between countries via extended exchange programmes for students and teachers. Research collaboration was undertaken with the United Kingdom and China and was very successful. The government therefore organized lectures on conservation genetics for post-graduate students and workshops for gene bank employees and government research staff across the country, focusing on the importance of local animal breeds. Training on measurement of phenotypic traits and breed characteristics according to FAO Guidelines was also provided. These courses offered new perspectives on thesis subjects to the students.

Courses and other training can provide experts with urgently needed skills to operate gene banks. However, achieving sustained human resources capable of solving any AnGR challenge and establishing programmes to conserve AnGR is possible only with well-educated students with a passion to conserve not only AnGR but also their agroecosystems. This must be complemented by the political will and commitment of governments to invest for the long term.

Source: Dr Sahar Albayatti

10.4 OUTREACH CAPACITY BUILDING AND FOR GENE BANK STAKEHOLDERS

In certain cases, the gene bank will benefit from provision of information and capacity building to others. As described in Section 1, communication with stakeholders is a critical aspect of a gene banking strategy. Keeping stakeholders informed about activities of the gene bank builds their trust and creates awareness about the importance of gene banking and opportunities for utilization of stored material. Communication with stakeholders is also important for quality management (see Section 2).

Capacity building of stakeholders can also increase efficiency and efficacy of the gene bank activities in terms of building and maintaining its collections of genetic material. If material is accessed from animals owned by stakeholders, training may be required on practices (such as feeding, health care) to ensure optimal yield of genetic material. Similarly, special procedures may need to be followed on-farm to ensure optimal sanitary hygiene for the material collection (see Section 7). Users of gene bank material may benefit from training in procedures that increase fertility and hence the number of live offspring from the material that they have obtained from the bank. Increased knowledge by breeders of

selection and mating strategies to optimize increasing productivity and maintaining diversity will help to expand the pool of potential gene bank donor animals. The bank may either provide such training directly or cooperate with a third party (e.g. veterinary clinic or research university).

An effective information system will most likely increase utilization of gene bank material (see Section 8). However, training in accessing and use of the system may be necessary to ensure its full exploitation. Finally, providers and users of gene bank material will have to be made aware of policies and regulations associated with exchange and utilization of genetic material, such as material acquisition and transfer agreements, and be informed about their roles and responsibilities (see Section 9).

10.5 SPECIALIZED TOPICS OF CURRENT IMPORTANCE FOR CRYOCONSERVATION

Regarding topics to be addressed in capacity building, subjects like general breeding and genetics and reproductive physiology and cryobiology will always be necessary for operating gene banks and cryoconservation programmes. More specific topics will change over time depending on development of technology, societal concerns and national and international policy. The following topics are of current importance:

Innovative uses of gene banks. As mentioned throughout these guidelines, gene banks should not only be used as resource to recover from breed extinction. University courses dealing with management of *in vivo* populations should be developed considering the option of using germplasm available in gene banks. The content of the courses should address both the collection of material for the bank and its utilization in subsequent generations. Capacity building and outreach with stakeholders should promote active interaction with the collections, for both provision and utilization.

Characterization of genetic diversity using genomic indicators. Recent advances in next generation sequencing and high-throughput genotyping platforms now allow for genomic characterization of the genetic diversity existing in collections to a breadth and level of precision which were previously impractical. Knowledge of these opportunities and the methods of analysis are of relevance for both university students and the staff members of gene banks who manage the diversity of collections. To support countries in these tasks, FAO has developed guidelines on genomic characterization (FAO, 2021).

Long-term maintenance of genetic diversity. New developments in reproductive technologies, genomic tools and in the theoretical framework underlying the management of small populations or those with low levels of genetic diversity provide novel opportunities for long-term maintenance of genetic variation both *in situ* and *ex situ* (e.g. Oldenbroek, 2017). Practical training in the use of software for population management such as MoBPS (Pook *et al*., 2020) should be incorporated where relevant (see section 5). These topics are relevant for both university curricula and breed associations and similar stakeholders.

Characterization and documentation of collections. For optimal utilization of gene-banked material, the metadata describing the stored material and the genetic resources they represent are critical (see Section 8). This undertaking requires training in data collection, organization, storage and retrieval. Proficiency in development and use of databases is critical. These skills will be of use in all

three categories of capacity building, i.e. university instruction and research, gene bank staff and stakeholders.

Legal Issues related with access and exchange of germplasm. As pointed out in Section 9, management of gene banks requires knowledge regarding national and international policies affecting the exchange of AnGR. Many stakeholders do not understand the implications of the Nagoya protocol on Access and Benefit-Sharing (CBD, 2020) as well as the importance and procedures for developing and using agreements stipulating the conditions for bilateral exchange of genetic material. These matters are of particular relevance for both gene bank staff and stakeholders.

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Annexes

Annex 2.1 Quality management checklist for animal gene banks

Gene banks with a full-fledged quality management system (QMS) should be able to answer yes to each of the following questions. Compilation of this questionnaire will allow identification of issues requiring further attention and consideration.

General gene bank management	**Yes**	**No**
1. Does the gene bank have a formally documented organizational and management structure?	□	□
2. Has the gene bank prepared formal cryoconservation goals?	□	□
3. Has the gene bank undertaken a stakeholder analysis?	□	□
4. Does the gene bank have a communication strategy and/or plan?	□	□
5. Have the major risks to the effectiveness long-term sustainability of the gene bank's operation been identified?	□	□
6. Has a mitigation plan been prepared for all major risks?	□	□
General quality management		
7. Has the gene bank established a Quality Management System?	□	□
8. Does the quality assurance involve a) formal certification or b) internal guidelines?	□	□
9. Has the gene bank established a formal quality policy?	□	□
10. Has the gene bank identified a specific employee to serve as the Quality Manager?	□	□
11. Have the key processes for the gene bank's operation been identified?	□	□
12. Are standard operating procedures documented for all key processes?	□	□
13. Does the gene bank maintain a collection of relevant regulation texts or references?	□	□
14. Does the gene bank have an up-to-date system for management of its quality system documentation?	□	□
Gene bank equipment		
15. Has the critical equipment for operation of the gene bank been identified?	□	□
16. Has the gene bank established standard operating procedures for regular maintenance of the critical equipment?	□	□
17. Does the gene bank have a system to record when critical equipment undergoes controls, routine maintenance and/or calibration?	□	□
Gene bank personnel		
18. Have the key personnel of the gene bank been identified?	□	□
19. Does the bank have a gene bank manager (i.e. a person who is responsible for overseeing all aspects of the gene bank)?	□	□
20. Do all key personnel have a formal job description?	□	□
21. Does the gene bank have an employee training programme?	□	□
Genetic material database		
22. Does the gene bank have a database system to record and trace the stored material?	□	□
23. Is write access to the data base restricted?	□	□
24. Is the data base backed up according to a regular	□	□

schedule?		
Genetic material acquisition		
25. Are formal contracts (such as Material Transfer Agreements) used when accessing material for the gene bank?	□	□
Material collection (for those gene banks that collect germplasm)		
26. Does the gene bank follow standard operating procedures for processing of and freezing of materials?	□	□
27. Does the gene bank have a quality control system for each collected sample of material?	□	□
28. Does the gene bank use a labelling procedure that uniquely identifies each unit of material in the gene bank?	□	□
Introduction of previously processed material (if relevant)		
29. Is there a specific policy for receiving genetic material for the bank?	□	□
30. Does the bank have a specific area dedicated to receiving incoming genetic material from outside sources?	□	□
31. Is there a system to ensure quality of material collected by other providers?	□	□
32. Is there a set of required quality control tests to be performed on all material from outside sources prior to being stored?	□	□
Material storage		
33. Is access to the storage area restricted?	□	□
34. Does the gene bank have a system to record the entry of persons into the storage area?	□	□
35. Does the gene bank have a system to store separately different types of material?	□	□
Material distribution		
36. Does the gene bank have a formal policy and procedure for providing access to stored samples for use by other persons or organizations?	□	□
37. Does the gene bank have a standard operating procedure for preparing samples for distribution to users?	□	□

Annex 4.1. Application of linear programming to design a material collection strategy

Linear programming (LP), or linear optimization, is a method to achieve the best possible outcome of a planning problem, such as maximum profit or least cost (Dantzig, 2002). The best outcome is achieved when the variables of the problem, called "decision variables" are optimal with respect to each other and/or under conditions where the range of some variables is limited. The rationale behind LP is that in real life problems, resources such as capital, labour, water, and energy are limited. Similar limitations often apply to gene banking. Consider the situation of multiple cooperating gene banks within a country or region. A LP study could suggest ways to collect and store genetic materials, in terms of number of semen doses, collection regions and in which gene bank to store, so that the current costs of operating gene banks could be reduced (De Oliveira Silva *et al.*, 2019).

In the case of a cryoconservation programme, the decision variable could represent the number of genetic samples to be collected for a given livestock breed, represented by the symbol X_b. X_b will be associated with a collection cost c_b and an expected economic return on the genetic material of that breed, represented by r_b. (In this case, "economic" return may refer to non-commercial value, expressed in monetary terms.) The limited resources (or model constraints) include the total capacity of cryotanks (C) and available budget of a gene bank (B). This rudimentary LP example can be represented by:

$$\text{Maximize } \sum_b r_b X_b \qquad (1)$$

Subject to

$$\sum_b c_b X_b \leq B \qquad (2)$$

$$\sum_b X_b \leq C \qquad (3)$$

$$X_b \geq 0 \qquad (4)$$

One can note that equations (1) to (3) are linear relationships, while (4) is mandatory requirement of LPs, i.e., that all decision variables are greater than zero. Equation (1) is called the "objective function", and represents the objective of finding the maximum expected economic return associated the collection of breeds, i.e., summing over a set of breeds (b), represented by the Greek letter for sums (Σ), while Equation (2) is total collection cost, which is constrained by the available budget; and Equation (3) says that the number of collected genetic materials, i.e., sum over Xb cannot be greater than the cryotank capacities.

In a more complex modelling exercise, seeking to rationalize *ex situ* collections, LP models can be used to frame the problem in terms of seeking to minimize collection costs and maximize diversity. The latter can be defined for example as a number representing collected breeds held in a gene bank network, subject to collective budget, distance between gene banks and collection regions, gene bank fixed and variable costs, and cryotank capacity (De Oliveira Silva et al. 2019). Optimization can be used for efficient reallocation of existing collections), or for planning future collections, for example by considering projected extinction risks.

A focus on breeds is a simplification, as genetic diversity rather than the number of preserved breeds might be more appropriate when data is available.

In the case of public conservation efforts, for example, national policies incentivizing the conservation of local livestock breeds (MAPA, 2020), another variable that might be considered, relates to current and future population status, i.e., “endangered” or “at risk”.

Probability of endangered levels can be estimated using census data and regression methods (De Oliveira Silva *et al.*, 2021). As resources are limited and *ex situ* conservation is a relatively expensive technology, it is rational for a problem to prioritize breeds that are more likely to be at-risk. In this case, cost effectiveness analysis (CEA) can be used to identify the trade-offs between costs (public or private) and extinction risks, genetic gain, or other attributes.

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Annex 4.2 IMAGE data collection list

What is the total quantity of each genetic material stored in your gene bank?

Material	**Number of samples**
Semen	
Embryos	
Ooctyes	
Somatic cells	

What is your total storage capacity?

Storage facility	**Number**	**Total capacity (samples)**
Liquid nitrogen tanks		
Freezers		

What are your fixed cost per storage facility per year?

Storage facility	**Fixed cost per storage unit**
Liquid nitrogen tanks	
Freezers	

What is the average cost of collecting genetic material from an individual donor animal on-farm per visit (excluding travel) for the following species?

	Material			
Species	**Semen**	**Embryos**	**Oocytes**	**Somatic cells**
Cattle				
Goat				
Sheep				
Horse				
Pig				
Etc.				

What is the average cost of collecting genetic material from an individual donor animal at a collection centre per visit (excluding travel) for the following species?

	Material			
Species	**Semen**	**Embryos**	**Oocytes**	**Somatic cells**
Cattle				
Goat				
Sheep				
Horse				
Pig				
Etc.				

What is the average number of samples you expect to obtain from each donor, according to species and material type?

Species	Material			
	Semen	**Embryos**	**Oocytes**	**Somatic cells**
Cattle				
Goat				
Sheep				
Horse				
Pig				
Etc.				

What is the number of donors needed per breed to reach your selection goal according to species and material type?

Species	Material			
	Semen	**Embryos**	**Oocytes**	**Somatic cells**
Cattle				
Goat				
Sheep				
Horse				
Pig				
Etc.				

What is the average failure rate (%) when collecting genetic material from an individual donor animal for the following species?

Species	Material			
	Semen	**Embryos**	**Oocytes**	**Somatic cells**
Cattle				
Goat				
Sheep				
Horse				
Pig				
Etc.				

What is the average distance between the donor farm and the collection centre and the average transport cost per km according to species?

Species	**Average distance (km)**	**Average cost (per km)**
Cattle		
Goat		
Sheep		
Horse		
Pig		
Etc.		

Annex 5.1 Multi-species SNP array

The genomic characterization of the gene bank collections within and between countries is important in decision making about additional samples needed to be stored for the future. A standardized SNP tool with information about all stored species can be a valuable decision-making -tool. Two open access multi-species SNP arrays for the main farm animal species (one array for cattle, pig, chicken, horse, goat and sheep; another array for buffalo, duck, quail, bee, rabbit and pigeon) have been designed to genotype genetic collections at a low cost (< US$20/sample). Around 9 000 SNPs per species (see table below) were collected from whole genome sequence (WGS) data generated within the IMAGE project as well as variation data obtained by IMAGE partners including publicly available data.

SNP selection for each species was performed based on:

1. overlap with existing arrays with a high allele frequency across populations
2. SNPs in genes affecting phenotypic traits
3. SNPs in the mtDNA
4. Ancestral SNPs
5. SNPs in the MHC region if available
6. random SNPs genes located in genes within QTL regions.

Both arrays can be purchased from commercial genotyping service providers worldwide. DNA samples can be sent out for genotyping after the purchasing agreement. Samples should preferably be submitted to Biosamples (EBI) and the genotyped data containing a BioSample identification number can be uploaded to the European Variation Archive (EVA) (https://www.ebi.ac.uk/eva/) (see more in Section 8). The genotypes can be compared with the other pre-loaded samples of the same species. The pre-loaded samples are derived from publicly available datasets which contain commercial as well as indigenous breeds. Protocols on how to submit and analyse data are available through the IMAGE portal (http://www.imageh2020.eu).

TABLE 5.1.1
Numbers of SNP per species and marker characteristic for the two multi-speccies SNP arrays designed in the IMAGE project

	IMAGE001 species						IMAGE002 species					
Marker characteristic	Cattle	Pig	Chicken	Horse	Goat	Sheep	Buffalo	Duck	Quail	Bee	Rabbit	Pigeon
Overlap existing arrays	7 817	6 173	7 366	7 748	7 979	9 583	7 991	0	0	0	7 897	0
Newly selected	0	0	0	0	0	0	0	7 900	7 901	7 901	0	7 901
Sex chromosome X/Z	240	539	635	368	200	134	131	0	474	0	296	0
Sex chromosome Y/W	50	26	100	80	69	50	17	18	2	0	7	36
mtDNA	13	36	90	0	170	136	198	7	4	7	11	0
Ancestral	974	2 000	2 361	322	1 043	256	573	1 426	335	0	0	0
Trait-related	73	107	32	50	1 164	80	201	251	1 751	0	0	202
MHC	134	9	63	203	0	0	0	0	0	0	0	0
Genes in QTL-regions	1 723	1 289	0	1 537	60	800	225	0	166	0	0	1 105
Total	10 093	10 107	9 308	10 114	9 993	10 111	7 901	7 900	7 901	7 901	7 897	7 901

Annex 6.1 Guidelines for semen cryopreservation

INTRODUCTIONS

Health accreditation of collection, storage and implementation operations INTRODUCTION. Due to National and International specific regulations and agreements, operators wishing to implement the following procedures are advised to approach the relevant authorities to consider the corresponding regulatory constraints.

Know-how is crucial for the success of the procedures. It is acquired with experience and time. Training is often required. We thus suggest contacting the drafters of the procedures before any use.

Semen quality evaluation. The following procedures will be successful if they consider the semen quality. Appendix B describes the basic methods of semen evaluation. Each of them must be adapted to the species.

Breeding factors and welfare. As explained in sections 3 and 6 of this guideline, semen cryopreservation must be conducted on males raised in the best welfare conditions, and semen collected in the best reproductive cycle. The conditions must be adapted to the species in question.

CRYOPRESERVATION OF CATTLE SEMEN

Bull (dairy, beef, water buffalo) semen collection, dilution, cooling, and cryopreservation
A high-quality source of information for freezing bull semen is the National Association of Animal Breeders - Certified Semen Services (https://www.naab-css.org/). This organization lists a variety of acceptable cryopreservation media (e.g. Tris-Egg yolk, milk and other acceptable freezing diluents) for use with bull semen as well as appropriate antibiotic treatments, and hygiene standards that must be used. However, if holding/shipping the samples is necessary then the use the Tris-egg yolk media (method listed below) is recommended, and the milk-based media and other diluents is discouraged because the post-thaw quality will be diminished. In addition, the sperm concentration specified in this protocol is meant to be used with bulls of unknown fertility. If the fertility of a bull is known, as determined with frozen-thawed semen and artificial insemination, then the sperm concentration when freezing can be adjusted lower and will result in the production of more semen straws per ejaculate.

The recommendations for the protocols in the semen collection

- Collect semen from sexually mature bulls and ensure the sample is free of urine and other contaminants.
- Determine the sperm concentration, ensure the sample has acceptable motility, and maintain samples at 37 °C.
- Add antibiotics to the neat semen and both cryopreservation media per the National Association of Animal Breeders (NAAB) Certified Semen Services standards (https://www.naab-css.org/). Current recommendations for antibiotics include the use of Gentamicin sulfate, Tylosin and Linco-Spectin.

- Dilute the samples in 15 or 50 ml tubes to 120 x 10^6 sperm/ml with 37 °C Tris-egg yolk A (TCA; see recipe below).
- Place the samples in a 37 °C water bath and cool to 5 °C in 2 hours.
- If the samples will be transported overnight then, after cooling, the samples are place in an insulated shipping container with enough cold packs to maintain the samples at 5 °C for at least 24 hours.
- Once the samples reach 5 °C, or when the samples arrive at the laboratory for freezing, samples are diluted 1:1 (volume to volume) with 5 °C Tris-egg yolk B (TCB; see recipe below) resulting in a final sperm concentration of 60 x 10^6 sperm/ml.
- Load the samples into 0.25 or 0.5 ml CBS or wick and powder (aka French) semen straws.

Two recommended freezing options for the semen

1) **Box freezing**: Place samples on a rack and freeze in liquid nitrogen vapor (4.5 cm above liquid nitrogen) for 10 to 15 min. Plunge the samples into the liquid nitrogen or storage.
2) **Programmable freezer**: Samples are placed on a rack and frozen with the following curve: 5 °C to -10 °C at 5 °C/min; -10 °C to -110°C at 40 °C/min; -110°C to -140°C at 20°C/min and then plunged into liquid nitrogen for storage.

Recommendation for thawing and insemination

- Thaw samples for 30 seconds in a 37 ºC water bath, ensure the straw is completely dry before use, and evaluate motility prior to insemination to ensure adequate numbers of sperm survived the cryopreservation process.
- Artificial insemination can be performed following estrous synchronization using single semen straws per insemination. However, either single or double (2 inseminations per estrus) inseminations may be performed.

Recipes

The preferred Semen Cryopreservation Media Recipe is from Purdy & Graham (2004). This media can also be purchased commercially and is known by the trade names Biladyl or Triladyl.

- Tris-Egg yolk A (TCA): 200 mM Tris, 65 mM citric acid monohydrate, 55 mM glucose
- Tris-Egg yolk B (TCB): TCA with 14% glycerol by volume

Both solutions can be frozen in aliquots, thawed, and used as described.

CRYOPRESERVATION OF BOAR SEMEN

Semen collection, dilution, cooling, and cryopreservation

Boar semen is routinely collected, diluted and held at 15 °C for multiple days prior to cooled semen insemination. Furthermore, it can be frozen immediately after collection or aliquots of those ejaculates intended for cooled semen insemination can be frozen 24 hours after collection. Those methods are described below as a supplement to the methods described in the 2012 guidelines.

- Prepare the semen shipping extender (e.g. Androhep Plus or Androstar from Minitube, Verona, WI or other commercially available media containing buffers, salts, sugars, and antibiotics designed to maintain a balanced pH while storing boar semen for extended periods of time) and warm to 37 °C.

- Collect the sperm-rich fraction of a boar semen sample using the hand-glove technique and remove the gel fraction with sterile gauze or a semen filter. Maintain the sample at 37 °C.
- Determine the sperm-rich ejaculate volume, sperm concentration, sperm count, and the volume of the ejaculate.
- Dilute the required sperm-rich fraction 1:1 (v:v) with 37 °C semen extender in a 37 °C beaker.
- Aliquot the sample into 50 ml centrifuge tubes or other appropriate sealable tubes that are labelled with the name and identification number of the boar.
- Cool the sample to 23 °C in 1 hour and shield it from light during this time.
- Cool the sample over to 15 °C in 1.5 hours and maintain it at this temperature during transportation. If the samples will be transported overnight then, after cooling, the samples are placed in an insulated shipping container with sufficient cold packs to maintain the samples at 15 °C for at least 24 hours.
- Upon receipt of the samples, centrifuged the diluted ejaculates for 10 minutes at 800 x g at 15 °C.
- Remove the supernatant, combine the pelleted sperm by boar, determine the sperm concentration and ensure the sample has acceptable motility.
- Dilute the samples to 600 x 10^6 sperm/ml with 15 °C Beltsville Freezing Extender 5 (BF5) cooling extender (CE; see recipe below), place the samples in a 15 °C water bath and cool to 5 °C over 2 hours.
- Once the samples reach 5 °C, dilute the samples ***drop-wise*** using 5 °C freezing extender (FE; see recipe below) over 5 minutes to 400 x 10^6 sperm/ml.
- Load the samples into 0.5 ml CBS or wick and powder (aka French) straws and freeze them using either:
  1) a programmable freezer (e.g. Cryo Bio System Mini Digitcool UJ400, IMV Corporation, Minneapolis, MN) and the following curve: 5 ºC to -8 ºC at -20 ºC per minute, -8 ºC to -120 ºC at -69 ºC per minute, -120 ºC to -140 ºC at -20 ºC per minute; *OR*
  2) box freezing by placing the straws on a rack in liquid nitrogen vapor (4.5 cm above the liquid) for 10 to 15 minutes.
- Plunge the samples into liquid nitrogen for storage.

Thawing and artificial insemination

Samples are thawed for 20 seconds in a 50 ºC water bath and the motility is evaluated prior to insemination to ensure adequate numbers of sperm survived the cryopreservation process.

Artificial insemination can be performed using either:

1) Standard intracervical insemination with 2 inseminations per sow or gilt and ≥ 1 x 10^9 motile sperm, per insemination. The sample should be diluted to a final volume of 80 ml in Beltsville Thawing Solution (Pursel and Johnson, 1975) per insemination;
   OR
2) Deep intrauterine insemination (1 insemination per sow or gilt; Martinez et al., 2001, Roca et al., 2003) with 1 x 10^9 motile sperm diluted in Beltsville Thawing Solution as described previously. After insemination flush the insemination catheter with an additional 2 ml of BTS to ensure complete deposition of the insemination dose.

Recipes

BF5 from Pursel and Johnson (1975)

Cooling extender (CE)

52 mM TES

16.5 mM Tris
178 mM D-glucose
20% Egg yolk, by volume
The CE should be centrifuged at 10,000 x g for 25 minutes to remove egg yolk particles

Freezing extender (FE)
The ingredients are by volume:
91.5% CE
6% Glycerol
2.5% Equex paste

CRYOPRESERVATION OF GOAT SEMEN

Semen Collection, Transportation, Processing and Cryopreservation Protocol
Because some bucks produce enzymes that cause egg yolk to coagulate upon dilution and incubation, centrifugation is commonly used to remove the seminal plasma and alleviate the problem. Cryopreservation media with low concentrations of egg yolk can also be used to circumvent the problem so centrifugation is not required. It is advisable to test bucks to determine if they produce the coagulating enzymes prior to collecting and freezing samples to avoid wasted efforts. Nevertheless, a low egg yolk cryopreservation medium such as the one presented here, can be used to ameliorate the deleterious effects.

- Collect semen from sexually mature bucks and inspect samples to ensure it is free of urine and other contaminants.
- Determine the sperm concentration, ensure the sample has acceptable motility, and maintain samples at 37 °C.
- Add antibiotics to the neat semen and the cryopreservation media per the National Association of Animal Breeders (NAAB) Certified Semen Services standards (https://www.naab-css.org/). Current recommendations for antibiotics include the use of Gentamicin sulfate, Tylosin and Linco-Spectin.
- Dilute the samples in a 15 or 50 ml tube to 400 x 10^6 sperm/ml with 37 °C Tris-egg yolk glycerol medium (TEYG; Mook and Wildeus, 2008, see recipe below).
- If coagulation is a concern, which may be the situation with bucks that are known to react with egg yolk or when collecting an undocumented buck under field conditions, then wash the sample to remove seminal plasma.
- Dilute the sample 4 to 5 times with egg yolk and glycerol free TEYG medium centrifuge at 800 x g for 10 min. Determine the sperm concentration and dilute with TEYG as described previously.
- Place the samples in a 37 °C water bath and cool to 5 °C over 2 hours.
- If the samples will be transported overnight then, after dilution and cooling place the samples in an insulated shipping container with enough cold packs to maintain the samples at 5 °C for at least 24 hours.
- Load the samples into 0.5 ml CBS or wick and powder (aka French) semen straws and freeze them using:
    1) Box freezing by placing the samples on a rack and frozen in liquid nitrogen vapor (4.5 cm above liquid nitrogen) for 10 to 15 minutes;
    OR
    2) A programmable freezer and the following curve: 5 °C to -10 °C at 5 °C/min; -10 °C to -110°C at 40 °C/min; -110°C to -140°C at 20°C/min.

- Plunge samples into liquid nitrogen for storage.

Thawing an insemination

Thaw samples for 30 s in a 37 ºC water bath and evaluate motility prior to insemination to ensure adequate numbers of sperm survived the cryopreservation process.
Artificial insemination can be performed following estrous synchronization or by identification of ewes in heat using a vasectomized male. Single semen straws are used per insemination. Either single or double (2 inseminations per estrus) cervical inseminations may be performed with the aid of a lighted sheep and goat speculum (Evans and Maxwell, 1987).

Recipes

Tris-egg yolk-glycerol (TEYG) diluent, for a 100 ml volume:

Tris	2.422 g
Fructose	1.0 g
Citric Acid	1.36 g
Penicillin G	0.006 g
Streptomycin sulfate	0.100 g
Egg yolk	2.5% by volume
Glycerol	2.0 % by volume

pH to 6.8-7.0

CRYOPRESERVATION OF RAM SEMEN

Many factors contribute to success/fertility with these techniques. It is recommended that rams used for semen collections should be at least 1-year-old, ewes should be 4 to 6 years of age, and if necessary, 2 to 3-year-old ewes may be used. Preferably, the ewes will have at least 1 parity. Timed inseminations should be performed between 47 and 55 hours post-CIDR removal. Ewe breed is a significant source of variation for fertility with these techniques and therefore it is recommended that a small initial fertility trial is performed testing multiple insemination times to identify an effective protocol that will result in the highest fertility possible.

Semen collection and processing

- Collect semen from sexually mature rams and inspect samples to ensure it is free of urine and other contaminants.
- Determine the sperm concentration, ensure the sample has acceptable motility, and maintain samples at 37 °C.
- Samples can be frozen using either skim milk egg yolk (SMEY) cryopreservation medium or Tris-egg yolk-glycerol (TEYG) cryopreservation medium. Both media produce acceptable fertility when non-surgical AI is performed but the TEYG medium (also known commercially as Biladyl or Triladyl from Minitube, USA) is less complicated to prepare (see Recipes section below) than the SMEY diluent. Moreover, samples can be held and transported in TEYG for at least 24 hours prior to freezing without detrimental effects on the sperm physiology, post thaw motility, and fertility whereas the SMEY diluent is not suitable for holding and transporting ram semen samples for long periods of time (> 3 hours) prior to cryopreservation.

Semen cryopreservation using the SMEY diluent

- Dilute ram semen samples to 1200 x 10^6 sperm/ml in 37 °C SMEY ***cooling*** media (recipe below) and place the samples in a 37 °C water bath. Cool the samples to 5 °C over 45 to 60 min.
- Dilute the samples drop-wise over 5 min (1:1; volume to volume) with 5 °C SMEY ***freezing*** media (recipe below) resulting in a final sperm concentration of 600 x 10^6 sperm/ml.
- Load the samples into 0.5 ml CBS or wick and powder (aka French) semen straws and freeze.

Semen cryopreservation using the TEYG diluent

- Dilute the ram semen samples drop-wise, in one step, with the TEYG freezing medium to 400 x 10^6 sperm/ml.
- Cool the sample to 5 °C over 90-120 minutes.
- If necessary, following cooling of the samples to 5 °C they can be maintained at this temperature for up to 48 hours prior to freezing. This will enable transportation via overnight courier to a laboratory for cryopreservation.
- Load the samples into 0.5 ml CBS or wick and powder (aka French) semen straws and freeze.
- Samples are frozen by:
  1) Box freezing by placing the samples on a rack and frozen in liquid nitrogen vapor (4 cm above liquid nitrogen) for 10 to 15 minutes;
     OR
  2) A programmable freezer and the following curve: 5 °C to -10 °C at 5 °C/min; -10 °C to -130°C at 60 °C/min.
- Plunge the samples into liquid nitrogen for storage.

Thawing and insemination

- Thaw samples frozen in either the SMEY or TEYG for 30 seconds in a 37 °C water bath and evaluate motility prior to insemination to ensure adequate numbers of sperm survived the cryopreservation process.

- Estrous synchronization and artificial insemination should be performed using the following protocols:
  - Estrous synchronization: administer CIDRs (e.g. 0.3 g progesterone in an inert silicone elastomer for 12 days; Pfizer Animal Health, New York, NY) followed by PMSG administration (400 IU, i.m.; total volume = 4 ml using an 18 gauge needle) 24 hours prior to, or at sponge removal;
  - Inseminations of 100 x 10^6 motile sperm are performed at 53- and again at 57-hours post CIDR removal. For identification of an optimal insemination time for a flock/breed, a low dose insemination of 70 x 106 motile sperm in a single dose can be used over a range of insemination times and ewe groups. However, this will not result in maximal fertility but will identify a range of times within which to inseminate in the future. Once the optimal insemination time is identified, a double insemination 4 hours apart can be used to maximize fertility.

- Artificial insemination is performed on a restrained ewe in a standing position (e.g. a sheep handling squeeze chute or haltered and held at the shoulder and hip next to a panel or wall) to minimize stress.

- Apply non-spermicidal lubricant to the tip of an AI gun loaded with a semen straw and place the lubricant on the bottom of the interior of the labia.
- Part the labia and insert the AI gun upward at a 45° angle through the lubricant.
- Tilt the AI gun into a horizontal position once contact is made with the top, interior of the vagina, and gently insert the gun through the vagina to the os cervix or into the cervix proper without force. Once resistance is observed, pull the gun back about 3 cm and probe with the tip to determine if the gun can be inserted further. Probing is attempted to determine if a deeper insemination may be achieved as the goal is to deposit the insemination dose as deep as possible in the vagina/cervix of the ewe without force.
- Deposit the insemination dose slowly when the maximum insemination depth is achieved and remove the gun from the ewe.

Recipes

Skim milk-egg yolk (SMEY) cryopreservation medium (Paulenz et al., 2007) for a two-step cryopreservation medium is produced as described below.

SMEY cooling medium

- Dilute 11 grams of non-fat dried skim milk into approximately 80 ml of distilled/deionized water and heat to 95 °C for 10 min.
- Cool the solution to room temperature and add 5 ml of egg yolk, at a minimum 1mg/ml streptomycin sulphate (or antibiotics according to Certified Semen Services standards (https://www.naab-css.org/) and water to bring the final volume to 100 ml.

SMEY freezing medium

- Mix 86% SMEY cooling medium by volume with 14 % glycerol by volume.

Tris -egg yolk- glycerol (TEYG) cryopreservation medium (Davis et al., 1963) is produced as follows:
This is a one-step cryopreservation medium and is also commercially available under the names Biladyl or Triladyl. The following is a recipe for 500 ml of ram semen cryopreservation diluent.

TRIS (MW 121)	12.112 g
Citric acid	6.8g
Glucose	5.0g
Glycerol	25.0 ml

- Fill to 400 ml with distilled, deionized water

Egg yolk 100 ml (20% by volume)
Antibiotics (either Certified Semen Services standards (https://www.naab-css.org/ or at least 500 mg streptomycin sulfate)

CRYOPRESERVATION OF HORSE SEMEN

Since the seventies, different methods of cryopreservation of stallion sperm have been developed (Pickett et al., 1975; Martin et al., 1979). The efficacy of the methods, evaluated by different parameters (sperm motility, viability, membrane integrity, etc.) or more rarely by fertility rate after

artificial insemination, varied among the labs and remained lower than chilling methods (Samper and Morris, 1998).
In France, the first freezing method was proposed by Palmer (1984) which was later modified by Vidament *et al. (2000).* This new freezing method allowed a significant increase in the fertility rate per cycle (Vidament, 2005). However, the protection of sperm cells against freezing damage remained to be improved. Therefore, a new freezing extender was developed (INRA Freeze) that, associated to the freezing steps, greatly improved fertility rate to reach chilled sperm level (Pillet *et al.,* 2008, 2011). The method that in mostly used in France is described below.

Freezing

INTRODUCTION
Stallions are submitted to regular sperm collection (3 times a week, every other day) using a closed artificial vagina one week before starting the freezing procedure. The same rhythm of semen collection is maintained during the stay of the stallion in the freezing lab. Just after collection, sperm is filtered through gauze (to eliminate the gel fraction and any debris) in a tube at 35-37°C then the concentration of raw sperm is evaluated using a photometer and immediately processed.

Freezing procedure
1. Sperm is diluted in 50 ml Falcon tubes at #50x10^6 spermatozoa per ml in the 1st extender namely INRA96 in the water bath at 37°C,
2. Diluted sperm is then transferred to 22°C (water bath) for 10 min before centrifugation at 600g (10min),
3. The supernatant is discarded and the pellet is re-suspended in INRA Freeze™ extender to obtain a concentration of 100x10^6 spermatozoa per mL,
4. Then the diluted sperm is cooled at 4°C for 75min before packaging in polyvinyl chloride 0.5mL straws and sealed,
5. Freezing of straws was achieved with a programmable freezer (automatic Minidigitcool) at -60°C.min^{-1} from 4°C to -140°C then stored in liquid nitrogen at -196°C until use.

Extenders
INRA96 and INRA Freeze extenders were patented by INRA and licensed to IMV-Technologies (L'Aigle, France). The two extenders are produced and commercialized throughout the world by IMV-Technologies.

Thawing
The thawing process consists of:
1. plunging straws in a water bath at 37°C for 30 seconds
2. wiping the straws before liberation of the content in a tube:
   - containing INRA96 extender when quality parameters of sperm cells are evaluated
   - without an extender if thawed sperm is inseminated.

A routine insemination artificial dose is composed of 8 straws (400x10^6 total sperm cells).

Sperm Evaluation
Sperm evaluation at thawing is mainly motility using microscope evaluation. However, an increasing number of laboratories are equipped with automated system (CASA: computer assisted sperm analysis). Other parameters such as measuring different sperm functions (membrane integrity and

organization, viability, acrosome integrity, oxidative level, DNA integrity, mitochondrial activity, *etc.*) can be analysed. Some of them are related to fertility (Barrier-Battut *et al.*, 2017).

CRYOPRESERVATION OF CHICKEN SEMEN

As indicated in Section 6, there are different methods to cryopreserve chicken semen. One of them, the glycerol method (Tselutin et al., 1999; Blesbois et al., 2007; Thelie et al., 2019) is standardized for males with different fertility levels while the others (Woelders et al 2006; Sasaki et al., 2010; Blesbois et al., 2011, Abouelez et al., 2017; Thananurak et al., 2020) are recommended for highly fertile males. The Annex describe the standardized "glycerol method" and one other simple method updated in 2020, the DMF method. For both methods, it was decided to keep the straws packaging that is the most practical for the cryobanking. Irrespective of the method used, great care must be taken regarding the steps before and after the cryopreservation (see section 6) since these steps must be rapid and involve a limited number of samples to be efficient.

Glycerol method for individual chicken semen cryopreservation

Read more about the protocol on the website of CRB-anim: https://www.crb-anim.fr/crb-anim_eng/).

Semen collection, treatment and freezing

1. Individual semen are collected from each male (Burrows and Quinn, 1937) in plastic tubes containing 200 µl LPC diluent at 20-25 °C (Lake and Stewart, 1978), containing for 1 L: Magnesium acetate (0.7 g), Sodium glutamate (5.0 gr), Potassium acetate (5.0 g), D-Fructose (8.0 g), BES (1 g), Polyvinylpirrolidone (10000: 3.0 g), NaOH 1N (4 ml). Diluent pH 7.1, Osmotic pressure 340 mosm. In case of semen with low expected fertility, add 10mM Valine (Bernal-Juarez et al., 2020). This is the dilution 1. The time of collection must be of a maximum 10 min for all the males, so usually it is not feasible to collect semen from more than 8 males.
2. Add LPC diluent at 20-25°C to each collected semen sample in order to reach a final dilution 1:1 (dilution 2) and then put the diluted semen in a fridge/cold room at 4-5°C for 10min.
3. Mix each sample with a volume of LPC (at 4-5°C) diluent equivalent to two initial semen volume containing 22% glycerol (Dilution 3). So, the final semen dilution will be 1:3, and the final glycerol amount 11%.
4. Equilibrate 10 min at 4-5°C with gentle shaking
5. Put the diluted semen in 0.5 ml straws previously identified per male, make a bubble at the top of the straws, seal the straws, transfer them in a programmable freezer
6. Freezing: -7°C/ min from + 4°C to -35°C, then – 60°C/ min from – 35°C to 140°C
7. Rapidly transfer the straws to liquid nitrogen tank
8. Clearly identified the place of the samples in the tank and register in archived documents.

Thawing and insemination

1. Remove straws from the liquid nitrogen tank (no more than 40 straws at the same time). Plunge the straws for 3 min in a 1L becker of water at 4°C. All the steps of thawing are at 4°C. Avoid any thermic shock.
2. Wipe the outside of the straws, liberate the semen in beckers identified per male and containing an amount of LC diluent (Lake and Stewart 1978) corresponding to dilution a (see Table 1). The composition of LC diluent is for 1L: magnesium acetate tetrahydrated (0.8 g), potassium citrate monohydrate (1.28 g), sodium glutamate (19.2 g), D-fructose (6 g), sodium acetate (5.1 g), TES

(5.0 g), NaOH 1N (5.2 ml). The volume of diluent depends on the number of 0.5 ml straws thawed per male.

3. Make the dilutions b to f (Table 1), each separated by 2 min. This permits slow removing of the intracellular glycerol.
4. Centrifuge each sample for 15 min at 500g, 4°-5 C.
5. Discard the supernatant (that now contain the glycerol) and replace it by an insemination diluent (e.g. L7.1, Lake and Ravie 1981).
6. If the thawing is followed by insemination, ensure rapid intra vaginal insemination of a mean of 200 to 400 million sperm/female and with great caution since frozen-thawed semen is more sensitive to all variations than fresh semen and since the receptivity of the female is a key factor of success. The success also depends on the female fertility level. The insemination must be conducted at least 3 hours before or 3 hours after the daily lay in order to avoid opposite vaginal peristalsis.

TABLE1

Dilutions before centrifugation and pellets resuspension after centrifugation

There are 6 successive dilutions of the thawed semen with LC diluent before the centrifugation (a to f). After the centrifugation, there is one resuspension in the L7.1 insemination diluent.

N straws (0.5ml)	2	4	6	10	12	20	24	30	40
a diluent vol (ml)	0,07	0,13	0,2	0,33	0,39	0,65	0,72	1	1,3
b	0,18	0,36	0,54	0,9	1,1	1,8	2	2,7	3,6
c	0,33	0,66	0,99	1,65	2	3,3	3,66	2 x 2,48	2 x 3,3
d	0,6	1,2	1,8	3	3,6	2 x 3	2 x 3,32	2 x 4,5	4 x 3
e	1,24	2,5	3,71	6,25	2 x 3,75	3 x 4,12	4 x 3,4	4 x 4,7	6 x 4,12
f	1,58	3,2	4,73	8	2 x 4,8	4 x 4	4 x 4,33	6 x 4	7 x 4,5
total diluent vol (ml)	4	8,05	11,97	20,13	24	40	43,94	60,3	80
straws vol (ml)	1	2	3	5	6	10	12	15	20
total vol (ml)	5	10	14,97	25,13	30	50	55,94	75,3	100
N tubes of 15ml	2	3	4	8	10	12	14	20	32
volume / tube (ml)	2,5	3,35	3,74	3,13	3	4,16	4	3,75	3,13
pellet resuspension in L7.1	100	100	100	100	100	100	100	100	100

DMF method of chicken semen cryopreservation

The second method of chicken semen cryopreservation is as simply as possible. It does not need a programmable freezer, and the internal cryoprotectant (DimethylFormamide, DMF) is not removed at thawing (Thananurak et al., 2019; 2020). This method is efficient for highly **fertile males**.

Semen collection, treatment and Freezing

1. Collect with precaution individual semen of each male (Burrows and Quinn, 1937) at room temperature (mean 25°C).
2. At room temperature, dilute 1:2 very rapidly the semen with BHSV-based diluent (5g glucose, 2.5g Inositol, 28.5g sodium glutamate, 0.7g magnesium acetate tetrahydrate, 5g potassium acetate, all of which were dissolved in 1,000 ml of double-distilled water, Schramm, 1991) supplemented with Serine 4mM and Sucrose 1mM (Thananurak et al., 2019; 2020).
3. Cool slowly the semen at 5°C by placing it in fridge with controlled temperature.
4. When the semen reach 5°C, add another fraction of diluent prepared at 5°C (vol1, equivalent to initial semen volume 1) containing DMF (24%). The final semen dilution is 1.3 and the final percentage of DMF in the diluted semen is 6%.
5. Load the diluted semen in 0.5 ml plastic straws sealed with PVP powders and equilibrate for 15 min at 5°C.
6. After equilibration, the filled straws are laid horizontally on a rack, 11 cm above the surface of LN_2 (-35ºC) for 12 min, then, placed 3 cm above liquid nitrogen vapor (-135ºC) for 5 min, and subsequently immersed in LN_2.
7. Transfer the straws to a liquid nitrogen tank

Thawing and insemination

1. Straws are thawed in a water bath at 5 ºC for 5 min
2. Wipe the outside of the straws, liberate the semen in beckers identified per male
3. Intravaginal inseminations are then conducted rapidly with the same precautions as for the glycerol method. The insemination must be conducted a minimum of 3 hours before or after the lay in order to limit the semen rejection due to the lay peristalsis

CRYOPRESERVATION OF GUINEA FOWL SEMEN

There are two methods for guinea fowl semen cryopreservation, one that uses the cryoprotectant DMF and the straws packaging (Seigneurin et al 2013), and the other using ethylene glycol and a packaging in glass ampoules (Varadi et al., 2013). We describe here the method of using straws since this packaging is the most popular for reproductive cryobanking.
The following protocol is detailed on the website of CRB-anim: https://www.crb-anim.fr/crb-anim_eng/).

Semen collection, treatment and freezing

1. Collect semen by massage (Burrows and Quinn, 1937)
2. Dilute 1:1 in BHSV diluent (5 g glucose, 2.5 g Inositol, 28.5 g sodium glutamate, 0.7 g magnesium acetate tetrahydrate, 5 g potassium acetate, all of which were dissolved in 1,000 ml of double-distilled water, Schramm, 1991) at room temperature (20-25°C).
3. Diluted semen was cooled to 4°C for 15 min (must be checked)
4. Add a second volume of BHSV diluent at 4°, containing 18% DMF. The final semen dilution is 1:2, with a final percentage of DMF of 6%.
5. Equilibrate for 4 min at 4°C and then load the diluted semen in 0.5 ml plastic straws sealed with PVP powder.
6. Freeze in a programmable freezer with the freezing rate 30°C/min from +4°C to -140°C
7. Transfer the straws in liquid nitrogen storage tank.

Thawing and insemination

1. Straws are thawed in a water bath at 40°C for 45 seconds.
2. Wipe the outside of the straws, liberate the semen in identified beckers.
3. Intravaginal inseminations are then conducted rapidly with the same precautions as for chicken sperm methods.

CRYOPRESERVATION OF GANDER SEMEN

Methods used for cryopreservation of gander semen (male geese) depend on species. Two different methods were proposed for Thaï or European ganders (Thaï et al., 2001, Lukaszewics 2002), which use different volumes of the same cryoprotectant (DimethylAcetamide: DMA) and gave equivalent results when adapted to Landese ganders (Dubos et al., 2008). Here we describe a simple method adapted by Thai et al. (2001) to Taïwan ganders.

Semen collection, treatment and freezing

1. Collect semen by massage of the gander (Burrows and Quinn, 1937)
2. Dilute 1:1 in Taï diluent containing 15 % egg yolk (Taï et al., 2001) at room temperature (20-25°C).
3. After 15 min at room temperature, add 2 new volumes of diluent containing 18% DMA. The final semen dilution is 1:3 and the final percentage of DMA is 9%.
4. Equilibrate for 10 min and then load the diluted semen in 0.5 ml plastic straws sealed with PVP powder.
5. Put on dry ice for 30 min or in nitrogen vapor.
6. Transfer the straws into a liquid nitrogen storage tank.

Thawing and insemination

1. Straws are thawed in a water bath at 40°C for 45 seconds.
2. Wipe the outside of the straws, liberate the semen in identified beckers.
3. Intravaginal inseminations are then conducted rapidly with the same precautions as for chicken sperm methods.

SEMEN OF OTHER DOMESTIC BIRD SPECIES

As described in Section 6, cryopreservation of turkey semen is not yet fully available but work by Ioro et al. (2020) has yielded encouraging results. Ducks represent a highly variable species in terms of the capacity of sperm to be efficiently cryopreserved and methods are not yet standardized. While cryopreservation of common duck and Pekin duck sperm follows the simple methods used for chicken semen freezing, the method used for Muscovy duck is different (Blesbois 2011). Standardization of Emu and Ostrich semen cryopreservation is also expected in the future.

CRYOPRESERVATION OF HONEY BEE SEMEN

Some of the most widely used honeybee semen cryopreservation procedures contain egg yolk in the cryopreservation media which is purported to play a role in protecting the cell membrane of the spermatozoa. However, since the sterility and quality of the raw yolk derived from chicken eggs

reared under varying conditions can vary, alternative yolk-free protocols have been explored. Presented below are two equivalent procedures published previously by Rajamohan et al. (2019) and Hopkins et al. (2012).

Cryopreservation with egg yolk-free diluent

The following protocol describes a method to cryopreserve ~50 µl of semen (25 µl of semen + 25 µl of cryoprotective diluent) in a 0.25 ml PETG sperm straw (CBS, 010261). However, the protocol may be modified to accommodate up to 75 µl of semen or more by adjusting the air spacing between the solutions in the straw while loading the semen. *Notes of caution:* The honeybee spermatozoa, which are extremely elongated and extremely fragile, move in an amoebic undulatory process. Therefore, it is prudent to desist from rapid pipetting, vortex mixing or centrifuging at unprescribed rates. Mixing is preferably done using a capillary with a blunt end that could be fabricated by melting one end of the glass capillary. Sterile laboratory practices are needed since the queen bee will store the semen after insemination in her spermatheca for months or even years. Therefore, contaminated semen would be deleterious to queen's health and hive's wellbeing.

Requisites

- ***Solution A*** - Semen extender (Table 2) without Dimethyl Sulfoxide (DMSO);
- ***Solution B*** – A 2x stock solution of semen extender containing 20% DMSO (**Table 2**) (keep chilled in a refrigerator)
- ***Solution C*** - Simple saline comprising of the 0.2M Tris buffer pH 7.2 with 1.12% potassium chloride and 0.88% sodium chloride;
- Harbo's syringe
- A programmable freezer set at 4°C and programmed to freeze at 3°C/min to -45°C.
- 0.25 ml sperm straws – the 133 mm straw used here has 115 mm usable space and ~ 12 mm of cotton plug at one end. A 50 µl sample will occupy 25 mm of the straw. From the open-end tip, using a permanent marker, mark a spot every 25 mm and also mark another spot at 15 mm from the open tip in a different color. There should be 5 spots on the straw as shown below.

FIGURE 1

Straw markers for drone semen freezing

- A 1 ml syringe barrel with appropriate silicone connectors to connect the straw (**Fig. 2**)
- Polyvinyl alcohol straw sealing powder.

TABLE 2

The content of 1 semen extender used for semen collection, storage and shipment

Constituents	**Concentration**	**Milligrams/100 ml of the extender**
Penicillin	10 kUnits	NA
Streptomycin	0.01%	10.0
Amphotericin	0.0025%	2.5
Gentamycin	0.005%	5.0
TRIS	50 mM	605.0
Sodium chloride	150 mM	876.0
Potassium Chloride	151 mM	1125.7
Trehalose	50 mM	1.711.5
Fructose	50 mM	901.0
EGTA	2.5 mM	76.0
EDTA	2.5 mM	73.1
Dimethyl sulfoxide		9.08 grams (or) 10 ml

*Rajamohan et al. (2019).

Semen collection, dilution, cooling and freezing

1. Collect semen from the eversion of the endophalis. The induced ejaculation procedure is done by applying pressure to the thoracic-abdominal segments. The pressure results in a partial eversion prior to the full eversion. In mature males this results in the extrusion of sub-microliter amounts of off-white colored semen floating on the bright white accessory gland secretions (mucus).
2. The semen is collected in sterile glass capillaries connected to a Harbo's syringe (Harbo, 1979, 1985). Prior to uptake of semen, the tube is lubricated with simple saline (Solution C) by simple suction and expelling of the solution. Between drones the tip of the capillary is kept immersed in an Eppendorf containing the extender Solution A.
3. The extender/semen proportions can range from 1:19 to 1:1 of semen without deleterious effects on the spermatozoa. Until cryopreservation or any other use, the semen could be stored at 14°C in dark for more than 30 days.
4. In two small sterile beakers or test tubes take sufficient amounts of 1x solution A and 2x solution B. *Solution B must be chilled*.
5. In a sterile 0.2 ml PCR vial, take 25 µl of semen and add 25 µl of solution B. The solution must be added 5 µl at a time and gently mixed with a heat-sealed capillary.
6. Connect the plugged end of the straw to a syringe tip using a silicone adapter (Fig. NN). Lubricate the straw by aspirating solution A all the way to marker 4 and expel the solution. Then aspirate solution A up to marker '0', lift the straw off the solution and continue aspirating air until the upper meniscus of the solution A is at marker '1'.
7. Now aspirate the semen solution until its upper meniscus reaches marker '1'. Aspirate air until the semen's upper meniscus reaches the marker '2'.
8. Begin aspirating solution A until it's upper meniscus reaches marker '1'.
9. At this point aspirate air until the first drawn solution A reaches the woven plug and the syringe struggles to further aspirate.

   Tap and press the tip of the straw into a vial of polyvinyl alcohol (PVA) until the tip of the straw is filled to about 5-10 mm with the powder. An illustration of the straw thus filled is shown in Figure 3

FIGURE 2
Syringe to load bee semen

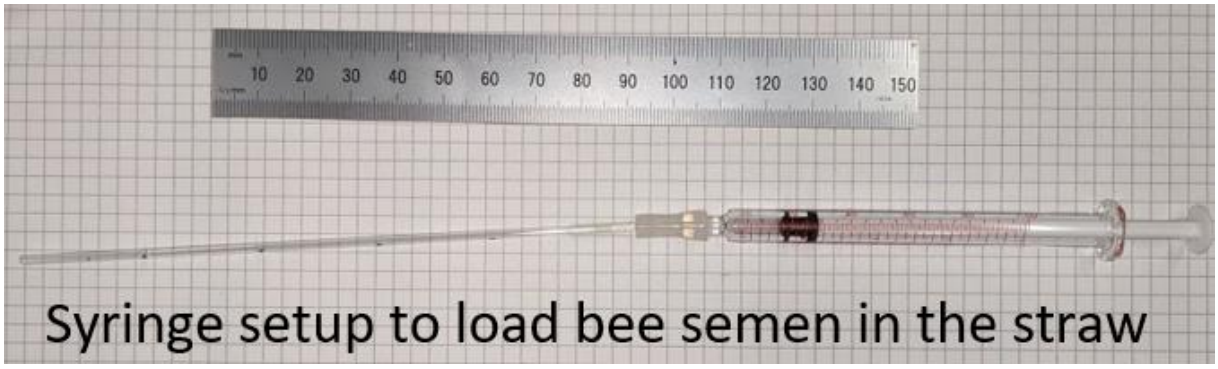
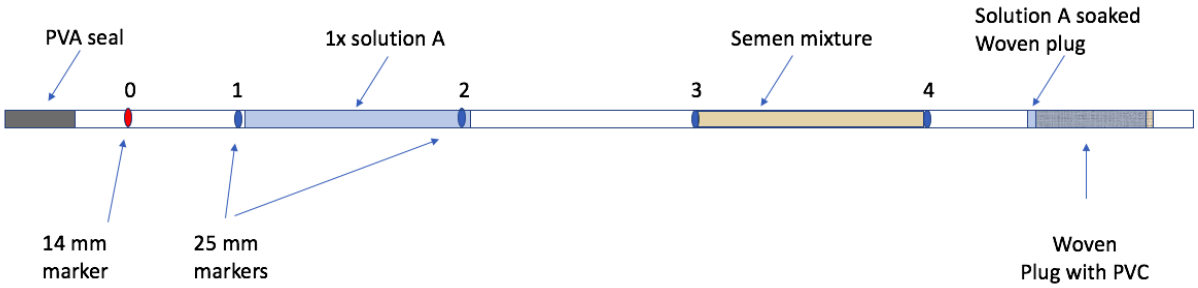


FIGURE 3
Straws filled with semen



10. Place the powder filled end of the straw in a 50-100 ml beaker with just enough water to not let the straw float and place the beaker in a refrigerator for 10 minutes to allow the PVA to solidify and the sample to equilibrate.
11. Transfer the straw to the programmable freezer set at 4°C and begin the programmed freezing to -45°C at 3°C/min.
12. When at -45°C, the straw is removed from the freezer and plunged into liquid nitrogen.
13. The straw is thereafter transferred to visotube which is placed in a daisy goblet (CryoBio Systems) and transferred as fast as feasible into a LN storage canister and container.

Thawing and insemination

1. The straw is rapidly retrieved from the LN and the daisy goblet and plunged and agitated in a 37°C water bath until the frozen contents have visibly thawed.
2. The contents are emptied into a sterile 0.2 ml PCR vial by cutting both ends of the straw. The vial is stored on ice or refrigerated until use.
3. Reconstitution of semen is another option to prolong the storage or transportation of semen. This is made feasible due to the absence of egg yolk components in the medium. The thawed semen is diluted further with 25 µl of 1x solution A and centrifuged at 1200 x g for 10 minutes. Most of the supernatant is removed and an equal volume of replacement solution 1 is added. The mixture is mixed very gently using a sealed tip capillary for not more than 20 seconds. The reconstituted semen sample is stored at 14 °C in the dark.
4. Artificial insemination is performed as previously described in the procedure **“**Conventional cryopreservation using egg yolk cryoprotectant diluent”

Conventional cryopreservation using egg yolk cryoprotectant diluent

Semen collection is carried out as described for the previous method by the eversion of the endophalis of the drone. The cryopreservation diluent includes three components: 50% Harbo's buffer, 25% DMSO cryoprotectant, and 25% chicken egg yolk.

- Harbo's Buffer - The buffer consisted of 79.7 mM NaH2PO4 (S-8282; Sigma-Aldrich) and 31.6 mM Na2HPO4 (S-7907; Sigma-Aldrich) in a final volume of 25 mL using double distilled water and pH adjusted to 7.2 using 6M NaOH.
- The three components are added to a 1.5 ml centrifuge tube and vortexed.
- In a separate 1.5 ml centrifuge tube a known volume of semen is added. The cryopreservation diluent is intended to be mixed at a ratio of 3 parts semen to 2 parts diluent. The diluent is then slowly pipetted into the semen while gently stirring.

Loading straws

The semen–diluent mixture is loaded into 0.25-mL plastic cryostraws (Cassou straws). Straws are cut to a length of 6.5 cm, including the cotton/gelatin plug. The straws are cut because of the small volume of semen (generally about 20 microliters) and to avoid using excessive fluid for backfilling. Straws are then loaded with 20 mL extender, an air space, 20 mL semen–diluent mixture, air space and approximately 20 mL extender or until the initial fluid sealed the plug (Fig 3). The ends of the straws are heat sealed or sealed using commercially available ultrasonic straw sealer and straws are labelled.

Slow cooling

The loaded and sealed straws are then placed in a room temperature water bath (suspended vertically). The water bath consists of 400 ml of water in a 600 ml glass beaker. The water bath with straws is placed in a standard refrigerator (4°C) for 2 hours.

Freezing

After two hours, straws are quickly dried and loaded into the programmable freezing unit. The unit is programmed to start at 4°C and ramp down to -40°C at 3 degrees/minute. Once the samples reach -40°C straws are removed one at a time and rapidly plunged into liquid nitrogen and packed in goblet for storage.

Thawing and insemination

- Straws are thawed in a 40°C water bath for 10 seconds. The sealed ends are cut off and the straw is ready to connect to the instrumental insemination system.
- Artificial insemination of virgin *Apis mellifera* honey bees is usually performed by restraining the queen in a Schley Instrument (Peter Schley, Germany) and mildly narcotizing the bee with flowing carbon dioxide (35 ml/min).
- Prior to manipulation of the queen, the semen is loaded in a Harbo syringe (Harbo, 1985, Cobey et al., 2013).
- Using the hooks in the Schley instrument the dorsal and ventral plates in the hind end of the queen are parted and approximately 5-7 microliters of the semen is placed directly in the median oviduct.
- After a brief recovery period, the queen bee is placed in a queen cage and introduced into either a nucleus hive or a queen bank. Queens require two doses of CO_2 narcotizing separated by 24 hours to initiate egg laying. Queens can either be dosed 24 hours prior to the insemination or 24 hours after, although it is best if they are dosed prior to the insemination event.

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Annex 6.2 Guidelines for Sperm Number and Quality Evaluation

INTRODUCTION

Semen cryopreservation is an important tool in ensuring the long-term survival of a given AnGR. However, these efforts will be futile and a waste of resources if the sperm is not fertile. Therefore, collected semen should be evaluated with classical *in vitro* tests evaluating the number and quality of sperm prior to processing and freezing (Jeyendran, 2000). Semen should be evaluated as soon as possible after collection. Exposure to temperature changes, light and contaminants are generally detrimental to semen quality. Specific equipment, such as computer-aided semen analysis (CASA) systems, can be used for automated evaluation of motility parameters; however, subjective evaluation under a microscope may also be used and technicians should be trained in this technique. Phase contrast microscope, fluorescence microscopy and flow cytometry may be used to evaluate plasma membrane integrity, mitochondrial function, acrosome integrity, DNA damage and chromatin maturation.

Five basic characteristics should be addressed routinely when evaluating semen number and quality:

1. sperm concentration;
2. motility;
3. viability;
4. morphological integrity; and
5. acrosome status.

In addition, sperm DNA damage is gaining interest as a potential cause of infertility.

SPERM CONCENTRATION

Evaluation by photometry

Sperm concentration is most accurately estimated with specialized equipment, such as a spectrophotometer. The sperm concentration is then evaluated by measuring the absorbance by sperm at a visible wavelength. It is recommended to use a wavelength between 550 and 650 nM. These values are species specific since the shape of the sperm may have an impact on the rate of absorbance.

Evaluation by heamocytometry

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

MOTILITY

The movement of the sperm should be checked: first, because movements indicate that sperm are alive; and second, because motility is related to sperm fertilizing capacity. Two types of motility are usually evaluated – gross motility and individual motility.

Gross motility subjectively evaluated under the microscope

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

Individual motility subjectively evaluated under the microscope

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

Sperm motility variables objectively evaluated by Computer Assisted Semen Analysis (CASA)

The percentage of total motile spermatozoa, the percentage of spermatozoa showing non-progressive motility, and the percentage showing progressive motility are recorded by CASA system. Sperm movement characteristics - curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement, beat-cross frequency – are also analyzed by CASA. Three progression ratios, expressed as percentages, are calculated from the three velocity measurements described above: linearity (calculated as VSL/VCL x 100), straightness (calculated as VSL/VAP x 100), and wobble (calculated as VAP/VCL x 100). A minimum of 500 sperm tracks should be evaluated.

VIABILITY/PLASMA MEMBRANE INTEGRITY

The sperm viability is evaluated by dyes related to the evaluation of **plasma membrane integrity/permeability/elasticity**, and may be analyzed by vital staining (e.g. eosin-nigrosin), hypo-osmotic swelling test (Jeyendran et al., 1984), or fluorescence microscopy (e.g. using the

fluorochromes propidium iodide and SYBR14). During sample staining with eosin-nigrosin, eosin Y dye enters dead sperm cells (i.e. cells with damaged plasma membrane) and stains them red.

Membrane integrity evaluated with Propidium Iodide

Propidium iodide (PI) is a DNA-intercalant fluorescent dye that cannot cross the intact plasma membrane; it therefore allows the identification of viable spermatozoa that exclude the dye in samples examined by epifluorescence (Soler et al., 2005). Sperm cells stained red by PI are considered dead while SYBR-14 green colored spermatozoa are deemed to be alive (Chalah and Brillard, 1998; Chalah et al., 1999).

Hypo-osmotic swelling test

The aim of this test is to evaluate the capacity of the sperm membrane to stay functional when the volume of the sperm is changed with osmotic variations of the medium, knowing that hyper and hypo osmotic shocks make part of the freeze-thaw process of cryopreservation.

1. 25µl of semen sample is mixed with 500 µl of a hypo-osmotic solution (100 mOsm/kg) prepared by adding 1g of sodium citrate to 100 ml of distilled water.
2. Incubation 30 min at 37º.
3. Fix the sample in buffered 2% glutaraldehyde solution at 37°C
4. Count 200 sperm and calculate the proportion of sperm with coiled tails (i.e. with functional membrane) under a phase-contrast microscope.

SPERM MORPHOLOGY

Abnormally shaped or damaged sperm are less likely to be capable of fertilization than normal sperm (Berndtson *et al.*,1981). Fixing samples in 2% glutaraldehyde buffered solution at 37 ºC using phase contrast microscopy or mixing the semen with a stain (e.g. eosin-nigrosin) highlights the sperm so that abnormalities can be readily identified under a microscope. Two kinds of abnormalities can be defined: primary abnormalities, which are assumed to have occurred in the testes; and secondary abnormalities, which arise later, in the epididymis or in the ejaculate. Usually, in most species, the proportion of sperm considered as normal should be > 70 percent. However, this may vary with the standard of the species.

MIXED EVALUATION VIABILITY-SPERM MORPHOLOGY

This is done using Eosin-nigrosin staining and counting of viable sperm (not red) and of normal and abnormal sperm.

Eosin-nigrosin staining (viability+ abnormal sperm measurements)

1. Place a drop or stripe of stain on a warmed microscope slide.
2. Add a small amount of semen.
3. Mix the semen and the stain with another slide and then use the narrow edge of the second slide to smear the mixture across the first slide.
4. Cover the mixture with a cover slip and examine under 1 000X magnification (oil immersion).
5. Examine the sperm for:
    a) viable sperm: white colour, whereas dead sperm are pink
    b) abnormalities, including the following:

- abnormally shaped (tapered or pear-shaped) or sized (too large or small) heads;
- missing or stump tails;
- coiled or bent tails;
- detached or creased (folded-over) acrosome;
- clumping of multiple sperm; and
- plasma droplets on tails

6. Count 200 sperm on 3 different places (600 sperm/slide) and calculate the proportion (%) of abnormalities.
7. Discard semen if the proportion of abnormalities is too high (e.g. more than 30%).

ACROSOME INTEGRITY

In mammals

In mammals the acrosome of the sperm is a specialized, cap-like structure that assists in assessing the ability of sperm fertilization and its reaction with the zona pellucida. An intact acrosome prevents loss of acrosomal enzymatic activity during acrosome reaction in the female genital tract prior to fertilization. The percentage of sperm with intact acrosome is a predictor of fertilizing capacity, and thus it is routinely used to assess semen quality. The percentage of spermatozoa with an acrosome showing an intact apical ridge is assessed using phase contrast microscopy.

The following protocol is recommended for mammalian species:

1. 25µl of semen sample is mixed with 500 µl of buffered 2% glutaraldehyde solution at 37°C.

   Buffered solution (BL-1):
   Glucose 2.9 g
   Sodium citrate $2H_2O$ 1 g
   Sodium bicarbonate 0.2 g
   Distillated H_2O: 100 mL
2. Count 200 sperm and calculate the proportion of sperm with alterations in the apical ridge: irregularly shaped apical ridge, no apical ridge, loose and vesiculated acrosomal cap.
3. Acrosomal status and sperm viability may be simultaneously evaluated by fluorescence microscopy (counting 200 cells) using a fluorochrome combination of propidium iodide (for sperm viability) and fluorescein isothiocyanate-conjugated peanut (Arachis hypogea) agglutinin (PNA-FITC) that binds specifically to the acrosomal membrane (Soler et al., 2005).

In birds

In birds the percentage of sperm with full acrosome may be determined by phase contrast microscope examining 200 sperm previously fixed with glutaraldehyde and stained with aniline blue (1000× magnification) (Santiago-Moreno et al., 2009). Sperm with abnormal morphology of the acrosome (form of a hook, swollen, thinned or absence of it) are considered as lacking acrosome integrity. The association of the fluorescent probes MITO and propidium iodide has been used to simultaneously assay the acrosomal, plasma, and mitochondrial membranes of rooster sperm using an epifluorescence microscope at 1000×. Bird acrosome reaction may be assessed by observation of sperm having completed their acrosome reaction after in vitro contact with standardized parts of perivitelline membranes (Lemoine et al., 2008). The capacity of chicken frozen-thawed sperm to undergo acrosome reaction is usually greatly affected by the cryopreservation process (Mocé et al., 2010).

DNA INTEGRITY

The chromatin integrity of spermatozoa is related to its fertilization potential. Cryopreservation can affect sperm DNA integrity and chromatin compaction. Several techniques to quantify DNA damage are now used to evaluate semen donors prior to processing and freezing and to analyze the effect of freeze-thawing process. These assays include toluidine blue and aniline blue staining (de Oliveira et al., 2013), sperm chromatin dispersion (SCD) (Enciso et al., 2006), chromatin structure assay status (SCSA; Ballachey et al., 1988), comet assay (Olive and Banath 2006), and terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) (Galarza et al., 2019). The TUNEL assay may be applied in both mammal and bird species using fluorescence microscope or flow cytometry.

In honey bees, Wegener et al. (2014) published a working protocol for TUNEL-assay for honey bee spermatozoa.

TUNEL assay for mammals and birds

1. Semen samples are fixed in 4% formaldehyde (v/v) in PBS solution at a concentration of 5×10^6 sperm/mL for 30 min.
2. 10 µl of sample is placed on a glass slide previously demarcated by Liquid-repellent slide marked pen for staining procedures and leave to dry on a thermic plate set at 37 °C.
3. The slides are permeabilized with 0.1% Triton X-100 (v/v) for 5 min in a humidified chamber at room temperature and washed with PBS.
4. Incubation for 1 h in a humidified chamber in the dark at 37 °C with the TUNEL reaction mixture, which contained terminal deoxynucleotidyl transferase (TdT) plus TMR-Red label.
5. Washing with PBS, staining with Hoechst 33342 (1 mg/mL), and mounting with Fluoromount aqueous mounting medium.
6. Count 200 sperm and calculate the proportion of sperm with damaged DNA (TUNEL +; sperm with red fluorescence) under an epifluorescence microscope.

TUNEL assay for bee sperm

(modified from Wegener et al., 2012):

1. Raw semen is diluted 1:500 in ‘simple saline’ (0.2M Tris buffer pH 7.2 containing 1.12% potassium chloride and 0.88% sodium chloride).
2. A thin layer of 20 µl of the above sample is smeared on a charged slide (Globe Scientific) and air dried.
3. A positive control is treated with DNAase I. A negative control is prepared with no treatments.
4. The slides are treated with 4% paraformaldehyde in PBS with pH adjusted to 7.4 for 1 hour and then gently rinsed in PBS at least thrice.
5. Permeabilization of the cells is done by immersing the slides for 2 minutes in a 0.1% solution of sodium citrate containing 0.1% triton X-100 on ice or in a refrigerator.
6. After the slides are washed 2-3 times in PBS and air dried, 50 µl of TUNEL reagent (Roche/Sigma-Aldrich) is added to each coverslip and incubated in a petriplate with a moist filter paper and in the dark at 37°C.
7. The slide are then rinsed three times in PBS and the site of the smear is covered with a drop of DAPI laced Everbrite (Biotium) and covered with a size ‘0’ coverslip. The slide is photographed on fluorescent microsocope with filters for 465 nm (exitation) and 525 (emission) or any GFP filter set. Automated microscopes such as the Biotek Lionheart can be programmed to count the

proportion of nuclei exhibiting green spotting. All the nuclei are stained blue and damaged sites are stained green.

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Annex 6.3 Guidelines for oocytes and embryos cryopreservation

INTRODUCTION

Health accreditation of collection, storage and implementation operations INTRODUCTION. To comply with National and International specific regulations and agreements, operators wishing to implement the following procedures are advised to approach the relevant authorities.

Know-how is essential for successful implementation of the procedures. This know-how is acquired with time through experience and relevant training. We therefore recommend contacting the authors of the guidelines if necessary.

Literature of embryo cryopreservation in mammalian species

Species	**Paper**	**Author(s), year**
Mouse	Survival of mouse embryos frozen to -196 °C and -269 °C	Whittingham et al. 1972
Cow	Experiments on the low-temperature preservation of cow embryos	Wilmut and Rowson 1973
Rabbit	Survival of frozen rabbit embryos	Bank and Maurer 1974
Sheep	Deep freezing of sheep embryos	Willadsen et al. 1976
Goat	*In vitro* culture, storage and transfer of goat embryos	Bilton and Moore 1976
Horse	Experiments in the freezing and storage of equine embryos	Yamamoto et al. 1982
Rat	Survival of rat embryos after freezing and thawing	Kasai et al. 1982
Baboon	Live birth following cryopreservation and transfer of a baboon embryo	Pope et al. 1984
Human	Two pregnancies following transfer of intact frozen-thawed embryos	Zeilmaker et al. 1984
African Eland antelope (*Tragelaphus oryx*)	Cryopreservation followed by successful transfer of African Eland antelope (*Tragelaphus oryx*) embryos.	Dresser et al., 1984
Cynomolgus monkeys (*Macaca fascicularis*)	Embryo cryopreservation in cynomolgus monkeys	Balmaceda et al. 1986
Marmoset monkey (*Callithrix jacchus*)	The effects of cryopreservation and transfer on embryonic development in the common marmoset monkey, Callithrix jacchus	Summers et al. 1987
Cat	First successful transfer of cryopreserved feline (*Felis catus*) embryos resulting in live offspring	Dresser et al. 1988
Rhesus monkey	*In vitro* fertilization and embryo transfer in the rhesus monkey	Wolf et al. 1989
Pig	Birth of piglets from frozen embryos	Hayashi et al. 1989
Macaque monkey [hybrid Pig-tailed macaque (*Macaca nemestrina*) + Lion-tailed macaque (*M.*	Macaque monkey birth following transfer of *in vitro* fertilized, frozen-thawed embryos to a surrogate mother	Cranfield et al. 1992

silenus)]		
Buffalo	Successful culmination of pregnancy and live birth following the transfer of frozen-thawed buffalo embryos	Kasiraj et al. 1993
Hamster	Containerless vitrification of mammalian oocytes and embryos	Lane et al. 1999
African wildcat (*Felis silvestris*)	Development of *in vitro* produced African wildcat (*Felis silvestris*) embryos after cryopreservation and transfer into domestic cat recipients	Pope et al. 2000
Ocelot (*Leopardus pardalis*)	Reproductive biotechnology and conservation of the forgotten felids – the small cats	Swanson 2001
European polecat (*Mustela putorius*)	Surgical recovery and successful surgical transfer of conventionally frozen-thawed embryos in the farmed European polecat (*Mustela putorius*)	Lindeberg et al. 2003
Camel (*Dromedary camel*)	Offspring resulting from transfer of cryopreserved embryos in camel (*Camelus dromedarius*)	Nowshari et al. 2005
Red deer (*Cervus elaphus*)	Successful use of oviduct epithelial cell coculture for *in vitro* production of viable red deer (*Cervus elaphus*) embryos	Locatelli et al. 2005
Mongolian Gerbil (*Meriones unguiculatus*)	Birth of offspring after transfer of Mongolian Gerbil (*Meriones unguiculatus*) embryos cryopreserved by vitrification	Mochida et al. 2005
Caracal (*Caracal caracal*)	*In vitro* embryo production and embryo transfer in domestic and non-domestic cats	Pope et al. 2006
Pigtailed macaque (*Macaca nemestrina*)	*In vitro* fertilization in the pigtailed macaque (*Macaca nemestrina*)	Kubisch et al. 2006
Domestic ferret (*Mustela putorius furo*)	Efficient term development of vitrified ferret embryos using a novel pipette chamber technique	Sun et al. 2008
Dog	Cryopreservation of canine embryos	Abe et al. 2011
Black-footed cat (*Felis nigripes*)	Applying embryo cryopreservation technologies to the production of domestic and black-footed cats	Pope et al. 2012
Sika deer (*Cervus nippon*)	First live offspring born in superovulated sika deer (*Cervus nippon*) after embryo vitrification	Wang et al. 2012
Djungarian Hamster (*Phodopus sungorus*)	Cryopreservation and *In vitro* culture of Preimplantation Embryos in Djungarian Hamster (*Phodopus sungorus*)	Brusentsev et al. 2015
Golden Syrian hamster (*Mesocricetus auratus*)	Effective cryopreservation of golden Syrian hamster embryos by open pulled straw vitrification	Fan et al. 2015
Alpaca (*Vicugna pacos*)	Birth of a live cria after transfer of a vitrified-warmed alpaca (*Vicugna pacos*) preimplantation embryo	Lutz et al. 2020

CRYOPRESERVATION OF PIG EMBRYOS

In pigs, due to a large amount of lipids inside the embryonic cells and to a cell cold-sensitivity, it is recommended to apply an ultra-rapid vitrification method to cryopreserve embryos. The vitrification procedure described here is practiced in several laboratories (Cuello et al., 2010) and was also validated in the H2020 EU project IMAGE European project (Guignot et al., 2019).

In vivo embryo production

Cyclic gilts aged 7 to 8 months should be used as donors. They should be synchronized and superovulated in order to obtain numerous embryos at the required developmental stage. Artificial insemination should be performed at 12 and 24 h after the onset of estrus with 3 x 10^9 spermatozoa per insemination per female.

Embryo collection and quality of the future cryopreserved embryo

Embryos are surgically recovered at day 6 after ovulation.

- Donors are sedated by administration of ketamine (20 $mg.kg^{-1}$ bodyweight, i.m.) and xylasine (2 $mg.kg^{-1}$ bodyweight, i.m.). The anesthesia is maintained by inhalation of 3% isoflurane.
- A midline incision is made on the white line and the reproductive tract is externalized. Embryos are collected by flushing the tip of each uterine horn with 40 mL of Talp-Hepes containing 0.1 $g.L^{-1}$ PVA. Embryos are transferred to a stereomicroscope and their developmental stages is examined. Only embryos at the blastocyst stage, 160 to 200 µm diameter, with a good or excellent morphological appearance and with intact zona pellucida must be selected for cryopreservation.

Embryo cryopreservation

It is very important that the temperature of media and embryos is maintained at 39°C with a heating plate during the entire procedure with the exception of straws plunging in liquid nitrogen. Groups of 4 o 7 embryos are vitrified together using a four-well multidish.

Vitrification

Embryos are washed twice at least 5 min twice in Talp-Hepes containing 0.1 $g.L^{-1}$ polyvinyl-alcohol (PVA). Then they are transferred in the same medium complemented with 5% ethylene glycol (EG) and 7.5% Dimethylsulfoxide (DMSO) (equilibration solution: ES) for 3 min. Finally, they are incubated in vitrification solution (VS), i.e. Talp-Hepes containing 0.1 $g.L^{-1}$ PVA, 16% EG, 16% DMSO and 0.4M sucrose for 1 min. During the last step, embryos are placed in a 1-µl droplet and then loaded together by capillarity into the narrow end of superfine open pulled straws (SOPS). Straws with embryos are then plunged horizontally into liquid nitrogen (Figure 1).

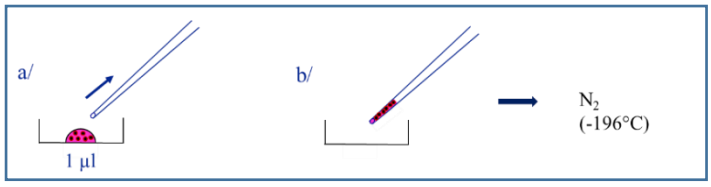

FIGURE 1
Vitrification in SOPS (Guignot, 2020). The solution containing embryos is in red.

Thawing

At thawing, straws are hold in the air for 2 sec and the narrow end is immerged in a well of four-well multidish containing 1000 µL Talp-Hepes containing 0.1 $g.L^{-1}$ PVA with 0.13 M sucrose. The embryos are pulled out of the straw by capillarity action, rinsed in this medium for 5 min, and then in Talp-Hepes 0.1 $g.L^{-1}$ PVA without sucrose for 5 min. After this, the embryos are ready for transfer.

Embryo transfer

Before transfer, host females should be synchronized with Regumate®. The embryo transfer is performed surgical 5 days after estrus. Recipients are sedated by administration of ketamine (20 $mg.kg^{-1}$ bodyweight, i.m.) and xylazine (2 $mg.kg^{-1}$ bodyweight, i.m.). The anesthesia is maintained by inhalation of 4% isoflurane. After a mid-ventral laparotomy, 30 blastocysts are transferred to the upper end of one uterine horn. Pregnancy can be assessed by ultrasonography around 25 days post estrus. Farrowing is expected around 115 days after estrus, i.e. 3 months, 3 weeks and 3 days.

CRYOPRESERVATION OF EQUINE EMBRYOS

Equine embryos are routinely produced in vivo and collected under field conditions using nonsurgical techniques. The embryo collection is usually performed on mare on day 7 or 8 after ovulation. An average embryo collection rate of about 75% can be obtained (90% for young maiden or fertile mares and 10%–20% for subfertile mares).

In equines, embryo cryopreservation by slow freezing or vitrification of early embryos <300 µm in diameter leads to high pregnancy rates, but cryopreservation of expanded blastocysts >300 µm in diameter needs some specific preparation and care. At this stage, the equine embryo has a specific acellular membrane around the blastocyst membrane (the capsule) and a large amount of fluid within the blastocoel. For these expanded blastocysts, it is recommended to empty the blastocoel cavity before cryopreservation and to cryopreserve the equine embryo with the ultra-fast vitrification method.

Embryo collection and quality of the cryopreserved embryo

Embryo collection is performed using a routine transcervical uterine washing procedure. Once the flush is completed, the remaining fluid in the filter is examined under a stereomicroscope. Once the embryo is found, the embryo is washed 10 times in embryo holding medium (EHM).

After washing, the stage of the embryo (morula, early or expanded blastocyst) and the quality of the embryo, according to the scale described by McKinnon and Squires (1988) (grade 1, best; grade 5, dead) should be recorded. Only embryos with grade 1 or 2 should be cryopreserved.

Embryo cryopreservation

Blastocoel fluid aspiration

The fluid aspiration can be done with micromanipulator equipment under a microscope (Figure 2). A micropipette linked to a syringe and a holding pipette are required. The embryo is placed in EHM and held under vacuum with the holding pipette. The glass micropipette, 20 µm external diameter with a beveled edge (20° angle) is positioned at the 3 o'clock position, against the embryo, and the inner cell mass of the embryo is positioned at 12 or 6 o'clock. The micropipette is gently introduced into the blastocoel cavity. Then the blastocoel fluid is aspirated through the micropipette by gentle suction until about 70% to 90% (Choi et al., (2011); Guignot et al. (2014); (2015): Diaz et al., (2016) and

Sanchez et al., (2017)). The micropipette is pulled out of the embryo and the embryo is ready for vitrification procedure.

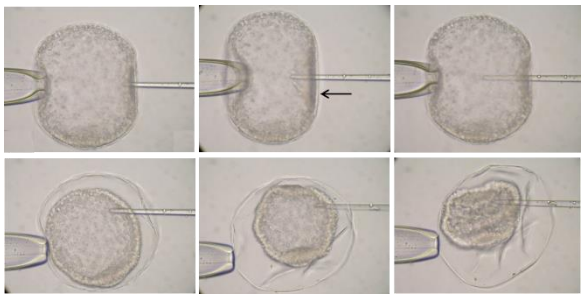

FIGURE 2
Fluid aspiration under microscope (Guignot, 2020)

The aspiration can be also done under a stereomicroscope, if there is no microscope and micromanipulator equipment in the laboratory. In this case, the glass micropipette for aspiration is positioned above the embryo (Figure 3) and the embryo is placed in a medium without protein, for example Ringer Lactate, so it will be attached to the bottom of the dish.

FIGURE 3
Fluid aspiration under stereomicroscope (Guignot, 2020)

Vitrification
The embryo is vitrified immediately after fluid aspiration using the open pulled straw (OPS) procedure, with one embryo per straw. Three steps are performed before vitrification, all are done at 38.5°C.

1) the embryo is placed in EHM supplemented with 20% of fetal calf serum (EHM serum) for 2 x 5 min.
2) the embryo is placed in EHM serum containing 1.5M ethylene glycol (EG) for 5 min
3) the embryo is placed in EHM serum containing 7M EG supplemented with 0.6M galactose for 30 sec.

During this last step, the embryo is loaded by capillarity in a 2-µL droplet into the narrow end of one open pulled straw. The OPS with the embryo is then plunged horizontally into liquid nitrogen (Figure 4). As OPS is not closed straw, it is possible to use another device, such as high security straw, which is sealed before plunging it into liquid nitrogen.

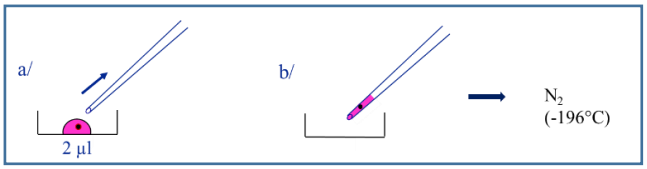

FIGURE 4
Vitrification in OPS (Guignot, 2020)

Thawing
At thawing, straws are hold in the air for 2 sec and the narrow end is immerged in a well of four-well multidish with 1200 µl EHM serum containing 0.22M sucrose. Then the embryo is pulled out of the straw by capillarity action, rinsed first in this medium for 3 min, and then in EHM serum with 0.13M sucrose for 3 min, and finally placed in EHM serum without sucrose. In case of closed straw, the extremity of the straw must be cut before immersion in medium. The embryo is ready for transfer.

Future improvement of this described technique of vitrification may be the possibility to use two cryoprotectants in order to reduce in the third step of the procedure the amount of cryoprotectant necessary to well vitrify equine embryo.

Embryo transfer
Embryo transfer is a commonly used procedure in equine breeding worldwide. The embryos are transcervically transferred to host felakes at day 5 or 6 after ovulation, one blastocyst per female. The embryos are loaded into an embryo transfer gun and transferred within 5 min into the recipient uterus. Pregnancies can be monitored by ultrasonography at day 14 post ovulation. At day 30, an embryonic heartbeat can be detected. Foals are expected 11 months later.

CRYOPRESERVATION OF BOVINE EMBRYOS

The method described here is a summary of the method described for bovine embryos in the appendix G of the previous 2012 FAO guideline on “Cryoconservation of Animal Genetic Resources”.

Embryo collection and treatment
1. Collect embryos non-surgically from a superovulated donor female at day seven of the oestrous cycle, evaluate them for morphological development, and assign an embryo-quality grade. The embryos should be at the compact morula and blastocyst stages..
2. Maintain the embryos in a clean environment and maintained at around room temperature (20 to 30°C). Freezing should be done as soon as possible after collection (i.e. within 4 to 6 hours).
3. While grading quality, check that the zona pellucida is intact on all embryos (under 50X light microscope) and that embryos are free from any adherent material.
4. Wash the embryos from one donor (no more than ten embryos) in 5 consecutive baths of phosphate-buffered saline (PBS) solution containing broad-spectrum antibiotic and 0.4% bovine serum albumin (BSA). Use different glass and plastic ware for each donor and new micropipettes for each subsequent wash.
5. Wash the embryos twice with trypsin (60 to 90 sec in total) to remove or deactivate any viruses. Trypsin wash is sterile porcine-origin trypsin (1:250) in Hank’s balanced salt solution at a concentration of 0.25%.

6. Wash the embryos an additional five times in PBS – antibiotic solution with 2% BSA.
7. Equilibrate the embryos at room temperature for ten minutes in PBS with 10% fetal calf serum and 10% glycerol.

Freezing

1. Place the embryos between 2 or 4 air bubbles in 0.25 ml sterile, pre-labelled plastic straws. Most often, one embryo is cryopreserved per straw. Place straws horizontally in a freezing unit and cool from room temperature to -7°C at a rate of 5°C/minute.
2. Induce seeding at -7°C by contact at the extreme end of the straw with liquid nitrogen-cooled tweezers, and freeze the embryo to -35°C at a rate of 0.5°C/minute.
3. Plunge the straws directly into liquid nitrogen and then store them at -196°C.

Thawing

1. Select the appropriate straw from the liquid nitrogen storage tank. Important! Do not bring the straws up above the frost line of the liquid nitrogen tank (the neck of the tank) until the correct straw is identified for embryo transfer.
2. Thaw the straw rapidly in a water bath at 20°C for 30 sec or 39°C for 8 to 25 sec, depending on the initial embryo-freezing rate. Then cut the ends of the straw and remove the embryo. Rehydrate the embryo in 1M (molar) sucrose solution for 10 minutes; then reduce the sucrose concentration in a stepwise procedure.

Embryo transfer

Prepare the Cassou gun and clean the perineal region of the recipient female. Transfer the contents of one straw (one embryo) into the uterine horn corresponding to the corpus luteum of a day-seven recipient female.

CRYOPRESERVATION OF GOAT AND SHEEP EMBRYOS

In vivo derived small ruminant embryos are routinely produced worldwide, whereas the embryo market for *in vitro* derived embryos is as yet practically insignificant (Souza-Fabjan et al., 2021). Overall, it is preferable to perform the slow freezing technique for *in vivo* embryos and vitrification for *in vitro* embryos. We describe here a slow freezing method that can be applied to both goats (Fonseca et al., 2018) and sheep (Figueira et al., 2019).

In goats, nonsurgical embryo recovery (NSER) is efficiently performed by several research groups, while for sheep, despite recent successes, the laparotomy procedure is still the technique of choice worldwide (Camacho et al., 2019; Fonseca et al., 2019). Of note, both techniques are similarly efficient when conducted by a trained technician but the NSER affects animal welfare to lesser extent (Santos et al., 2020). Embryo collection is usually performed from day 5.5 to 6.5 after ovulation. The embryo recovery rate may reach around 70% for both NSER and laparotomy techniques and the number of viable embryos recovered after superovulation varies considerably, on average 4 to 6 per female.

***In vivo* embryo production**

Small ruminant females may be used as embryo donors either in the breeding or anestrus season. In any case, they should be heat-synchronized and superovulated, to obtain a reasonable number of

embryos. When in estrus, they must be either naturally mated or artificially inseminated, twice, at 12 and 24 hours after estrus.

Embryo collection and quality of the cryopreserved embryo

Prior to embryo recovery, the ovaries may be evaluated by either ultrasonography or laparoscopy in order to check the ovarian response to superovulation and perform corpora lutea count.

Laparotomy

- Donors must be fasted for 18 to 24 hours before surgery, for water and food, respectively.
- Donors may be sedated by administration of ketamine (2 $mg.kg^{-1}$ bodyweight, i.v.) and diazepam 0.2 to 0.5 $mg.kg^{-1}$ bodyweight, i.v.). After intubation, the anesthesia is maintained by inhalation of 1.5% isoflurane. Lidocaine (5 $mg.kg^{-1}$)diluted up to 1% in physiological saline solution may be administered intraperitoneally to increase the analgesia during visceral manipulation.
- A paramedian incision is performed on the skin (approximately 5 cm long and 5 cm cranial to the udder) and the uterine horns are gently externalized. Note that there is no need to exteriorize the ovaries.
- Fix the uterine horn with fingers and puncture the uterine wall at a point on the horn adjacent to and below the uterus bifurcation. A sterile catheter with balloon such as the two-way Foley (number 8 or 10) is used for uterine flushing. Insert the catheter at the base of each horn through the puncture in the uterine wall. Place the open end of the cathether firmly into a 90-mm petri dish or 50 mL Falcon tube.
- At the utero-tubal junction, place a catheter through an incision made by a white tip into the lumen. Inject at least 30 mL/uterine horn (totaling 60 mL) of prewarmed flushing medium into a hypodermic syringe without plunger. During the entire procedure, it is essential to maintain the uterus hydrated with prewarmed saline solution.

NSER

- Donors do not need to be fasted before NSER.

- A hormonal relaxation treatment is needed. In sheep, 1 mg estradiol benzoate i.m. and 37.5 µg cloprostenol l.v. is administrated at 16 hours prior to NSER, followed by 50 IU oxytocin i.v. 20 minutes before NSER; in goats, only injection of 37.5 µg cloprostenol l.v. must be performed 16 hours before NSER. Depending on the sheep breed, no estradiol benzoate is needed.
- Dipyrone and n-butyl hyoscine bromide solution (5 mL/animal i.v. and 5 mL/animal i.m.) are administered 20 minutes before flushing. Donors may be sedated by administration of 1% acepromazine maleate (0.1 $mg.kg^{-1}$ bodyweight, i.m.) 20 minutes before flushing followed by 2% lidocaine (2 mL/animal) via the epidural (S5-C1) route 5 minutes before flushing. Right after specula introduction, a 2% lidocaine (3 to 5 mL/animal, intravaginal) should be added into a gauze under the cervical ostium.
- A Collin speculum is inserted into the vagina and the cervical os may be identified with a flashlight. Two Pozzi forceps are inserted laterally to the cervical os, and the cervix is retracted to allow easier manipulation per vagina or rectum.
- A Hegar dilator should be introduced into the cervical os and fingers can be placed above and under the cervix, to help traversing the first cervical rings.

- After cervical penetration, the Hegar dilator is replaced by a catheter equipped with a mandrel and cervical penetration is again performed.
- The catheter is directed to the first uterine horn and the mandrel is gently removed as the catheter is gradually advanced. The catheter is then connected to the flushing circuit. Each uterine horn is flushed with successive 10 mL injections of PBS solution, totaling 180 mL per horn.

Embryo assessment

Embryos are examined under a stereomicroscope and classified according to their developmental stages and quality. Each embryo is washed 10 times in embryo holding medium. Only Grade 1 and 2 blastocysts with intact zona pellucida must be selected for cryopreservation. Alternatively, embryos at the stage of compact morula can also be cryopreserved, but the pregnancy rate after thawing is usually lower.

Embryo cryopreservation (slow freezing)

Freezing (Figure 5)

- Embryos collected from a donor female must be kept at room temperature for 10 minutes in PBS with either 0.4% of BSA or 10% of fetal calf serum.
- Equilibrate the embryos using to 1.5 M of ethylene glycol (diluted in PBS) in one-step for a minimum of 10 and maximum of 20 minutes. Ethylene glycol can be used as cryoprotectant for both sheep and goats.
- One or two embryos should be loaded into the central part of the 0.25 mL sterile prelabelled plastic straw, separated by two air bubbles from columns containing PBS plus 20% fetal calf serum.
- Place the plastic straws in the freezing equipment and cool from room temperature to -6°C at the rate of 3 °C/minute.
- After 5 minutes at -6°C, induce seeding and after 10 minutes freeze embryos to -30/32°C at a rate of 0.5°C/minute.
- After 10 minutes at -30/32°C, plunge straws directly into liquid nitrogen. Store the straws in liquid nitrogen at -196°C.

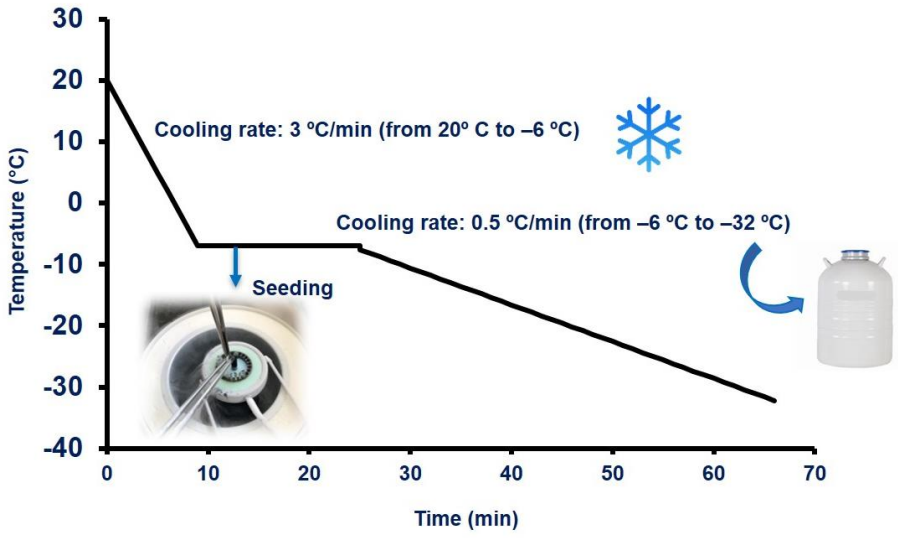

FIGURE 5

Cooling rates during slow freezing of small ruminant embryos

Thawing

- Select the appropriate straw from the liquid nitrogen storage tank. Importantly, do not bring the straws up above the frost line of the liquid nitrogen tank (neck of the tank) until the correct straw is identified for embryo transfer. Thaw one straw at a time and transfer the embryo(s) before thawing the next straw.
- Embryos may be maintained at room temperature for 5 to 10 seconds and then immersed in a water bath at 20°C for 30 seconds. Then, cut the end of the straw and remove the embryo.
- Embryos can be either evaluated at this stage or directly transferred to the prepared recipient.

Embryo transfer

Embryo transfer by a semi-laparoscopic procedure is a used worldwide in small ruminants. At Day 5.5 or 6.5 after ovulation, either ultrasonography or laparoscopy is performed in order to detect the presence, number and viability of corpus luteum. On the selected recipients, 24 hours of fasting is recommended. Through a tiny abdominal incision made by a white tip or stilete, the anterior portion of the uterine horn ipsilateral to the ovary containing at least one functional corpus luteum should be exteriorized. The whole content of the straw containing one or two embryo(s) may be deposited by a simple system of tom-cat catheter connected to a 1 mL syringe. Pregnancy can be assessed by ultrasonography around 25-30 days post estrus. Progenies are expected around 150 days after estrus.

CRYOPRESERVATION OF HONEY BEE EMBRYOS

As previously mentioned in Section 6, the method to cryopreserve honey bee embryos is technically strenuous and uses non-standardized components to assist in the process. While this method is still at the experimental stage, below is outline of the protocol that is currently being used to obtain viable honey bee embryos and larvae that are destined to be either grafted into a hive or reared in vitro (Rajamohan et al., 2020).

Requisites

- Scalvini cages
- Sterile 3 cm and 9 cm petri plates and a glass petri plate
- Cell filtration baskets such as the Falcon cell strainer (<50 µm mesh) or fabricate one as shown in the figure 6 below.
- Single filament brush to handle the eggs.
- Capillary tubes with ID of 1 or 2 mm and capillary tube fillers (Fisher microhematocrit capillaries are perfect).
- Nuclepore polycarbonate membrane (Millipore, 0.2 µm, PVP coated)

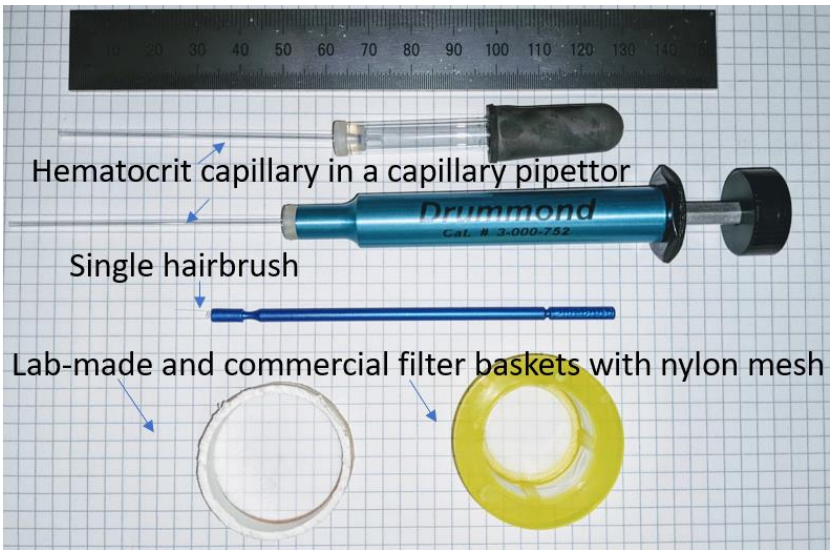

FIGURE 6.
Equipments for honey bee embryo preparation for freezing

- **Chemicals**: Sodium hypochlorite (10% active; Sigma-Aldrich), 2-propanol (<99% water), heptane, trehalose dihydrate, ethane-1,2-diol (ethylene glycol), Schneider's Cell Culture Medium (SCCM) with calcium chloride and sodium bicarbonate, antibiotic-antimycotic mixture (Sigma-Aldrich)
- **Wash/culture medium:** Prepare Schneider's CCM with 50 µl of antibiotic-antimycotic solution per liter
- **Thawing medium**: As above with 0.5M trehalose (18.5 g/100 ml).
- **Cryoprotectant pretreatment:** Schneider's CCM with 10% ethane-1,2-diol
- **Vitrification solution:** Schneider's CCM with 0.5M trehalose and 36.5% ethane-1,2-diol.
- Suitable foam container to hold liquid nitrogen (LN) with undisturbed layer of vapor nitrogen for at least 10 centimeters above the surface of LN. the LN must not boil vigorously during use.

Embryo collection

Embryo collection has been described elsewhere in **Section 6.** Briefly, the Scalvini cages as shown in **Figure 7** are left over night in the hive affixed to a frame. The next day, the queen is gently caught and transferred to the cage and left undisturbed in the hive for 60-120 minutes. The queen is later on released and the cage is brought back to the lab cradled in a moist tissue paper. Once in the lab, the cage is opened, inverted and gently tapped 2-3 times over a 9 cm sterile petri plate to permit the embryos to dislodge from the cage and fall on to the petri plate. Using a dissection microscope the eggs are checked for damage and the damaged eggs could be removed or marked. The plate is incubated at 34°C, 60% humidity and in the dark for 62-65 hours before assessment and treatment.

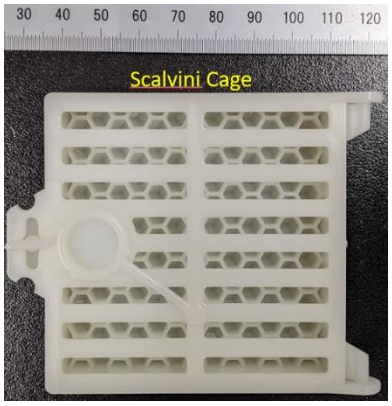

FIGURE 7
Scalvani cages

Embryo cryopreservation

The basic steps for cryopreservation include:

- Stage selection
- Dechorionation
- Permeabilization
- Cryoprotectant loading and dehydration
- Vitrification
- Thawing
- Rehydration and detoxification
- Embryo culture

Stage selection: Studies indicate that the most treatment tolerant stage of the honey bee is a pre-cuticular stage at about 68 hours of development. The stage is ascertained by checking the anterior embryonic morphology which would resemble as shown in the Figure 8. The arrows in the figure point to the outer vitellin membrane and the inner serosal cuticle. Of critical importance is the state of development of the cephalo-thoracic components and the embryonic segmentation as well as the distribution of the yolk material.

FIGURE 8.
Microscopy of the anterior embryonic morphology at 68 h of development

Embryo transfer: handling and treatment

All the treatments are done in capillaries which when combined with a pipettor to aspirate and expel the embryo rapidly, enables precise timing of the treatments.

1. Take 100 µl of distilled water in the middle of a 3 cm petri plate.
2. Aspirate 25% diluted sodium hypochlorite (in tap water) in the hematocrit capillary to about one centimeter. Place it under a dissection microscope with the capillary tip clearly visible as shown in the figure below.
3. Using a wet single hair brush carefully remove the egg by touching its adhesive pedestal.
4. *Dechorionation*: Introduce the egg into the capillary (**Figure 9**) while observing the introduction under a microscope.

FIGURE 9

Dechorionation and observation of the embryo when the chorion begins to peel

5. When the chorion begins to peel, quickly expel the contents into the water drop in the petri plate. Using a pipette remove and replace the water continuously for about a minute and a half.
6. Gently pick up the floating embryo back on the single hair brush. Touch the tip of the brush carefully without losing the egg to a lint free tissue paper to remove excess water from around the embryo.
7. De-wetting: Load another fresh capillary with 2-propanol (isopropanol). Introduce the embryo into the capillary just as described above and in less than 5 sec, expel the embryo on to filter basket. Quickly blot the basket especially in the vicinity of the embryo with a tissue paper to remove excel 2-propanol and then ‘blow’ air for 1 minute on the basket and the embryo to quickly dry isopropanol from the embryo.
8. Fill a shallow glass petri plates with heptane and another sterile 3 cm disposable petri plate with Schneider’s CCM at room temperature.
9. Permeabilization: Place the embryo with basket slowly in heptane ensuring that the embryo does not move from its position on the mesh. Remove the basket after 15 sec and ‘Blow’ air to dry for 1 minute. Quickly place it in the petri plate with the cell culture medium. If the embryo does not float, remove the basket from the medium and place it back in the medium again forcing the embryo to float. DO NOT handle the embryo with the brush tip.
10. Cryoprotectant pre-treatment: Remove the basket with the embryo from the Schneider’s CCM and blot away excess medium from the bottom of the basket. Prepare another 3 cm sterile

disposable petri plate with 10% ethane diol in Schneider's CCM. Place the basket in the solution ensuring that the embryo is floating. Incubate at room temperature for 5 minutes.

11. Vitrification solution treatment: Remove the basket and blot away excel medium and place the basket in yet another petri plate containing cold vitrification solution. The embryo must float on the solution. This treatment must be done on ice or in a refrigerator for 4 minutes.
12. As the embryo is incubating in the vitrification solution, (a) prepare a 2-3 mm wide and 2 cm long strip of nuclepore polycarbonate membrane. Note the shiny side which is the polymer coated side. (b) Add about 5 cm of liquid nitrogen to the freezing container for embryo vitrification and place a mesh basket in the LN.
13. Using a forceps, place the shiny side of the membrane on the floating embryo and slowly lift the membrane removing the embryo from the vitrification solution. Quickly place the back side of the membrane (the side without the embryo) on a lint-free tissue paper and gently drag it to remove excess vitrification solution adhering to the membrane and the embryo.
14. Hold the embryo about 1 cm above LN in the vapor phase for 1 minute. Place a dry mesh basket in the LN and drop the membrane with the embryo into the basket ensuring that the membrane is not lost in the LN. If long term storage is intended, a histological cassette is used to trap the membrane containing the embryo and clasped shut under LN.
15. Thawing: Fill a 3 cm petri plate with thawing medium (0.5M trehalose in Schneider's CCM). Keep it close to the freezing container. Remove the embryo from the liquid nitrogen and hold it one cm above the surface in the vapor phase for one minute. Note the side where the embryo is stuck on the membrane. Rapidly transfer the embryo side of the membrane on to the surface of the thawing medium. Gently plunge the membrane into the solution. Shake the membrane slowly to dislodge the embryo into the solution. Within one minute, using a pipette drain most of the solution and replace it with Schneider's CCM. Repeat the process every 7 minutes for 3 more time. Let the embryo float on the SCCM for 24 hours in the incubator until the larva hatches.
16. Larval rearing: Viable larva is scooped from the surface of the Schneider's CCM and placed in 3 cm petri plate and on a 100 µl drop of a mixture of royal jelly, glucose, fructose, yeast extract and water as described by Kaftanoglu et al. (2011). In brief, the diet mix contains 53:4:8:1:34 parts of royal jelly, glucose, fructose, yeast extract and water, respectively. On day 2, the viable larva is transferred to a 24 well plate containing 2.5 ml of the above diet. On day 6, the viable larva is transferred to a 3 cm plate with a lint-free tissue paper to allow the larva to pupate. The larva/pupa should always be kept in a 70% humidified chamber at 34°C until emergence. It should be noted that the diet shown here only yields workers.

VITRIFICATION OF PORCINE IMMATURE OOCYTES

We describe here the method recently developed in the studies by Somfai et al., (2014); Appeltant et al., (2018); Nguyen et al., (2018). It should be stressed that this method is still at the experimental stage.

Oocytes and zygotes collection

Porcine ovaries are collected from pre-pubertal cross-bred gilts and transported to the laboratory in Dulbecco's phosphate buffered saline at 35°C within 1 hr. Cumulus-oocyte complexes (COCs) are then aspirated from 3–6 mm follicles, and vitrified immediately. Porcine zygotes are obtained by in vitro maturation and in vitro fertilization of COCs as described in Nguyen et al., (2018).

Vitrification

1. Wash the cumulus-enclosed oocytes/zygotes in a HEPES-buffered base medium (BM) which can be either TCM-199 or NCSU-37 supplemented with 4 mg/ml bovine serum albumin (or alternatively polyvinyl pyrrolidone).
2. Equilibrate oocytes/zygotes in BM supplemented with 2% (v/v) ethylene glycol and 2% (v/v) propylene glycol at room temperature for 13-15 min.
3. Wash oocytes/zygotes in 3 drops (50 µl) of a vitrification solution within 40 sec and drop them into liquid nitrogen in 2 µl vitrification solution (microdrop vitrification) or place them on Cryotop devices (10 oocytes/device) and plunge them into liquid nitrogen. The vitrification solution consists of 17.5% (v/v) ethylene glycol + 17.5% (v/v) propylene glycol + 0.3 M sucrose + 50 mg/ml polyvinyl pyrrolidone (FW = 40000) in BM.
4. Place microdrops with pre-cooled forceps into cryovials (microdrop vitrification) or cover Cryotop devices with sheath within liquid nitrogen.
5. Store cryovials/Cryotop devices in liquid nitrogen until use.

Thawing

1. From liquid nitrogen, place microdrops/Cryotop devices in 1 sec into 2.5 ml of a warming solution in a 35 mm petri dish at 42 °C and keep them there for 2 min. The warming solution is 0.4M sucrose in BM.
2. Gather oocytes/zygotes in a group and transfer them stepwise into reduced concentration of sucrose (0.2 M, 0.1 M and 0.05 M for 1 min each) at 38°C. Finally, keep oocytes/zygotes in BM for 3 min.

Use of frozen/thawed immature oocytes and zygotes

To utilize vitrified immature oocytes in vitro oocyte maturation and fertilization (or alternatively ICSI) is required. This method is still at the experimental stage.

VITRIFICATION OF MATURE BUFFALO OOCYTES

The method described here has been developed following the studies of Liang et al, (2011), (2012), (2018), (2020), Parnpai et al., (2016).

Oocytes collection and in vitro maturation

Buffalo ovaries obtained from a slaughterhouse are transported within 4 h to the laboratory in physiological saline solution (0.9% NaCl) at room temperature. Cumulus-oocyte complexes (COCs) are collected from 2–8 mm diameter follicles and washed in Dulbecco's phosphate-buffered saline (mDPBS) supplemented with 0.1% polyvinyl pyrrolidone (PVP). Each of 20 COCs is cultured in 100 µL IVM medium at 38.5 °C under a humidified atmosphere of 5% CO_2 for 23 h. The IVM medium consist of TCM199 supplemented with 10% fetal bovine serum, 0.02 AU/mLFSH, 50 IU/mL hCG and 1 µg/mL estradiol-17β.

Vitrification of matured oocytes

1. After dilution in IVM, COCs are partially denuded by gentle pipetting with a pulled-pipette using 0.1% (w/v) hyaluronidase.

2. Place a group of five oocytes in base medium (BM: TCM199-Hepes + 20% FBS) supplemented with 10% (v/v) dimethylsulfoxide (DMSO) and 10% (v/v) ethylene glycol (EG) for 1 min.
3. Expose oocytes in vitrification solution (BM supplemented with 20% (v/v) DMSO, 20% (v/v) EG, and 0.5 M sucrose) at 22–24°C for 30 sec.
4. Place oocytes onto the end tip of Cryotop (Kitazato Supply Co., Tokyo, Japan) in a small volume of vitrification solution, and within 30 sec directly plunge into liquid nitrogen (LN2).

Thawing vitrified matured oocytes

1. From LN_2, direct immersion the tip of Cryotop into 3 mL of 0.5 M sucrose in BM at 38.5°C for 5 min.
2. Transfer oocytes into BM without sucrose for 5 min.
3. Place vitrified-warmed oocytes in BM under humidified atmosphere of 5% CO_2 at 38.5°C for 1 h.

Evaluation of oocyte viability

Viability of the oocytes can be examined using fluorescein diacetate (FDA) staining as described previously (Mohr & Trounson 1980).

1. Treat oocytes with 2.5 µg/mL FDA in phosphate buffered saline (PBS) supplemented with 5 mg/mL BSA at 38.5°C for 2 min in dark room.
2. Wash oocytes three times in PBS supplemented with 5 mg/mL bovine serum albumin (BSA).
3. Examine oocytes under fluorescent microscope with UV irradiation and, an excitation wavelength of 460–495 nm and emission of 510 nm. Oocytes displaying a bright green fluorescence are considered to be alive and will be used in subsequent experiments.

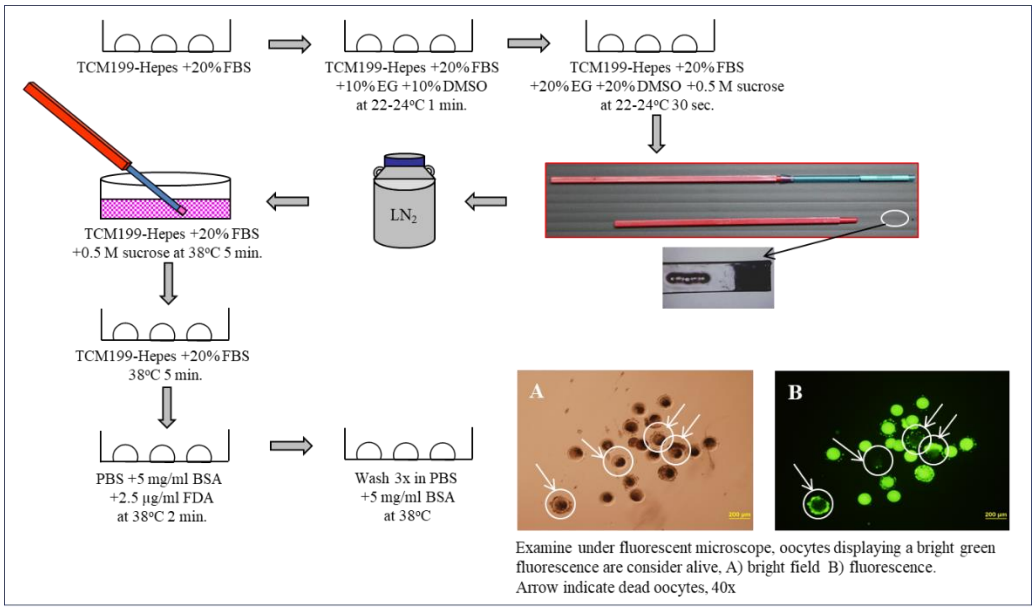

Figure 10
Vitrification, thawing and viability evaluation of mature oocytes

Use of vitrified/thawed matured oocytes

To utilize vitrified/thawed matured buffalo oocytes, including in vitro fertilization, ICSI and SCNT must be developed. This method is still at the experimental stage.

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Annex 6.4 Guidelines for gonadic and peri-gonadic tissue cryopreservation

INTRODUCTIONS

Health accreditation of collection, storage and implementation operations INTRODUCTION. Due to National and International specific regulations and agreements, operators wishing to implement the following procedures are advised to approach the relevant authorities in order to take into account the corresponding regulatory constraints.

Know-how is very important for the success of the procedures. This know-how is acquired with the experience and the time. A training is often required. We thus suggest contacting the drafters of the procedures before any use.

CRYOPRESERVATION AND USE OF GONADAL TISSUE IN POULTRY

Vitrification proved to be a successful freezing method of newly hatched chick, Japanese quail, and turkey gonadal tissues (Liu *et al.*, (2010); (2017); Liu, (2013); Barna *et al.*, (2020); Liptoi *et al.*, (2020)). This procedure is quick and requires an insulated box for liquid nitrogen, laminar flow hood, suitable scissors and forceps for micromanipulations, acupuncture needles, cryotubes or cryostraws for storing the organs, and a fixed-temperature heating plate. Gonadal transplantation can be performed on a disinfected table using a heated pad and infrared lamps to ensure the suitable temperature. Applying electrocautery can assure the complete ablation of the recipients. A stereo/dissecting microscope eases the removal of connective and other tissues from the gonad.

Chicken (*Gallus gallus domesticus*)

Preparation and vitrification of donor tissues

- Prepare the vitrification solutions prior to the collection of gonads as follows:
  - Basal solution: Dulbecco's phosphate-buffered saline (DPBS) with 20% fetal bovine serum (FBS). Can be stored for up to 1 hour at 0 °C if necessary.
  - Vitrification Solution 1: Basal solution with 7.5% dimethyl sulfoxide (DMSO) and 7.5% ethylene glycol (EG).
  - Vitrification Solution 2: Basal solution with 15% DMSO and 15% EG and 0.5 M sucrose.
- Euthanize 1-day-old chicks via cervical dislocation.
- Remove feathers from the abdomen and clean the abdomen with 70% ethanol.
- Perform the following under sterile conditions in a laminar flow hood:
  1. Make a 2 to 3 cm transverse incision on the left side of the chick and remove the yolk sac.
  2. Open the incision further to facilitate the removal of the gastrointestinal tract from the cavity.
  3. Remove the air sacs and serous membranes.
  4. Remove the ovary or testes in the caudocranial direction with fine forceps or microsurgical scissors (e.g. iris scissors).
  5. Place the donor organs into Basal solution, in antiseptic Petri dishes on ice. Maintain in this solution for no longer than 30 minutes.

- Once removed, and depending on the size, organs can be halved and three of these gonads (halved or whole) are placed onto a human acupuncture needle (e.g. 0.18 x 38 mm — Dongbang Acupuncture Inc, Korea).
- The needles are then placed into successive vitrification solutions (Vitrification Solution 1 then Vitrification Solution 2) in sterile Petri dishes for 5 minutes each at room temperature (Liptoi *et al.,* 2020). *Note:* Wang *et al.* (2008) treated the donor organs in two solutions for 10 and 2 minutes, respectively.
- Vitrify the samples by plunging the needles into liquid nitrogen and placing them in labeled 1 mL cryovials (Labsystem Comp., Hungary).
- Close the cryovials under liquid nitrogen with a forceps and place them in a liquid nitrogen tank for long-term storage.

Thawing of donor organs – performed immediately prior to implantation

- All media should be prepared, warmed, and maintained on a fixed-temperature heating plate in sterile, closed, labeled vials in a laminar hood.
- Prepare the following solutions, sterilize using a 0.2 µm filter
    - Basal solution: DPBS with 20% FBS. Can be stored for up to 1 hour at 0 °C if necessary.
    - Thawing Solution 1: Basal solution with 1M sucrose.
    - Thawing Solution 2: Basal solution with 0.5 M sucrose.
    - Thawing Solution 3: Basal solution with 0.25 M sucrose.
- Wash acupuncture needles with vitrified gonads through three different thawing solutions at 38.5 °C, in order 1 to 3 (decreasing amounts of sucrose), under sterile conditions for 5 minutes each (Liptoi *et al.,* 2020).
- The gonads must be completely submerged in the thawing solutions using at least 3 mL of sterile-filtered solution at the proper temperature for each needle (Barna *et al.,* 2020).
- Following thawing, cut each organ into 2-4 pieces under a stereomicroscope using an iris scissor.

Preparation of the recipient animals

- Determining the sex of the chicks prior to surgery makes the interventions more efficient.
- Maintain chicks in a heated room, under infrared heating lamps for pre-, peri-, and postoperative care.
- Administer local anesthesia, 0.1 mg xylazine (Narcoxyl 2) and 0.5 mg ketamine (CP Ketamin 10%), i.m., per chick.
- Place chick in a recumbent position on a heating pad and administer general anesthesia (isoflurane (Forane)) using a mask during the procedure.
- Remove feathers from the abdomen and washed the surgical site with 70% ethanol.
- Open the abdomen using a 2 cm transverse incision and a 1 cm incision to the last rib.
- Tie off the yolk sac and remove it from the chick.
- Push aside the gastrointestinal tract and locate the ovary which is located on the left part of the abdominal cavity, enclosed by air sacs, adjacent to the left kidney and the mesentery of the colon. In newly hatched chicks it is a triangular organ, 5-6 mm long, 1.5-2 mm wide, and yellowish-white in colour.
- Remove the native ovary by excising small pieces, in a caudocranial direction. Extreme care should be taken when performing the ovariectomy because the ovary is adjacent to the abdominal aorta and vena cava and damage to this vasculature will result in excessive bleeding (Buda *et al.,* 2019). The ovariectomy can be done precisely applying electrocautery (Kentamed 1E) with 16 Watts (Liptoi *et al.,* 2020). *Note*: The suitable wattage may vary

depending on the equipment. Clean the original location of the recipient's ovary with sterile cotton.

- *Note:* Testes removal is performed in a similar manner (Song & Silversides, 2006, 2007a, 2007b; Liu, 2013; Liptoi *et al.*, 2013). In this case removing of testes can be done with a fine scissor and forceps.
    - Testes are located below the cranial division of the kidney. The mesorchium is responsible for attaching them to the dorsal abdominal wall. Their form is oblong oval and structure is compact, because serous membrane and connective tissue cover them.

Transplantation

- Place donor ovary as close as possible to the natural anatomical location.
- Cover the transplanted tissue with the parietal layer of the abdominal air sac.
- Close the abdominal incision with two layers of polyglactin suture (Safil 3.0).
- *Note:* If transplanting testes then the donor tissues are positioned under the mesenterium.

Postoperative treatments

- 0.05 mg dexamethasone intramuscularly immediately after surgery to reduce edema and inhibit an immune response
- Mycophenolate mofetil, aka CellCept, 100 mg/kg body weight orally daily for 2 weeks, then once/week for 6 weeks to support gametogenesis and inhibit B and T cell activation thus facilitating implantation of donor tissue (Song & Silversides, 2006; 2007ab; Song *et al*., 2012; Liptoi *et al.*, 2013; Barna *et al*., 2020).
- *Optional antibiotic*: Ceftiofur, Excenel ubcutaneously., 2.55 mg, may not be this unnecessary as it does not influence the mortality or the adherence of the grafted organs (Song & Silversides, 2006; 2008; Song *et al.* 2012; Liptoi *et al*., 2013, 2020).

Japanese quail (*Coturnix japonica*)

Live progeny is obtained from transplanted, frozen thawed gonads of week-old Japanese quails (Liu *et al*., 2010; Liu, 2013). The procedure is similar to that described above for to the chicken with a few differences that are highlighted below.

Preparation and vitrification of donor tissues

- After euthanasia ovaries are immersed in Dulbecco's modified Eagle medium (DMEM) with 10% FBS on ice up to 4 hours.
    - Note: Testes are placed into Basal solution on ice (Liu, 2013).
- Four to five tissue pieces are put on a single acupuncture needle (Cloud & Dragon Medical Device Co. Ltd. Jiangsu, China).
- The needles are placed into Vitrification Solution 1 then Vitrification Solution 2 for 10 and 2 minutes, respectively, at room temperature.
    - Remove the vitrification solution from the organs by blotting with a piece of gauze.
- Place the needles with vitrified gonads into cryovials under liquid nitrogen and close with precooled caps.

Thawing of donor organs

- Wash the needles with vitrified gonads through the three thawing solutions at room temperature in order 1 to 3 for 5 minutes each.

    - Increase the temperature of the solutions for testes to 40°C instead of room temperature.
- Place the ovaries into DMEM and the testes into Basal solution on ice for storage up to 4 hours.

Preparation of the recipient animals and transplantation

- Administer local anesthesia, 0.1 mg of ketamine (Ayers Laboratories, Guelph, ON, Canada) and 0.05 mg of xylazine (Bayer Inc., Toronto, ON, Canada), subcutaneous per chick.
- Administer isoflurane gas
- Place the chick on a heating pad and open the left side the abdominal cavity distal to the last rib.
- Ovarian transplantation:
    - Push aside the gastrointestinal tract and locate the ovary.
        - The ovary has an irregular oval shape with a granular surface and is located dorsal to the body cavity and cranial to the left kidney in week-old quail chicks.
    - Remove the host ovary with fine forceps.
    - Place the donor ovary in the anatomical site and cover with the parietal layer of the abdominal air sac.

- Testes transplantation:
    - Make a 1.5 cm incision 1 cm left of the medial plane. Displace the abdominal organs to expose the testes. Remove the whole testes by cutting the mesorchium with fine forceps. Two testes from two different males can be inserted under the dorsal skin of each recipient through a small incision, which is closed by a single stitch (Liu, 2013).
- Close the skin with 4-5 interrupted sutures.

Postoperative treatments

- Mycophenolate mofetil (CellCept; Hoffmann-LaRoche Ltd., Mississauga, ON, Canada) orally 100 mg/kg body weight daily for 2 weeks.

Turkey (Meleagris gallopavo**)**

In turkeys, only the vitrification and warming methods of ovarian tissue were published (Liu *et al*., 2017).

Preparation and vitrification of the donor ovarian tissues

- Prepare the vitrification solutions prior to beginning the collection of gonads. The content of the Basal and Vitrification solutions is the same as the chicken.
- Immediately after euthanasia recover the ovaries with fine forceps (Fine Science Tools, Foster City, CA, USA) and place them in DPBS
    - Remove the surrounding connective tissue
    - Place the gonads in Basal solution
- Place the organs on an acupuncture needle (J-type, Size No. 2 (0.18)×30 mm, SEIRIN-America, Weymouth, MA, USA)
    - Note: Utilizing a dissecting microscope may help to minimize handling damage
- The needles are placed into Vitrification Solution 1 for 10 minutes then into Vitrification Solution 2 for 2 minutes at room temperature.
    - Blot the ovaries briefly with a piece of gauze.

- Place the needles with vitrified gonads into cryovials under liquid nitrogen and close with precooled caps.

Thawing of donor organs

- Immerse the needles into successive thawing solutions (Thawing Solution 1, Thawing Solution 2, Thawing Solution 3, and Basal solution) for 5 minutes each at room temperature.
- Remove the needles and wash the organs in fresh Basal solution.
    - Gonads can be stored in Basal solution on ice until use.

CRYOPRESERVATION OF HONEY BEE TESTICULAR AND SEMINAL VESICLE TISSUE

The protocol below describes cryopreservation of testicular and seminal vesicle (SV) tissue of honey bee drones.

Requisites

Tissue collection and culture: Leibovitz L15 (modified with 0.25% glucose, 0.25% trehalose and 10% fetal bovine serum) cell culture medium; sterile filtered simple saline (0.2M Tris buffer pH 7.2 with 1.12% potassium chloride and 0.88% sodium chloride); sterile pipettes; sterile 3 cm petri plates; and dissection tools.

For cryopreservation: To cryopreserve, CBS tissue cryopreservation straws (CBS; 018960) or cryovials (Nunc; 368632). Any programmable freezer capable of 0.3°C/min cooling to -80°C.

Marked drone cells in the hive are monitored for emergence of drones and the drones are marked. The testis will regress progressively after day 3 and would become nearly vestigial thereafter. Therefore, depending on whether the testicular tissue is required for future tissue culture or the SV are required to obtain semen, due consideration must be paid to the age of the drones as source of tissue for cryopreservation.

Tissue harvesting

1. Drones of specific ages are brought to the lab and anesthetized using carbon dioxide. The wings and the limbs are clipped and the drone is quickly rinsed in chilled 5% dilute Clorox™ followed by immersion in chilled 70% ethanol.
2. The abdominal organs are accessed using a midline dorsal incision. The organs are loosened by dropping sterile simple saline on the tissue.
3. The testis + seminal vesicle complex is excised at the base of SV just before the accessory glands and transferred to a drop of modified L15 medium in a cavity slide. The slide is always kept chilled on ice.
4. In drones that are less than 3-day old, the testis is separated from the SV and transferred to another drop of L15 medium for testicular cryopreservation. In bees older than 3 days, the testis that is shrunk is left intact on the SV.

Cryopreservation

1. The L15 medium is replaced with ice cold L15 with 12% DMSO and after 5 minutes, the medium is again replaced with ~200 µl of the L15. The excised testicular tissue does not require any further manipulation; however, the SV is cut into either 2 or 3 separate segments with a sharp sterile razor.

2. The entire 200 µl suspension is aspirated into a 2 ml tissue cryopreservation straw (Cryo Bio System, France). After a gap of the same length as the loaded suspension, aspirate 0.5 ml of L15 medium without DMSO. Center both the suspension and the solution with equal gaps on either side using a heated forceps to heat seal the straw. Alternatively, the straw could be sealed using an electric heater or a plastic bag sealing equipment.
3. If using a cryovial, pipette the contents using a sterile silanized widened micropipette tip into a cryovial.
4. After incubating on ice for 10 minutes, place the straw or the vial in a programmable freezer at 4°C. The cooling curve is from 4°C to -10°C at 1°C/min. In the case of the cryostraw, the sample is manually seeded with a forceps tip with condensate ice gathered by dipping in liquid nitrogen. With respect to the cryovial, the ice crystals are added into the vial using sterile forceps.
5. After seeding, the program continues the cooling at 0.3°C/min to -40°C followed by 5°C/min to -80°C.
6. The straws are transferred to a visotube in a daisy goblet (Cryo Bio System, France) and placed in the LN container. The cryovial on the prelabeled cryocane is directly transferred to a marked canister in the LN storage container.

Thawing and assessment

1. Thawing is performed by swiftly transferring the straw to a 37°C water bath. The entire content (tissue suspension + L15 medium) is expelled into an Eppendorf tube. Another 250 µl of L15 medium is added to the suspension. The contents are centrifuged at 1200xg for 10 minutes and the supernatant is replaced with fresh L15 medium after which the contents are gently resuspended.
2. The cellular viability is assessed using a live-dead cell kit (Invitrogen/Thermo Fisher) by adding 1 µl of 10x diluted stock Sybr14 per 250 µl of the sample and incubating the sample at room temperature in the dark for 10 minutes. This is followed by adding 1 µl of stock propidium iodide per 250 µl of the suspension and continued incubation for another 10 minutes in dark. The cell viability is assessed using a fluorescent microscope to obtain the proportion of the green/(green+red) nuclei using GFP and RFP or Texas red filters.

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Annex 6.5 Guideline for diploid germcells cryopreservation: Isolation, establishment, cryopreservation and use of *in vitro* propagated chicken primordial germ cells

INTRODUCTION

Health accreditation of collection, storage and implementation operations preamble. Due to National and International specific regulations and agreements, operators wishing to implement the following procedures are advised to approach the relevant authorities in order to take into account the corresponding regulatory constraints.

Know-how. The Know-how is very important for the success of the procedures. This know-how is acquired with the experience and the time. A training is often required. We thus suggest contacting the drafters of the procedures before any use.

COLLECTION AND IN VITRO CULTURE OF PRIMORDIAL GERM-CELL (PGC)

Fertile eggs of the breed to be preserved must be transported to a laboratory equipped with cell culture facilities and egg incubator. The eggs are placed in the incubator and incubated under standard conditions (see below) until PGCs collection.
PGCs can be collected from multiple sites within the avian embryo (Barna *et al*., 2020, Nakamura et al. 2013, Song et al., 2014), however, from the following two embryonic sites are preferred (Nakamura, 2016):

1. The blood at the stage when PGCs circulate and migrate towards forming gonads (HH stages 13-17; cPGCs, Hamburger & Hamilton, 1951) At this stage approximately 100-200 PGCs circulate in the vascular system and represent 0.02% of blood cells.
2. The embryonic gonads colonized by PGCs (HH stages 27-31). Gonadal PGCs (gPGCs) represent 2% of gonadal cells.

Embryonic blood can be used directly for subsequent derivation of PGCs culture. The number of PGCs and their proportion in the gonads is higher than in the blood; however, a gonad dissociation step is needed before starting *in vitro* culture, which is impractical when high numbers of embryos have to be sampled.

We describe here two protocols, of which the first, Protocol 1, is the reference protocol. Protocol 2 may be applied in specific circumstances.

Protocol 1

Required equipment: egg incubator, laminar flow hood, CO_2 incubator, binocular magnifier or stereomicroscope, fibre optic light source, sterile pulled glass microcapillary, mouth pipette apparatus, Petri dishes, small scissors, small forceps, non-adhesive, or cell culture coated 48-well and 24-well plates and pipettes.
Renewable supplies: sterile phosphate buffered saline (PBS) solution and culture medium for PGCs (Whyte *et al.,* 2015)

PGCs culture medium: a custom basal medium (a modification of knockout DMEM [250 mosmol/L, 12.0 mM glucose, and $CaCl_2$-free; ThermoFisher Scientific]) supplemented with 0.15 mM $CACl_2$, 1 × B-27 supplement, 2.0 mM GlutaMax, 1 × NEAA, 0.1 mM β-mercaptoethanol, 1 × nucleosides, 1.2 mM pyruvate, 0.2% ovalbumin (Sigma), 0.01% sodium heparin (Sigma) ~~5 µg/mL~~ in a basal medium, human Activin A or BMP4, 25 ng/mL (Peprotech), human FGF2, 4 ng/mL (R&D Biosystems), 0.2% chicken serum (Biosera) or 10 µg/mL ovotransferrin (Sigma).

Collection of PGCs from the blood

The following procedures require adequate technical expertise. Blood is collected from individual embryos at HH stages 13-17 (52-58h of incubation).

1. Fertilized chicken eggs are incubated for 52-58 hours at 37.7°C and 45-51% humidity until the embryo reaches the embryonic stage HH 14-16. Wipe the egg surface with 70% ethanol to sterilise. The incubation period may slightly vary depending on the breed and on the progress in the laying period.
2. Carefully break the eggshell and pour the contents into a Petri dish so that the embryo is situated on the top. Alternatively, position the egg so that the blunt end is on the top. Make a window in the eggshell at the blunt end. Position the egg under the binocular magnifier, carefully remove the shell membrane on the bottom of the air chamber. Place the optic fibre light source so that the embryo is well lit.
3. Carefully introduce the sterile pulled glass microcapillary (~ 10 µm ±0.5 internal diameter at the end) in the dorsal aorta and take in 1-3µL of blood using a mouth aspirating device.
4. The blood is immediately placed into a single well of a 48-well cell culture plate containing 300 µl PGCs culture medium. (Whyte *et al.*, 2015).
5. Tissue samples for sex-determination are collected from each isolated embryo and stored at -20 °C until further use (Lázár *et al*., 2020).

Derivation of PGC cultures

1. *In vitro* culture of sampled blood using PGC culture medium is a necessary step which allows propagation of PGCs and eliminate other cell types. Established homogeneous PGC culture can also be used for cryopreservation. The isolated blood from single embryos, which contains circulating PGCs, is cultured in PGCs culture medium at 37°-37.7C with 5% of CO_2.
2. One-third of the medium is changed every 2 days. 100 µl of the medium is carefully removed and replaced by fresh medium. Be careful not to remove PGCs during this procedure, since PGCs are non-adherent cells.
3. PGCs start to divide and blood cells are progressively lost through the culture. A homogeneous PGCs population can usually be obtained after 2-4 weeks of culture. PGCs are round big cells with a large nucleus and granular cytoplasm. They can be observed after approximately 3-5 days after the onset of culture. When the wells fill with PGCs and most blood cells are lost, the cells are counted and transferred in the wells of 24-well culture plate containing 500 µl of PGC medium. Usually ~50,000 or more PGCs are present at this stage.
4. The whole volume of the medium is changed every two days. The cells are centrifuged at 400 g for 8 min. The medium is carefully removed, and the cell pellet is resuspended in 200 µl of fresh culture medium. The cells are counted and seeded in the wells of 24 well plate at a concentration of 1.26 $x10^5$/ml. PGCs are propagated until their desired number is obtained. This number depends on the number of cryovials/individual cultures that are planned to be frozen. If the number of PGCs from one embryo reaches 1.0 x 10^5 cells in 3-4 weeks, the establishment of the permanent culture is considered successful (Lázár *et al*., 2020).

Protocol 2

Protocol 2 may be recommended if the embryo is at a later stage of development (5-7 days of incubation). In this case, the PGCs are extracted from the embryonic gonad.
Required equipment: egg incubator, laminar flow hood, CO_2 incubator, stereomicroscope, Petri dishes, pipette, scissors, forceps, centrifuge tubes and non-adhesive 48-well and 24 well plates.
Renewable supplies: phosphate buffered saline without Ca^{2+} and Mg^{2+} (PBS [-]), culture media for PGCs as described in **protocol 1**.

Collection of PGCs from embryonic gonads
This procedure is based on the method described previously by Nakajima et al., (2011).

1. Fertilized eggs of chicken are incubated about 5-7 days at 37.7°C (HH 27-31).
2. After incubation, both right and left gonads are isolated from the embryos.
3. The isolated gonads are placed in a 1.5 mL centrifuge tube containing 500 µL of phosphate buffered saline without Mg^{2+} and Ca^{2+} (PBS [-]).
4. The centrifuge tubes are placed in an incubator maintained at 37.8℃ for 1 – 1.5 hours.
5. Gonads are pipetted gently in the centrifuge tube and 100 µl of cell suspensions is placed into wells on 48-well plate, containing 200 µL PGC culture medium as described in **protocol 1**. After 24-48 hours of culture non-attached cells are transferred to new wells. These cells are PGCs and can be cultured as described in **protocol 1**.

Freezing
The cryopreservation strategy and protocol are essentially as described by Nandi et al. (2016).

Required equipment: Cell culture 24-well plates, thermostat, freezer (Mr. Frosty™), laboratory centrifuge, pipette, Eppendorf tubes, Nunc cryotubes, - 80 °C freezer.

Renewable supplies: Avian DMEM containing 4% DMSO and 5% chicken serum (Freezing medium), or STEMCELL BANKER, liquid nitrogen storage.

1. PGCs are suspended carefully from the bottom of the culturing well, and then pipetted into 1.5 mL Eppendorf tubes.
2. After centrifugation (1000 g, 3 minutes) the supernatant is removed.
3. 5-6x10^4 cells are slowly resuspended in 250 μl of DMSO-free freezing medium for PGCs and pipetted into labelled cryotubes.
4. 250 μl of freezing medium for PGCs is added slowly dropwise, and then the cryotubes are placed in a freezing box and into the freezer at -80 °C.
5. Alternatively, the cell pellet is resuspending in 250 µl of STEMCELL BANKER before transfer to the freezing box.
6. After one night, the samples are moved into the freezer at -150 °C or into liquid nitrogen.

Thawing
Required equipment: water bath, thermostat, laboratory centrifuge, non-adhesive 24 wells cell culture plates, pipettes.

Renewable supplies: culture medium for PGCs described in **protocol 1**.

1. For thawing of PGCs, water bath at 37 °C is used.
2. 2 mL of culturing media for PGCs is slowly pipetted into the thawed tubes.

3. Samples are centrifuged (1000 g, 10 minutes) and the supernatant is removed.
4. The cells are resuspended in fresh culture medium for PGCs, placed into 24 well cell culture plate and cultured as described in **protocol 1**.

Transfer into host embryos
Required equipment: egg incubator, chick hatcher, laminar box, binocular magnifier or stereomicroscope, fibre optic light source, dry block heating thermostat, sterile pulled glass microcapillary, mouth aspirating device, Eppendorf tubes and pipettes.

Renewable supplies: 70% alcohol, laboratory parafilm, sterile phosphate buffered saline solution (PBS), DMEM high glucose, no glutamine, no calcium (Gibco) and sterile water (ratio 2:1).

The following procedure require high technical expertise:

1. Fertilized recipient eggs are incubated for 52-58h (HH 14-16, Hamburger & Hamilton, 1951) at 37.7°C.
2. After centrifugation in a mixture of DMEM and sterile water (ratio 2:1) the PGCs pellet is resuspended in DMEM. One-two µL of 1% solution of vital dye Fast Green in ultra-pure distilled water can be added to the cell suspension to better control the injection procedure.
3. The disinfection of the recipient eggs, transfer is started by opening a 10 mm diameter "window" at approximately one-third of the egg, closer to the pointed end.
4. 1 µl (~ 3-5 000 PG cells) of the prepared cell suspension is injected into the heart or the dorsal aorta of the 52-58h day recipient embryos.
5. Following the injection, ~ 50 µl of sterile prewarmed 1xPBS is dripped onto the embryo.
6. The hole is sealed with two- layers of melted laboratory parafilm or sealed with 1 cm of leukosilk tape. Alternatively, the hole is covered with egg shell membrane and sealed with melted parafilm.
7. The egg is placed into a stationary egg incubator for 24 h. After 24hours, the incubator is rocked at 45 until day 18 of incubation. The egg is then transferred to a hatcher until hatching occurs at 21 days. (Barna *et al*., 2020).

Sanitary Status
The equipment and solutions used in the protocols described above must be sterile and free of all avian pathogens. However, if chicken serum is used for culture of the PGCs it must be certified pathogen and mycoplasma-free.

Protocol of livestock parent stocks free from pathogens
Vaccination protocol is used to prevent viral and bacterial diseases, through development of maternal immunity that is transferred to the embryo developing in the eggs.

Vaccination prevention / domestic fowl species	
Viral diseases	Bacterial diseases
Marek's disease	*Salmonella enteritidis*
Newcastle disease	*Salmonella typhimurium*
Infectious bursitis	
Infectious bronchitis	
Avian encephyalitis	
Fowl pox	

Mycoplasma infection is prevented through antibiotic treatment of the laying hen or of the recipient eggs in the last third of hatching.

Protocol for treatment of egg with antibiotics*:*

- Preparation of the solution required for treatment: mix 5 ml of 10% Baytril solution in 200 ml of physiological saline.
- Eggs must be disinfected with iodine solution before injection.
- Injected amount of Baytril solution: 0.1 ml / egg (recommended with an automatic syringe).
- Injection above the air chamber at a maximum depth of 5 mm.
- After the injection, the hole is sealed with glue.

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ANNEX 9.1 – Potential set of elements in the material acquisition agreement

An example of MAA is provided in the GUIDELINES FOR THE DEVELOPMENT OF MATERIAL ACQUISITION AGREEMENTS (MAA) on the website of the European Regional Focal Point for Animal Genetic Resources, which is a regional platform for the support of management, conservation and sustainable use of animal genetic resources: https://www.animalgeneticresources.net/wp-content/uploads/2019/11/MAA-final-version.-04_11_19.pdf

The guidelines provide a potential set of elements, which could be incorporated in a MAA:

Part 1. IDENTIFICATION OF THE PARTIES

Part 2. PREAMBLE

Part 3. DEFINITIONS

Part 4. SPECIFIC OBJECTIVES OF THE MAA

Part 5. DESCRIPTION OF THE MATERIAL

Part 6. FINANCIAL ARRANGEMENTS AND OWNERSHIP

Part 7. RIGHTS AND DUTIES OF THE PROVIDER

Part 8. RIGHTS AND DUTIES OF RECIPIENT

Part 9. WARRANTY

Part 10. LIABILITY

Part 11. GOVERNING LAW

Part 12. DISPUTE SETTLEMENT

Part 13. ARRANGEMENTS ABOUT GENERAL DATA PROTECTION

Part 14. EFFECTIVE DATE AND VALIDITY

ANNEX 1 TECHNICAL INFORMATION ON THE ACQUIRED BIOLOGICAL MATERIAL AND ITS LEGAL STATUS

ANNEX 2 PIC / MAT AND IRCC (IF RELEVANT)

ANNEX 9.2 Potential set of elements in the material transfer agreement

An example of MTA is provided in the GUIDELINES FOR THE DEVELOPMENT OF MATERIAL TRANSFER AGREEMENT (MTA) FOR CONSERVATION AND BREEDING on the website of the European Regional Focal Point for Animal Genetic Resources, which is a regional platform for the support of management, conservation and sustainable use of animal genetic resources: https://www.animalgeneticresources.net/wp-content/uploads/2019/11/MTA-Conservation-and-Breeding-final-version.-04_11_19.pdf

The guidelines provide a potential set of elements, which could be incorporated in a MTA:

Part 1. IDENTIFICATION OF THE PARTIES

Part 2. PREAMBLE

Part 3. DEFINITIONS

Part 4. SPECIFIC OBJECTIVES OF THE MTA

Part 5. DESCRIPTION OF THE MATERIAL

Part 6. FINANCIAL ARRANGEMENTS AND OWNERSHIP

Part 7. RIGHTS AND DUTIES OF THE GENE BANK

Part 8. RIGHTS AND DUTIES OF RECIPIENT

Part 9. MTA COMMITTEE

Part 10. WARRANTY

Part 11. LIABILITY

Part 12. GOVERNING LAW

Part 13. DISPUTE SETTLEMENT

Part 14. ARRANGEMENTS ABOUT GENERAL DATA PROTECTION

Part 15. EFFECTIVE DATE

ANNEX I. – Description of the Material

ANNEX II. – Description of the Activity

ANNEX III. – Technical specifications and protocols