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Innovations in cryoconservation of animal genetic resources

Practical guide

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Foreword

The livestock sector faces a range of challenges, including climate change, emerging diseases, competition for natural resources and evolving demand for animal-source foods, which is increasing globally, especially in developing countries. Genetic diversity of livestock is a key resource for allowing livestock keepers to address these challenges, but this diversity has been in a state of decline. The diminishing genetic diversity thus represents yet another obstacle for sustainable livestock production. Cryoconservation (i.e. *ex situ – in vitro* conservation) of genetic resources through gene banking provides one of the most powerful tools governments and other stakeholders have to manage genetic diversity in both the short and long term and thereby provide future generations with the tools to meet the challenges ahead.

Gene banking genetic resources fits within the context of the *Global Plan of Action for Animal Genetic Resources*,[1] which was developed and adopted by FAO Member Nations. Specifically, Strategic Priority 9 of the Global Plan of Action is "Establish or strengthen *ex situ* conservation programmes" and Strategic Priority 11 urges countries to "Develop approaches and technical standards for conservation. To assist countries in the implementation the Global Plan of Action, FAO worked with experts from around the world to prepare technical guidelines. In 2012 FAO published FAO Guidelines on *Cryoconservation of animal genetic resources*.[2]

Gene banking is a long-term effort that needs to be viewed in terms of decades rather than years, as demonstrated by similar systems for agricultural crops. The responsibility for establishing such resources lies squarely within governments' roles of providing public goods and food security. Gene banking of animal genetic resources is a technology-intense undertaking and the associated technologies are in a continual state of research and development. The livestock sector also continues to evolve rapidly. Since the development and release of the previous guidelines, numerous changes have taken place. Critical among these is a greater appreciation of the opportunities for actively utilizing cryopreserved material to enhance management of *in vivo* populations, rather than as simply an "insurance policy" to protect breeds against extinction.

This key development has led to further changes in gene bank management. First, interaction with users of the stored material has increased. This in turn has created a need to involve stakeholders more closely in the management of genetic collections and to better monitor and document the processes of gene banking to ensure quality management. Measures have been developed to help ensure high health and sanitary standards. New models and methods have been proposed for utilization of collections in the management of genetic diversity *in vivo*. Greater potential demand for stored material has also increased the need for expanded procedures and legal instruments, in some instances, to ensure

[1] **FAO.** 2007. *Global Plan of Action for Animal Genetic Resources and the Interlaken Declaration.* Rome. www.fao.org/3/a1404e/a1404e.pdf

[2] **FAO.** 2012. *Cryoconservation of animal genetic resources.* FAO Animal Production and Health Guidelines No. 12. Rome. www.fao.org/3/i3017e/i3017e.pdf

exchange of material is equitable for all parties and does not compromise long-term goals for management of animal genetic resources. In terms of policy, the Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization[3] of the Convention on Biological Diversity has come into force (ratified by more than 125 countries) since the release of the previous guidelines. Assisted reproductive technologies and cryobiology have improved and their application for germplasm collection and storage has expanded. Advances in genomics have increased the opportunities to evaluate gene bank collections at the molecular level, increasing the precision and power for management of genetic diversity. Finally, in nearly all aspects of daily life, recent years have continued the ongoing trend in the increased quantity and importance of information. Gene banking is no exception. Data about stored samples should be considered an integral aspect of the collection. Modern systems and tools for management of these data, including their integration with other sources of complementary information, and sharing with stakeholders are becoming more and more fundamental features of gene banks.

The European Union has recognized the importance of cryoconservation of animal genetic resources and the continual need for associated research. Through its Horizon 2020 research-funding programme, the European Union financially supported the "IMAGE" project (Innovative Management of Animal Genetic Resources – grant number 677353)[4] with a focus on animal gene banks. IMAGE addressed a wide range of issues, including gene bank management, reproductive physiology and cryopreservation, genomic characterization of stored material, data management, and utilization of stored material in management of *in vivo* populations. FAO was among the partners in the IMAGE project.

One of the deliverables[5] of IMAGE was a review of the previous guidelines and recommendations for content in a revised document. This publication addresses these recommendations, while also considering the results from other IMAGE deliverables. To ensure a comprehensive and global perspective, further expertise was gathered through the contributions of scientists from all FAO regions. The document was then reviewed by members of the Commission on Genetic Resources for Food and Agriculture and its Intergovernmental Technical Working Group on Animal Genetic Resources for Food and Agriculture. The result is a comprehensive and up-to-date guide to aid gene bank managers, policymakers and other relevant stakeholders for decision-making in the optimal use of cryoconservation as a tool in the management of genetic resources for food and agriculture.

[3] **CBD.** 2011. Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits Arising from their Utilization. Montreal. Cited 4 March 2022. www.cbd.int/abs/

[4] **Innovative Management of Animal Genetic Resources Project (IMAGE).** 2020. Project funded by the Horizon 2020 Research and Innovation Programme of the European Union under Grant Agreement Number 677353. Cited 31 October 2020. www.imageh2020.eu

[5] **Honkatukia, M. & Boes, J.** 2020. *IMAGE – Innovative Management of Animal Genetic Resources, Deliverable D 7.13: Guidelines for the management of gene banks*. Paris. Cited 3 February 2022. www.imageh2020.eu/deliverable/D7.13.pdf

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This document includes numerous text boxes to provide detailed information about specific topics. Many of the authors listed above contributed text boxes, in addition to the following people: Sahar Albayatti, Iraq; Sheila Butungi, Uganda; Ngo Thi Kim Cuc, Viet Nam; Isidore Houaga, Burkina Faso; Lilian P. Villamor, Philippines; and Xueming Zhao, China.

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Goal and structure

This document has been developed to complement and update the existing FAO Guidelines on *Cryoconservation of animal genetic resources*[2]. Although research and development in cryoconservation advances rapidly, the content of the 2012 guidelines generally remains valid. For example, methods of cryoconservation described in the previous guidelines can still be applied successfully, whereas the comparable approaches provided in this document will generally represent an update in the science and in practical experiences in the context of gene banking. Content that is repeated in this document was done so either to provide the necessary context or to underline its importance. This document also complements other FAO guidelines on the management of animal genetic resources, particularly FAO Guidelines on In vivo *conservation of animal genetic resources*[6] and on *Breeding strategies for sustainable management of animal genetic resources*[7].

The document is intended to serve as a decision aid for gene bank managers and other stakeholders with respect to the various objectives for cryoconservation and the options for its implementation. In general, the information provided will be relevant for all species of domestic livestock, but species-specific guidance is given where appropriate.[7]

The document is pertinent for different types of stakeholders, ranging from policymakers seeking information on the opportunities offered by cryoconservation to staff of gene banks wishing to learn about new techniques. Although reading all sections is recommended, certain sections are aimed at specific stakeholders with specific technical interests and responsibilities.

Section 1 reviews the possible objectives for banking of animal genetic material and highlights the need to undertake gene banking in a strategic manner. Cryoconservation involves the interests of multiple stakeholders with varying priorities and multiple scenarios for utilization of stored material. These factors imply the need for governance, periodic review and assessment of gene bank collections and for consideration of ethical issues. Aspects of multicountry gene banking are also addressed.

Section 2 reviews the preparation, implementation and organization of gene banks and introduces the concept of quality management in a gene banking context.

Section 3 summarizes the types of biological material that may be preserved in gene banks, presenting the latest developments for commonly used materials like semen and embryos, and describing the potential benefits of utilizing novel materials such as primordial germ cells and gonadic tissues.

Section 4 reviews the details of economic issues when operating a gene bank, discussing not only aspects of the fixed and variable costs, but also considering the values and benefits of cryoconservation and formal economic approaches that can be used to balance costs and benefits.

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[6] **FAO.** 2013. In vivo *conservation of animal genetic resources*. FAO Animal Production and Health Guidelines. No. 14. Rome. www.fao.org/3/i3327e/i3327e.pdf

[7] **FAO.** 2010. *Breeding strategies for sustainable management of animal genetic resources.* FAO Animal Production and Health Guidelines. No. 3. Rome. www.fao.org/3/i1103e/i1103e.pdf

Section 5 provides an overview of the new developments and insights in developing and using gene bank collections. An emphasis is placed on utilization of stored material for purposes beyond reconstitution of extinct breeds. Several case studies are included.

Section 6 reviews and updates the common methods for collecting, cryopreserving and thawing for utilization of various types of genetic material from various species of livestock and introduces new approaches.

Section 7 addresses the health and sanitary issues that must be considered when establishing and operating gene banks for animal genetic resources, including regulatory issues.

Section 8 describes documentation and database requirements for storing information on individual animals and on the samples of genetic material stored in the gene bank. It also addresses the integration of gene bank data with other relevant publicly available data systems, while making key information available to stakeholders.

Section 9 addresses the legal issues associated with cryoconservation. The section considers the legal aspects of cryoconserved germplasm as a private and public good and outlines the issues to be accounted for in the agreements that govern transfer of material to and from the gene bank, including transfer across national borders.

Section 10 discusses priorities for capacity building in relation to gene banking and considers the differences in the demands of the wide range of stakeholders.

The main sections are followed by a series of **Annexes**, which provide complementary information to the main sections, including example documentation of interest for gene banking and step-by-step instructions on procedures for collection and cryopreservation of germplasm.

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
API	application programming interface
ART	assisted reproductive technologies
BCn	backcross (*n* indicates the generation number)
CBD	Convention on Biological Diversity
CBA	cost-benefit analysis
CEA	cost-effectiveness analysis
CIDR	controlled internal drug release
CNA	competent national authority
CPA	cryoprotectant (or cryoprotective agent)
C:P	cholesterol to phospholipid ratio
CSV	comma separated values
DAD-IS	domestic animal diversity information system
DBMS	database management systems
DMF	dimethyl formamide
DMP	data management plan
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
doi	digital object identifier
DPBS-FBS	Dulbecco's phosphate-buffered saline – fetal bovine serum
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	Germplasm 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
MHC	major histocompatibility complex
MSC	mesenchymal stem cells
MTA	material transfer agreement
NAAB-CSS	National Association of Animal Breeders – Certified Semen Services
NAGP	National Animal Germplasm Program (of the United States of America)
OPU	ovum pick-up
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PGC	primordial germ cells
QMS	quality management system
QTL	quantitative trait locus
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
WOAH	World Organisation for Animal Health

SECTION 1
Building a gene banking strategy

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1.1 INTRODUCTION

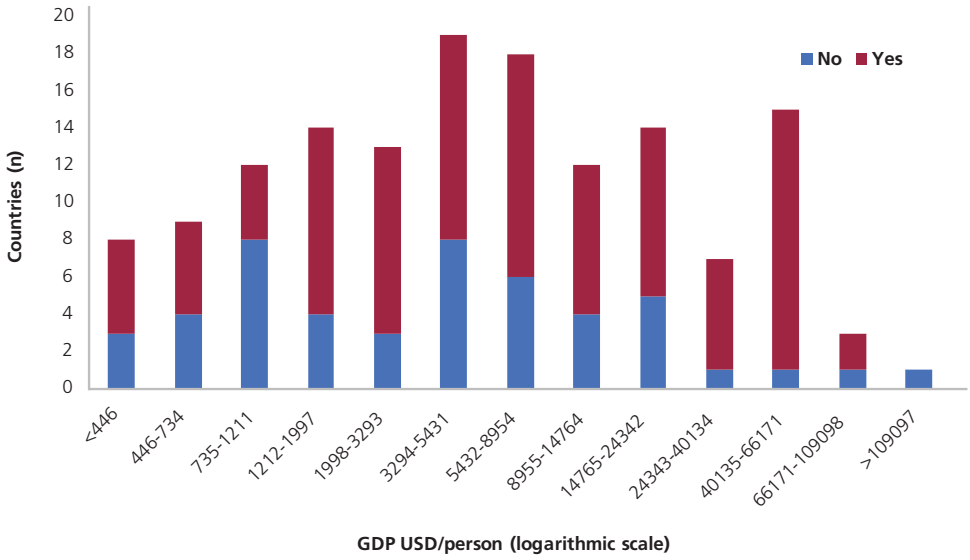
Gene banking is a powerful tool for the management of animal genetic resources for food and agriculture (AnGR). Although gene banking is technically demanding and can require substantial initial investments, once genetic materials are cryopreserved, properly stored and documented, they can remain viable 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.

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 USD). 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,

FIGURE 1.1
Numbers of countries reporting that they have a gene bank (yes/no) according to level of economic development (GDP/person in USD)



Source: Authors' own elaboration.

due to genetic drift, demographic factors, or suboptimal 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 banking 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 management of genetic variation

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 the 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 provides more detail on the economic aspects of cryoconservation programmes.

Many public gene banks provide stored material free of charge to users who can demonstrate a justifiable need. However, gene 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 communications 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 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.

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, Purdy and Blackburn (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 (Sonesson, Goddard and Meuwissen, 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 may still benefit from cryoconservation activities. See FAO (2013) for information about breed risk criteria. The earlier a material collection programme starts, the larger and more diverse it will be in the future. It will also 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 reintroduce 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 which include 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 obligations with respect to international agreements for management of biodiversity, e.g. the Convention on Biological Diversity (CBD, 1992) or the SDGs (UN, 2020). 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 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 banking 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 developing 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 he or she may have to consult with various stakeholders for the long-term decisions. The establishment of a multiactor stakeholder committee to provide input and support to the gene bank is recommended.

This stakeholder committee could have several responsibilities, in particular, in assisting the development of gene banking strategy, and monitoring its implementation and rationalization 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 to be maintained, and how to be 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 that consists 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.

1.4.1 Sampling

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

1.4.1.1 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).

1.4.1.2 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 banks 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, Mariante and Blackburn, 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, Hiemstra and Blackburn, 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 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.[1, 2] 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 percent chance of capturing the allele. Utilization of this equation is a cost-efficient approach for building collections.

[1] **FAO.** 1983. *Animal genetic resources conservation by management, data banks and training.* Proceedings of the Joint FAO/UNEP Expert Panel Meeting, Part 1.

[2] **Smith, C.** 1984. *Genetic aspects of conservation in farm livestock. Livestock Production Science,* 11(1): 37–48. https://doi.org/10.1016/0301-6226(84)90005-8

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 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.

1.4.1.3 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. 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.

1.4.1.4 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 type and number of gametes 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, Purdy and Blackburn, 2012). More recently, cryoconservation of primordial germ cells has been developed and validated to revive a chicken breed (Woodcock *et al.*, 2019).

1.4.1.5 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 farm collection, private sector artificial insemination (AI) facilities and research institutions, which may differ in sanitary status and regulations. Section 7 provides more information.

1.4.2 Storage organization

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

1.4.2.1 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 systems 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.

1.4.2.2 Duplication and backup

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 centre 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 backup 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.

1.4.2.3 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.

1.4.2.4 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 (see Section 8).

1.4.3 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.

1.4.3.1 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 the country's 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 the 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?

1.4.3.2 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 of being able to reconstitute breed(s) that may become threatened or extinct.

1.4.3.3 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 11 000 animals since 2004. The data also shows that samples of animals of nearly all birth years are being continually used by the various 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 (see Box 1.3). Furthermore, such examples are extremely useful in articulating the value of gene banking to the stakeholders, administrators, and the public at large. One option to facilitate tracking is to use the digital object identifier (doi) system 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 1.2

Using banked material to reconstitute lost Holstein sire lineages

Yue, Dechow and Liu[1] 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 1970s 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.[2] 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 artificial insemination (AI) centre.

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 populationcan be done much more quickly and efficiently than previously thought.[3]

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[1] **Yue, X.P., Dechow, C. & Liu, W.S.** 2015. A limited number of Y chromosome lineages is present in North American Holsteins. *Journal of Dairy Science,* 98(4): 2738–2745. https://doi.org/10.3168/jds.2014-8601

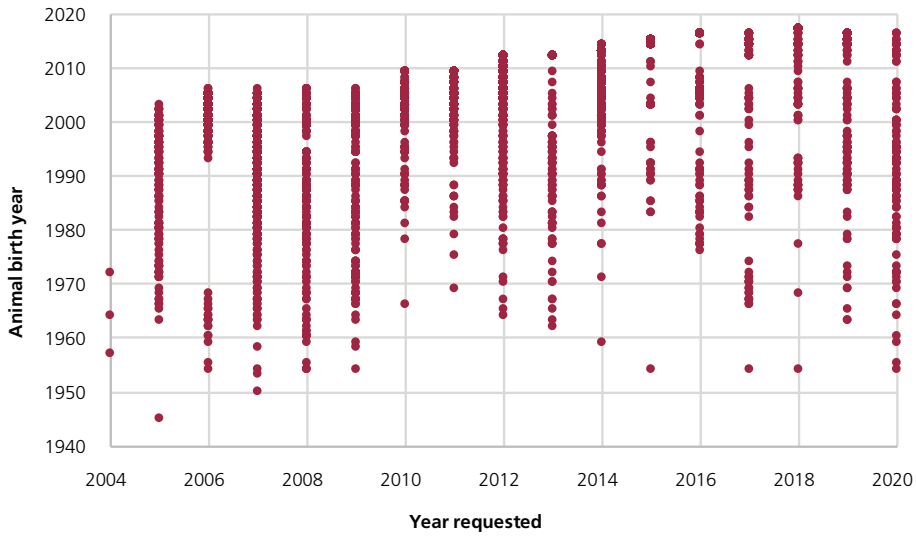
[2] **Dechow, C.D., Liu, W.S., Specht L.W. & Blackburn, H.** 2020. Reconstitution and modernization of lost Holstein male lineages using samples from a gene bank. *Journal of Dairy Science,* 103(5): 4510–4516. https://doi.org/10.3168/jds.2019-17753

[3] **Leroy, G., Danchin-Burge, C. &Verrier, E.** 2011. Impact of the use of cryobank samples in a selected cattle breed: A simulation study. *Genetic Selection Evolution,* 43(36). https://doi.org/10.1186/1297-9686-43-36

1.4.4 Rationalization

Gene banks should regularly evaluate and rationalize their scope, policies, implementation strategies and protocols to optimize and further to develop the gene banking strategy. By doing so, countries will contribute to Strategic Priority 12 of the *Global Plan of Action* (FAO, 2007) 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."

FIGURE 1.2
Samples released from the US gene bank between 2000 and 2020 among species and germplasm types



Source: Authors' own elaboration.

BOX 1.3

Genomic use – Experience from the United States of America and the Netherlands

In the United States of America, a major breed association 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 USD 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 USD 4 billion per year to the US dairy industry.[1]

In the Netherlands, the Holstein and Het Maas-Rijn-IJsselvee (MRIJ) gene banks and DNA collections have shown to be very valuable in the initial phase of implementation of genomic selection by Dutch cattle breeding industry. Moreover, 50 000 single nucleotide polymorphism (SNP) data of all gene bank bulls of native Dutch cattle breeds were 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.[2]

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[1] **Rexroad, C., Vallet, J., Matukumalli, L.K., Reecy, J., Bickhart, D., Blackburn, H., Boggess, *et al.*** 2019. Genome to phenome: Improving animal health, production, and well-being – A new USDA blueprint for animal genome research 2018–2027. *Frontiers in Genetics,* 10: 327. https://doi.org/10.3389/fgene.2019.00327

[2] **Hulsegge, I., Calus, M., Hoving-Bolink, R., Lopes M., Megens, H.J. & Oldenbroek K.** 2019a. Impact of merging commercial breeding lines on the genetic diversity of Landrace pigs. *Genetic Selection Evolution* 51(60). https://doi.org/10.1186/s12711-019-0502-6

For example, the notion of "sufficient" material in SDG Indicator 2.5.1b is motivated by one purpose of 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 banking strategies should be done both *ex ante* and *ex post*, and involve 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 demands 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.

1.4.4.1 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 1 000 animals of local breeds) and US collections (over 6 000 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).

1.4.4.2 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

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.[1] 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 and Landrace) and breeding goals, the finite populations mean that genetic drift will separate those populations over time, leading to unique subpopulations. 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 normal practices for local breeds. Gene banking 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.

[1] **Wood, R. & Orel, V.** 2004. *Genetic Prehistory in selective breeding: A prelude to Mendel.* Oxford University Press.

of its key collections, or should develop partnerships with relevant stakeholders (i.e. breeders and breed associations, research institutes and breeding companies) to obtain such data.

1.4.4.3 Projections toward the future

Quantifying the different attributes of a gene banking strategy is difficult, especially in a long-term perspective. First, stakeholders' strategies are evolving (see Box 1.4). Moreover, future scenarios have a higher or lower uncertainty, while conservation decisions must be made in the short term, also taking the budget constraints into account. Gene banks should anticipate changes in future demand, e.g. motivated by possible changes in climate, production systems, markets, consumer preferences and possible calamities such as diseases and 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 preferences and strategies. Alternative strategies and future scenarios should be compared before the final strategy selection.

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 strengths, weaknesses, opportunities and threats (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 banking strategy goes together with a policy for training its staff and informing its users (those who provide or utilize the material) on the latest methods and techniques.

1.4.5 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* (FAO, 2007), 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.

1.4.5.1 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.

1.4.5.2 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.

1.4.5.3 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.

1.4.5.4 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 policymakers 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.

1.5 STRATEGIC CONSIDERATIONS FOR MULTICOUNTRY GENE BANKING

Most of the world's gene banks for AnGR are national or subnational in their scope (Zomerdijk *et al*., 2020). However, under certain circumstances and for specific objectives, multicountry gene banking may be the preferred option, with increased economic efficiency or complementary technical expertise serving as key drivers in such cases.

Possible examples include the following:

- a group of countries pooling resources (which may include project funds from an outside donor) to establish a single physical gene bank that serves all countries;
- a donor country offering gene banking services to one or more less developed countries as a means of providing technical and/or financial assistance;
- several countries agreeing on a common strategy for cryoconservation of a shared transboundary breed; and
- two or more countries with operational gene banks that agree to use each other's facilities to store backup/duplicate material.

1.5.1 General considerations

For certain aspects, the national cryoconservation strategy of a country storing material in an externally hosted gene bank may not differ substantially from a strategy based on within-country storage. The main difference is simply the physical location of the bank. The species and breeds to be conserved and their characteristics will be mostly invariant, as will many of the stakeholders of interest. Data management will remain a critical aspect of the cryoconservation programme. Periodic monitoring of the *in situ* population and rationalization of the banked collection will continue to be necessary. Institutional commitment is no less important.

However, some additional factors must be taken into consideration:

- Cryoconservation objectives may need to be altered somewhat, depending on ease of access to the collection. Conservation of material in another country may decrease access relative to local storage, making the utilization of material to actively manage an *in situ* population more difficult. Insurance against breed extinction may thus be a more feasible objective.
- Cooperation with another country that includes technical assistance may increase the range of tissue types that can be collected and stored.
- For sanitary concerns (see Section 7), it will likely be necessary to apply the standards of the strictest country to all samples, regardless of the origin. A host country may jeopardize its collection, if the material from a country with less stringent rules is added. On the other hand, if a country stores its samples in the facility of a neighbouring country with lower standards, it may be impossible to repatriate them.

- Long-term institutional commitment remains critical. Establishing a national gene bank requires a substantial initial financial investment, which may help ensure *psychological investment* for the bank's long-term sustainability. Countries that have material hosted elsewhere will not have made this investment, and must understand that continuous institutional support will be required for the foreseeable future. Multicountry gene banking may start as part of a development project (e.g. AU-IBAR, 2020), but gene banking is an ongoing process that will require financial maintenance long after such projects end.
- Because multiple countries are involved, governance issues will be more complex.

1.5.2 Governance

If a multicountry gene bank consists simply of one country storing material from other countries in its bank, governance will be relatively straightforward for providers. Countries providing material will simply require a governance structure that pertains to their respective collections of stored genetic resources, which would be similar to that for a national gene bank. The host country would merely act as a service provider, and would need to agree on a cost recovery framework with the provider country or countries. (Economic aspects of gene banking are addressed in Section 4). A quality management system (see Section 2), although recommended for all gene banks, may be especially important in the multicountry context. When countries agree to host each other's backup collections, they may simply agree to undertake this on a cost-free basis, especially if the amounts of material stored by each country are similar.

Governance will be more complex, if a multicountry gene bank is geographically located in a single country, but owned and managed by a multicountry body such as a regional economic community. In this case, each country may continue to independently manage their respective collections of genetic material, but an overarching governing body with representatives from each country will likely be required to oversee the operation of the gene bank.

The cooperative cryoconservation of transboundary breeds and/or cross-country utilization of the stored material will add yet another level of intricacy. For transboundary breeds, a joint cryoconservation programme implies the necessity to establish a multicountry advisory group or similar body for management of the shared genetic resources, both *in situ* and *ex situ*. This body would need to establish a common breeding goal for the breed, as well as to manage both the development and utilization of the collection. If the gene bank is used as a mechanism to facilitate cross-country utilization of material from native breeds from participating countries, legal issues pertaining to international access and benefit sharing would need to be considered as well (see Section 9).

The potential benefits of multicountry gene banking should be given strong consideration by countries that lack national gene banks and/or countries that could substantially increase their cryoconsevatoin efficiency through international cooperation (such as through sharing backup collections). However, the added complexity of governance cannot be ignored. Through a project funded by the European Union, the African Union – Interafrican Bureau of Animal Resources (AU-IBAR, 2020) has been able to establish five subregional gene banks in strategic locations throughout the continent. However, various governance issues remain to be worked out before the banks can be made operational.

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SECTION 2

Quality management for improved organization and implementation

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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 (Kelderman, 2021):

1. **Quality policy and objectives.** The gene bank should write a quality policy that defines what *quality* means to the gene bank and establish 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), and refer to quality control procedures and policies being followed.

3. **Organizational structure and responsibilities.** An organigramme should be drafted, that illustrates the organizational structure including personnel and governing bodies, and explains 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

Countri22 and gene bank managers around the world have recognized the benefits offered by Development of an international standard for biobankseither 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).

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 2.1

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[1] 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 the requirements for QMS.

[1] **International Organization for Standardization (ISO).** 2018. *ISO 20387: 2018 Biotechnology – Biobanking – General requirements for biobanking*. Geneva. Cited 20 January 2021. www.iso.org/standard/67888.html

BOX 2.2

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

The European Commission[1] 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 European Union and cooperating research infrastructures and the strategy for their utilization, and has encouraged European Union 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).

[1] **European Commission.** 2020. *European Research Infrastructures.* Brussels. Cited 20 December 2020. ec.europa.eu/info/research-and-innovation/strategy/european-research-infrastructures_en

2.2 QUALITY MANAGEMENT FOR GENE BANKING

2.2.1 Defining the quality policy

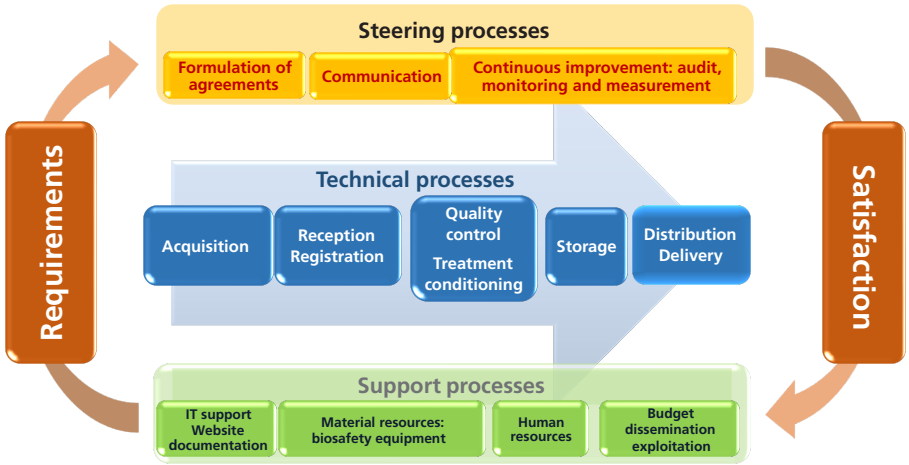
As a foundation for its QMS, a gene bank shall compile in a single document its main objectives, commitments and plans to reach its objectives, addressing issues described in Section 1. This quality policy document should be brief, formally institutionalized and made freely available to the public. Commitment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

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* (FAO, 2007) and the national strategy and action plan, sanitary regulations, animal breeding regulations, access and benefit sharing, intellectual property, at the national and international level.

FIGURE 2.1
Mapping the processes of a gene bank quality management system (QMS)



Source: Authors' own elaboration.

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, 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, and/or documentaries produced by government). This activity must not be overlooked since the support by the general public is important for policymakers.

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 programme; 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	backup 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	backup or duplicate collections; selection of storage sites
customer relations	dissatisfaction of users or stakeholders	establish a multiactor board; satisfaction scores and complaints; corrective measures in case of complaints

Source: Authors' own elaboration.

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 strengths, weaknesses, opportunities and threats (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 (ISO, 2018), 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 European Union project Innovative Management of Animal Genetic Resources (IMAGE, 2020) to help gene banks in the development of their QMS (see Annex 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 banking 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 and operational safety (see Box 2.3);
- ensuring implementation and overseeing day-to-day operation of the gene bank, including incoming and outgoing 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;

BOX 2.3

Maintaining operational safety in gene banks

As noted in Table 2.1, factors that may compromise hygiene and safety are among the important sources of risk to staff and to regular gene bank operations. A QMS must consider specific sources of risk and measures to control them. The two most important hazards to staff involve their interaction with donor animals and liquid nitrogen.

Interaction with animals (and their tissues) is associated with two potential dangers: (i) injury during animal handling; and (ii) transmission of zoonotic disease. For both cases, the most effective measure is to ensure that all staff are sufficiently trained and/or experienced. For animal handling, additional prevention measures include appropriately designed and maintained animal housing and material collection facilities. Actions to improve staff safety will also tend to maximize animal welfare. With regard to zoonotic disease transmission, a first step is to ensure that donor animals are healthy and that their source herds are free from disease (by veterinary inspection and/or testing) before bringing them to the collection facility or performing collection in the field. Upon arrival at the collection facility, animals may be additionally subject to quarantine. Then, high sanitary standards must be applied for collection, and all persons handling the collected material must be properly clothed with proper personal protective equipment. All safety measures associated with handling of animals and their biological materials must be recorded in the QMS documentation. In addition, whenever facilities and equipment are inspected, the results should be recorded for the QMS.

Liquid nitrogen vaporizes at -196 °C and therefore poses two major safety risks: (i) freezing or "burning" of skin upon contact; and (ii) hypoxia (lack of oxygen) and respiratory distress. Prevention of contact injury is achieved by using protective clothing, including specifically designed gloves, lab coats, closed shoes and safety goggles. Both the liquid nitrogen itself and the stored materials and associate storage vessels must only be handled with equipment that is designed to resist extreme cold. The risk of hypoxia can usually be avoided by ensuring that the material storage rooms are well-ventilated, and outfitted with an oxygen monitoring system with alarms that are activated when the oxygen level falls below a certain threshold (e.g. 19.5 percent). As with animal handling, all safety measures associated with liquid nitrogen must be fully described in the QMS documentation, including maintenance and inspection routines and their results.

- 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.

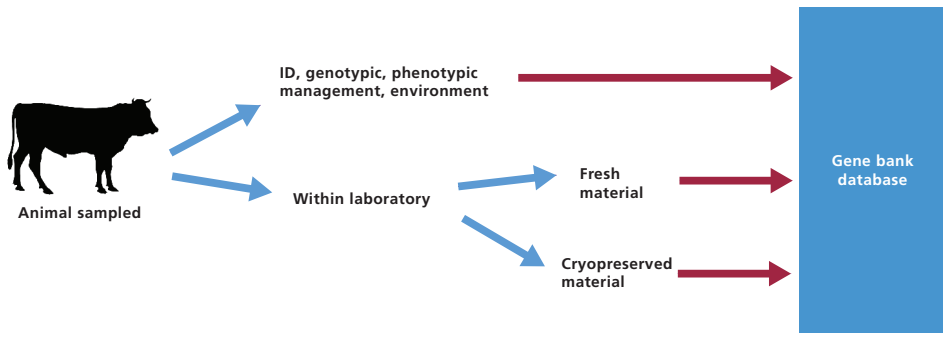
2.3.4 Genetic material database

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.
- Spreadsheets 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 the geographic information system (GIS) coordinates for where the animal was born, and management system (extensive, mixed crop-livestock, or intensive) the animal was produced.

FIGURE 2.2
Data stream of information captured by a gene bank



Source: Authors' own elaboration.

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).

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 Section 3).

Gene banks often acquire cryopreserved material that has already been fully processed by a third party. The gene 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 live 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," 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, with regard to 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.

The gene bank's primary concern is to ensure viability of the stored material, i.e. 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.

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 must be 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 may be 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 if their expectations are fulfilled.

Each process should regularly be reviewed to check indicators, confirm or revise objectives, update the SWOT, and 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 if 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, and 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

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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 resistant to harsh environmental conditions, and that are appropriate for the type of germplasm collected;
- 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;
- 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, introduction of genes to enable adaptation to climate change or production demands, research, evaluation of animal health evaluation – see Section 1);
- 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 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. In some instances, repopulation of a line or breed based solely on semen will require initial mating with females of a different population, 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 routine breeding and reproductive programmes in almost all major 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),

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 a male, into the vagina, cervix or uterus of a female.
- assisted reproductive technologies (ART) – the collection of methods for artificially improving the reproductive performance of animals, which may include AI, *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 an artificial environment (literally "in glass") 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 co-culture of 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 immature oocytes are placed in culture to achieve a specific developmental state which allows them to be fertilized.
- *in vitro* production (IVP) – the process of creating embryos via IVF.
- 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 follicles from ovaries with an ultrasound guided device for 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.

intracytoplasmic sperm injection (ICSI), or for DNA extraction. Therefore, this subsection will provide an overview of the current state of: (i) the advantages and limits of semen collection, cryopreservation and utilization for the major agricultural species; (ii) address some improvements to aspects of these processes; and (iii) discuss how these developments increase the value of this form of germplasm.

Semen collection is a simple procedure for the major livestock species. When performed correctly, for many species, it is minimally invasive (for example, by using an artificial vagina in cattle and horses) and causes no apparent physical discomfort, thus usually resulting in the collection of high-quality samples with significant quantities of sperm. Males of other 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 used. 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, 2018, 2019) 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 *et al*., 2017), roosters (Villaverde-Morcillo *et al*., 2016), rams (Bergstein-Galan *et al*., 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 and 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 and the integrity of the plasma membrane and acrosome. In addition, the semen quality can be assigned by kinetics or cytometric, 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. Flexibility with these thresholds may be considered if the animal is from a particularly valuable population or if the acquisition of additional samples will be difficult. The threshold may also differ depending on the potential uses for the semen sample (for example, with 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 to inhibit cellular damage during freezing and thawing). The samples are then stored in liquid nitrogen (see Section 6). The goal is for the sperm to 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 gene banking. 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.*, 2020a, 2020b). Recent reports have documented the financial savings of storing samples in this format while obtaining acceptable cleavage and blastocyst development rates, thus 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, because they are gametes like semen, 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 re-animated 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.

3.3.1 Oocyte cryopreservation techniques

Slow freezing or vitrification are both options for cryopreservation, depending on the species and developmental state as described in Section 6, i.e. 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 properties 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 subsequent 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 the costs of the media (reagents, serum, hormones) may be considerable. Moreover, collection of these types of germplasm may also be challenging and expensive if superovulation or embryo flushing is utilized. When the success rates for producing live animals from these germplasm sources are low, then the cost of these activities may not be justified.

Equine oocytes can be harvested from live mares using ultrasound-guided ovum pick-up (OPU). Mares do not respond to superovulation like other mammals, 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 after embryo transfer (ET) (Maclellan, *et al.,* 2010). However, collection of *in vivo* matured oocytes and the current efficiency of the vitrification technique is still 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 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, Kikuchi and Nagai, 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 developmental competence than those vitrified at the mature stage (Egerszegi *et al*., 2013). Subsequently, use of vitrified immature oocytes for *in vitro* embryo production results has allowed the production of live piglets using surgical ET (Somfai *et al.*, 2014; 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.

3.3.2 Embryo cryopreservation techniques

Slow freezing methods have been successfully used for ruminant embryos in breeding and conservation schemes for decades. Acceptable pregnancy rates have been obtained with intact *in vivo* produced (IVP) embryos. However, pregnancy rates have not been satisfactory after slow freezing of OPU derived IVP embryos, especially if biopsied for genomic selection or some other purpose. Embryo vitrification has had more promising results, especially for IVP and biopsied embryos, but additional research is still needed 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 (labour 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 144–156 h 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 problematic. Reduction of the blastocoel cavity fluid by aspiration via micromanipulation, followed by vitrification, has provided a breakthrough in this field, and pregnancy rates are 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 IVC. The advantage of IVP is that the embryos can be selected for cryopreservation upon blastocyst formation when they are still small and have no glycoprotein capsule. Consequently, cryopreservation of equine IVP blastocysts via either vitrification (Choi and Hinrichs, 2017) or slow freezing (Lazzari *et al.,* 2020) is very successful, with pregnancy rates similar to the rates obtained by fresh transfer. 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 when they have reached the blastocyst stage, which occurs between day 6 and day 10 following fertilization, adding 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*,* Claes and Stout, 2019). Comparison of the current techniques shows that 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 and Silversides (2006, 2007a, 2007b) 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 and Silversides (2007a) also demonstrated that testes of a young chick could be vitrified and transferred, and at the time of puberty, the host would produce fertile sperm. More recently, Liptói *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 × 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 (Liptói *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 anaesthesia, 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 and 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, personal communication, 2020), and agricultural species (Devi and Goel, 2016). However, the success is highly variable and influenced by the type of tissue (e.g. whole, hemi-, pieces or 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 or ICSI) and the interaction of these effects (Devi and 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, STEM AND SOMATIC CELLS

3.5.1 Preservation of diploid cells for cloning or *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 (e.g. cattle, sheep, horses, pigs and goats) by replacing the oocyte nucleus with a somatic cell nucleus (Wilmut *et al.*, 1997; Cibelli *et al.*, 1998; Baguisi *et al.*, 1999; Polejaeva *et al*., 2000; Galli *et al*., 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 ET.

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-Hamilton) embryos (which amounts to approximately 2.5 days of embryo incubation), and they can then be successfully cryopreserved or vitrified (Nakamura *et al.*, 2011) or cultured prior to transplantation to increase the quantity of viable cells for more than 50 percent success rate (Whyte *et al*., 2015; Nandi *et al.*, 2016; Wang *et al.*, 2017; 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, a *DDX4* knockout chicken has been 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) 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 could be cultured and transplanted into sterile hosts, and those hosts (goat, mouse and pig) would 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 have been demonstrated in other animals (e.g. quail, trout, salmon and zebrafish), the novelty to the research reported by Ciccarelli *et al.* (2020) is that it was based on sterility induced by using CRISPR/Cas9 editing of the NANOS2 gene (knockout), rather than through chemical, environmental or radiological means. 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 RECOMMENDATIONS

3.6.1 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 endeavour 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.

3.6.2 Oocytes and embryos

The costs associated with preservation of oocytes and embryos (labour, laboratory facilities and 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.

3.6.3 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.

3.6.4 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. The methods to utilize these types of germplasm 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.

3.6.5 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.

3.7 SUMMARY

Successful collection, preservation and utilization of germplasm depends on numerous factors. However, even though variable results should be expected when utilizing any assisted reproductive technology, a secure and 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 3.1 to aid with decision making.

TABLE 3.1
A comparison of germplasm types according to various factors influencing their utility

Type of germplasm	Ease of acquisition	Cryopreservation expertise	Collection costs[a]	Utilization expertise	Utilization costs	Proportion of desired breed in 1st generation offspring (percent)
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]	50–1100

Note: 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. The information in this table generally refers to conventional collection from live animals. Collection of epidymal sperm and immature oocytes from animals after slaughter is a fairly simple and inexpensive procedure, but may not be routinely applicable in gene banking for conservation, which usually involves healthy animals of high genetic and/or financial value.

[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 (< USD 0.50/straw), if a sample is collected and then sent to a gene bank for cryopreservation (~ USD 1.00/straw), or if the gene bank travels and performs the collection on-site and cryopreserves the samples (~ USD 15.00/straw). These factors can be applied to any type of germplasm, 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.

Source: Authors' own elaboration.

3.8 STRATEGIC CHOICES OF STORED MATERIAL TYPE TO FIT NATIONAL NEEDS

The wide range in the types of tissues and cells available for cryoconservation offers 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, Canada, China, France, Thailand and the United States of America describe their choice of stored materials in the context of their most important livestock species and national goals for management of AnGR.

3.9 EXAMPLES OF THE CHOICE OF GERMPLASM BY COUNTRY

The Brazilian Animal Gene Bank

Samuel Rezende Paiva

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*[1] (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*.[2]

The Canadian Animal Pedigree Act and cryoconservation activities

Carl Lessard

Production of Canadian livestock used for food relies on the producers and industries. To assist them with breed improvement, Canada's government[3] 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) – is available for livestock associations or industries with mission to preserve germplasm or gonadal tissues from Canadian purebred animals.[4] Typically, frozen semen or embryos are sent to AnGRC's facility at no cost to the

1 **Embrapa (Brazilian Agricultural Research Corporation).** 2021. Alelo Animal/Animal GRIN. Brasilia. Cited 8 February 2021. aleloanimal.cenargen.embrapa.br/

2 **FAO.** 2007. *Global Plan of Action for Animal Genetic Resources and the Interlaken Declaration.* Rome. www.fao.org/3/a1404e/a1404e.pdf

3 **Canada.** 1988. Animal Pedigree Act. Ottawa. Cited 8 February 2021. https://laws.justice.gc.ca/eng/acts/A-11.2/page-1.html

4 **Canada.** 2021. Animal Genetic Resources of Canada. Ottawa. Cited 8 February 2021. https://agriculture.canada.ca/en/agricultural-science-and-innovation/agriculture-and-agri-food-research-centres-and-collections/animal-genetic-resources-canada

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.

The Chinese animal germplasm collection

Xueming Zhao

The Chinese animal germplasm collection[5] (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, 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.

[5] **China.** 2021. Chinese animal germplasm collection. Beijing. Cited 10 February 2021. db.cngb.org/

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, and 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.

The French National Cryobank of Domestic Animals

Elisabeth Blesbois

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.[6] 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, aiding in genetic selection activities, creating new resources, and developing 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.[7] This portal offers the opportunity to view the

[6] **France.** 2007. Code rural et de la pêche maritime. France. Cited 12 February 2021. www.legifrance.gouv.fr/codes/article_lc/LEGIARTI000006596187

[7] **CRB-Anim.** 2020. Access to collections. Paris. Cited 10 February 2021. https://crb anim.fr/access-to-collection/#

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 predict the level of inbreeding when a particular sample of germplasm is used.

Ex situ conservation in Thailand

Rangsun Parnpai

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:

- 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.
- Frozen embryos are conserved from dairy and beef cattle, native cattle, buffalo and goats for genetic improvement and maintenance of genetic diversity.
- Tissue and DNA samples are collected for both research and biodiversity needs from all livestock species. DLD also cooperates 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.[8] The permanent DNA storage facility is managed by the National Science and Technology Development Agency.

The National Animal Germplasm Programme of the United States of America

Harvey Blackburn

The National Animal Germplasm Programme (NAGP) is the programme of the government of the United States of America charged with the conservation of animal genetic resources. Since the programme's inception in 1999, the government has 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 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.

[8] **Biotec.** 2021. Biotec culture collection laboratory. Pathum Thani, Thailand. Cited 8 February 2021. www.biotec.or.th/bcc/index.php/service

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.[9]

The National Livestock Cryobank of the Philippines

Lilian P. Villamor

The National Livestock Cryobank (NLC) was established by the Department of Agriculture in 2012 and is located at the Philippine Carabao Center in the Science City of Muñoz. The Korean International Cooperation Agency provided financial support. The NLC supports the existing genetic improvement programmes and underlies the livestock sector's response to future threats posed by climate change.

The NLC's strategy includes (i) the collection and preservation of genetic material, (ii) data banking, (iii) provision of access to stored samples, (iv) and dissemination of information. The NLC aims to preserve the diversity of native breeds, and oversee the introduction of exotic breeds that may be economically beneficial while still exhibiting resilience towards endemic diseases and the local environmental conditions. The collection currently consists of semen and oocytes and emphasizes buffaloes (91 percent), but also includes cattle (8 percent), goats and swine (<1 percent). Whole blood cells and DNA from various species are also preserved for research opportunities. The NLC envisions establishing a national repository of samples from the diverse range of livestock breeds and species, as well as threatened and wild animals in the Philippines.

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[9] **United States Department of Agriculture (USDA).** 2021. National Animal Germplasm Program. Fort Collins. Cited 8 February 2021. agrin.ars.usda.gov/collection_overview_page_dev?language=EN&record_source=US

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SECTION 4

The economics of gene banking

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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 minimize 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 and 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 programme. Specific cost categories may require *long-term investment* and can be considered as *fixed* or *variable* costs (see Table 4.1). As a general principle, maintaining infrastructure and staff can be considered *fixed* costs, while 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 programme, which should target at least twenty years of operation. The physical plant 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 (e.g. 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 programme. 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 (see 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 analyser 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 categories/items classified as fixed costs are annual costs of cryopreservation lab facilities and cryostorage 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 cryocontainers. Thus, the annual amount of liquid nitrogen should not vary substantially over the years, facilitating the estimation of this cost when preparing a multiyear 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 programme 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 evaluation framework for gene bank operations

Cost category / item	Fixed (annual costs, 20-year horizon)	Variable (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/m^2) • receipt of material • preparation and evaluation • processing • packaging • freezing • computer workstation	X	
Equipment • maintenance and repair • processing equipment (microscope, centrifuge, spectrophotometer, counter chamber, haemocytometer, pH meter, osmometer, water bath, straw filling equipment, straw printer, styrofoam box, programmable freezer, quarantine tank)	X	
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 and disposables • reagents • portable equipment		X
Processing material • consumables and disposables • reagents		X
Long-term storage • tanks • liquid nitrogen		X

Source: Authors' own elaboration.

BOX 4.1

Investments of Canada to preserve its animal resources

Carl Lessard

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 programme 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.
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:

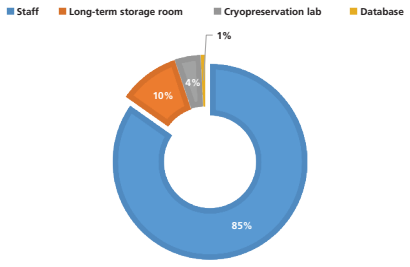
- **Infrastructure:** The animal national gene bank is located in a refurbished room at an existing federal centre in Saskatoon (Saskatchewan, Canada). This room has all the monitoring equipment to detect liquid nitrogen spills or a low level of oxygen. In addition, an industrial dehumidifier ensures 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 programme, but used existing facilities to accommodate the needs of AnGRC.
- **Equipment:** AnGRC acquired computer-assisted sperm analysis (CASA), 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, United States of America 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.

(Cont.)

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

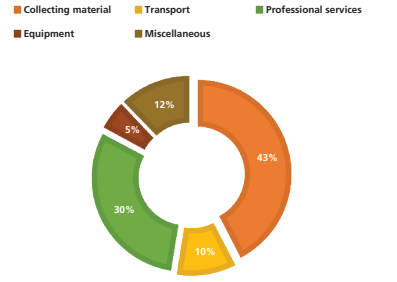
- **Fixed costs:** Staff salaries account for around 85 percent of the fixed costs (see Figure 4.1). A significant amount of liquid nitrogen is required to fill all the cryotanks located in the storage room, which requires 10 percent of the fixed costs budget every year, because the number of cryotanks 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 program updates or upgrade of the informatics system.
- **Variable costs:** Each year, several livestock producers and industries donate frozen germplasm to the AnGRC programme, 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 (see Figure 4.2). Field collections are a major activity for the Canadian preservation programme, 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.

FIGURE 4.1
Distribution of fixed costs (Canada)



Source: Authors' own elaboration.

FIGURE 4.2
Distribution of variable costs (Canada)



Source: Authors' own elaboration.

BOX 4.2

The Dutch farm animal gene bank

Sipke Joost Hiemstra

The Dutch gene bank for farm animals is managed by the Centre for Genetic Resources, the Netherlands (CGN), of Wageningen University and Research. Development and maintenance of the gene bank collections for farm animals is part of the Statutory Research Tasks programme 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 backup gene bank collections of all breeding programs; and
3. To stimulate the use of gene bank collections in breeding and research.

Long-term investment:

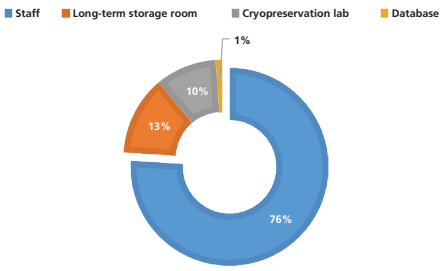
- **Infrastructure:** The national Dutch gene bank for farm animals is located at the Campus of Wageningen University and Research, and a duplicate collection is maintained at the Veterinary Faculty of Utrecht University. The gene bank consists of a cryopreservation laboratory facility and two storage 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 (see Figure 4.3). 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 (see Figure 4.4). 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.

FIGURE 4.3

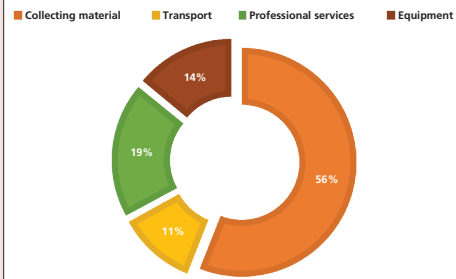
Distribution of fixed costs (Netherlands)



Source: Authors' own elaboration.

FIGURE 4.4

Distribution of variable costs (Netherlands)



Source: Authors' own elaboration.

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 public 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)" (i.e. 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 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.

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 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

BOX 4.3

Types of "values" for cryoconserved genetic material

Current use value – the value derived from immediate exploitation of the 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 when choosing one alternative over another.

Source: Modified from **OECD.** 2018. Cost–Benefit Analysis and the Environment: Further Developments and Policy Use, OECD Publishing. Paris. Cited 18 October 2020. https://doi.org/10.1787/9789264085169-en

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 non-market 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, policymakers 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 Section 4.5) are useful for evaluating public and private investment decisions (Riegg Cellini and 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 guarantee the same outcome. The outcome does not have to be expressed in 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 (e.g. 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 conceptualized 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.

This document 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 maximize 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 maximize 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 bank 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 re-allocation 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.

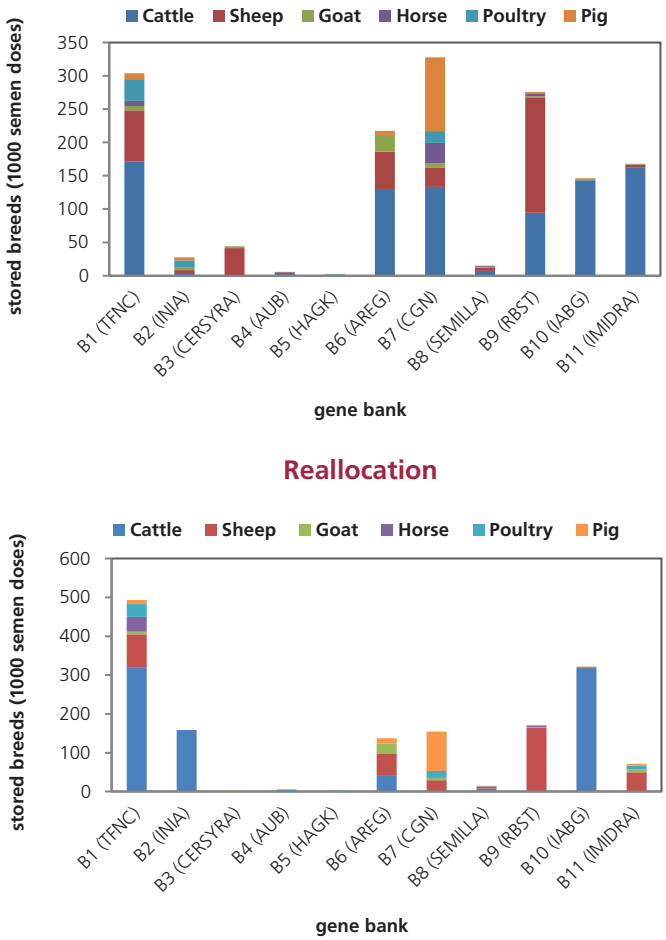
The probability of endangerment can be estimated using census data and regression methods (De Oliveira Silva *et al*., 2021). As resources are limited and *ex situ* conservation is a relatively expensive technology, it may be rational to prioritize breeds that are more likely to be at risk. In this case, 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 4.5 is an example of how information from LP can be used to inform strategic *ex situ* collections for a gene bank network with single objective. 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 illustrated in 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 4.5
Distributions of semen doses per breed before and after cost-effectiveness analysis



Notes: An example of a CEA 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, R., Vosough Ahmadi, B., Hiemstra, S.J. & Moran, D.** 2019. Optimizing ex situ genetic resource collections for European livestock conservation. *Journal of Animal Breeding and Genetics*, 136(1): 63–73. https://doi.org/10.1111/jbg.12368

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 European Union project Innovative Management of Animal Genetic Resources (IMAGE, 2020), the following basic recommendations for performing a full cost analysis of collection enrichment, maintenance and future regeneration steps can be given:

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 OF, 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. **Collection 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 4.5). MO allow for deriving cost-curves of diversity vs expected costs, or extinction risks vs costs, for example.

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 such as 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

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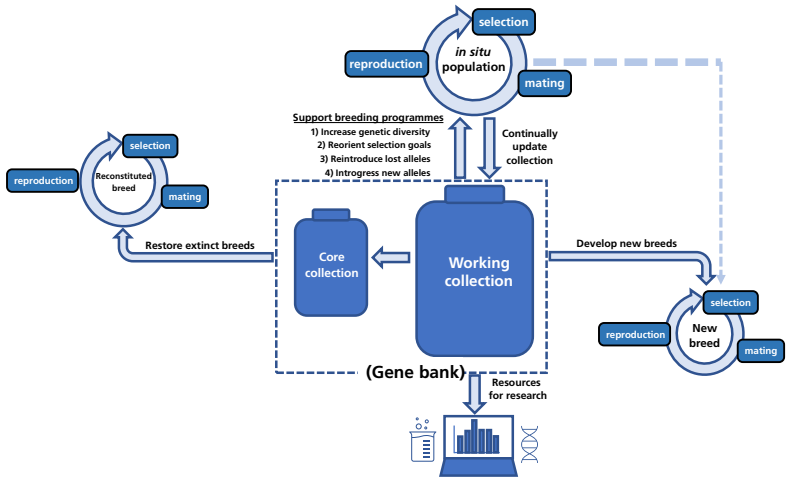
5.1 INTRODUCTION

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 breeding programmes in existing *in situ* populations, to develop new populations, and to support research. In contrast to live populations, a gene bank collection does not "evolve". Once a collection is established, genetic drift and associated loss of alleles does not occur, nor does adaptation to the environment. Consequently, a collection can play different roles in the conservation of a breed (see 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 continual. As a result, alleles can be present in a gene bank that had been lost from the live population, and gene banks thus can help to restore genetic diversity in the live population (Dechow *et al.*, 2020).

FIGURE 5.1
Uses of gene bank collection (*ex situ in vitro* cryocollection)



Source: adapted from Berg P. & Windig J.J. 2017. Management of cryo-collections with genomic tools. In K. Oldenbroek, ed. *Genomic management of animal genetic diversity*, pp. 155–178. Wageningen, Netherlands, Wageningen Academic Publishers; and Tixier-Boichard, M. & Crooijmans, R. 2019. A universal tool to share knowledge about animal gene banks. Press release 5 of IMAGE project. Cited 22 September 2020. www.imageh2020.eu/Publications/PR5.pdf

Figure 5.1 and the previous FAO guidelines for *Cryoconservation of animal genetic resources* (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 supporting breeding programmes. The previous FAO guidelines (FAO, 2012) contain detailed instructions on the amounts of genetic material 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 single sufficiently large collection and to manage it strategically to serve multiple purposes. With this in mind, gene bank managers and stakeholders for animal genetic resources (AnGR) are advised to consider their national *in vitro* collections to be dynamic resources and to routinely identify and develop opportunities for continual exploitation and replenishing of those collections.

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 and Oldenbroek, 2007).

In the first case, conserved genetic material can be used to enlarge the effective population size of the living population. Because populations of breeds at risk of extinction are small by definition, the probability that a long-term viable effective population size of at least 50 animals can be maintained *in situ* is small. Breeds with a low effective population size may consequently 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 potential breeding population consists not only of the individuals from the current population *in vivo*, but also of the individuals with material in the gene bank. The impact of gene bank material on the effective population size can be estimated depending on the proportion of the population whose parents have material stored in the gene bank (Sonesson, Goddard and Meuwissen, 2002). The inbreeding level of offspring from animals in the gene bank sampled k years ago will reduce to the level of inbreeding in the population at that point in time, and the effective generation interval will consequently increase to a maximum of k years.

Gene bank collections play an important role in the long-term conservation of AnGR. The previous guidelines (FAO, 2012) contain recommendations on how to develop gene bank collections. These recommendations start with (i) assessing the status of AnGR populations; and (ii) determining which populations should provide material to be conserved in the gene bank. The latter step considers 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.

BOX 5.1

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

Steffen Weigend

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 second population. The use of genetic markers can accelerate this process. Within the Horizon 2020 European Union project Innovative Management of Animal Genetic Resources,[1] the marker-assisted transfer of a specific trait, the blue eggshell colour, from a donor population, the Araucana breed of chickens, to a commercial 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 of blue shelled eggs.

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

Selection criteria were heterozygosity or homozygosity for blue eggshell colour locus, high similarity to the WL and high genetic diversity. On average, marker-assisted selection increased the proportion of the White Leghorn genome in the BC2 generation by 4.4 percent relative to the expectation (87.5 percent), thus decreasing the donor genome of Araucana accordingly. In 2019, the IC population hatched, of which 188 animals were homozygous carriers for the blue eggshell 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 IC compared to the White Leghorn. Homozygous IC hens and cocks are the basis for a high performing blue egg layer line that is highly similar to the White Leghorn.

[1] **Innovative Management of Animal Genetic Resources Project (IMAGE).** 2020. Project funded by the Horizon 2020 Research and Innovation Programme of the European Union under Grant Agreement Number 677353. France. Cited 26 September 2020. www.imageh2020.eu

Once the decision is made to conserve a population, one must decide which type of genetic material (such as semen or embryos – see Section 3) should be conserved, and 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. The size needed for the collection depends on how intensively the stored material is used. A good practice is that, whenever 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 sufficiently large, but also that it keeps pace with changes in the *in situ* population.

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 also increased, and software has been developed to better predict the impacts 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 *in situ* population. Numerous scientific publications (e.g. Maignel, Boichard and Verrier, 1996; Gutiérrez and Goyache, 2005) are available on indicators calculated for a wide array of breeds and species, with different methods described in the previous 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, assuming that pedigree data are available. A drawback of pedigree analysis is that estimates of inbreeding and other paramters are based on theoretical expectations, and deviation from these expectation (i.e. due to random assortment of chromosomes) can only be determined with genomic data (see Subsection 5.2.2 below).

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 (where kinship, also referred to as "coancestry", defined as the the probability that single alleles drawn from the same locus of each of two individuals are identical by descent from a common ancestor). For instance, one could calculate the kinship of all males with semen in the gene bank versus males in the living 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 materials from the descendants of ancestors that are not yet well-represented in the gene bank.

Pedigrees can also be used to guide collection development by calculating the genetic relationships among animals in a breed's *in situ* population and gene bank donors. Cluster analysis can then be applied to the resulting data (i.e. a genetic relationship matrix) to group closely related animals, by using statistical methods like Ward's Minimum-Variance. Once the two populations are clustered, the gene bank collections and *in situ* populations can be compared to assess the gene bank for completeness and identify potential *in situ*

donors that would contribute the greatest amount of genetic variation to the gene bank collection (Blackburn, 2009).

5.2.2 Molecular analysis

Molecular analysis generates more accurate estimation than pedigree analysis, and is particularly recommended for any breed with unavailable and/or unreliable pedigree data. The disadvantage is the frequent scarcity of available DNA samples from animals in the living population and almost complete absence of samples from previously living individuals. 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 utilized 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 *in situ* breeding males or the main breeding males at the time of the study (see Eynard *et al.*, 2015 for an overview of different methods). In addition to the effective population size, changes in allele frequencies and inbreeding over time are also parameters that may be used for managing collection. 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 briefly describes how molecular data is currently being used for management of the collection in the French gene bank.

BOX 5.2

Utility of genomic analysis for an existing collection

The French National Cryobank was set up in 1999 and currently (2022) preserves reproductive biological material from 241 breeds of 12 different species of livestock (in addition to material from nine aquaculture 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 have been 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.

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. Box 5.3 includes a glossary of commonly used methods in genomic analysis. Prior to this, molecular analyses were based on single loci or small sets

BOX 5.3

A glossary of the most commonly used methods in genomics

Whole genome shotgun sequencing: Whole genome shotgun sequencing (WGS) or 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: This method aims at sequencing of an individual's genome in order to detect differences between the individual and the 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 (i.e. 2022) used in genomic selection belong to the middle category (around 50 000). The typing of an animal costs about USD 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.

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 can 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 are the estimation of the genetic value of an individual by using thousands of typed markers and the subsequent use of these estimated breeding values in efficient selection programmes, better known as "genomic breeding value estimation" and "genomic selection" (Meuwissen, Hayes and Goddard, 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 genetic relationships derived from the marker data (VanRaden, 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 – Practical guide.* (FAO, 2023) provides more detailed information about the utilization of genomic data in the assessment of the diversity of animal genetic resources. Box 5.4 provides two examples of the use of pedigrees and/or genetic markers in evaluation of gene bank collections.

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 under-representation of globally rare variants on SNP arrays compared to whole genome resequencing data, a phenomenon known as SNP "ascertainment bias" (Nielsen, 2004; Lachance and Tiskoff, 2013). Some array design schemes further increase this bias by intentional over-representation of common SNPs with regard to the natural background distribution. To a degree, this bias can be addressed by constructing subpanels composed of minor alleles (Blackburn *et al.*, 2014). A direct implication for monitoring genetic diversity is that this bias towards common alleles increases heterozygosity estimates, and populations therefore show an upwardly biased amount of genomic variation, particularly for the populations from which the SNP were ascertained.

Within this design process, such target breeds are screened for variant sites that are especially informative in the target breeds. Such sites may not necessarily be as informative in other, non-target breeds, 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 reality, which is not the case with whole genome sequencing (WGS) data (Malomane *et al.*, 2018).

This population-specific ascertainment bias also 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. Whole genome sequencing offers a mechanism to alleviate biases that are introduced with smaller SNP panels (Eynard *et al.*, 2015).

BOX 5.4

Complementary use of pedigrees and molecular information to evaluate the genetic variability in Holstein cattle gene banks

A. Analysis of Holstein gene banks

France, the Netherlands, and the United States of America 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.[1] 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 US, French and Dutch collections seems to be genetically similar, the US 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 genome-wide inbreeding and kinship.[2, 3] 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 in the estimated trend 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, corresponded closely with the adoption of genomic selection. The observed trends in genetic diversity reflect major changes in the Dutch-Flemish HF breeding programmes over the past 30-plus years.

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[1] **Danchin-Burge, C., Hiemstra, S.J. & Blackburn, H.** 2011. *Ex situ* conservation of Holstein-Friesian cattle: Comparing the Dutch, French, and US germplasm collections. *Journal of Dairy Science*, 94(8): 4100–4108. https://doi.org/10.3168/jds.2010-3957

[2] **Doekes, H.P., Veerkamp, R.F., Bijma, P., Hiemstra, S.J. & Windig J.J.** 2018a. Value of the Dutch Holstein Friesian germplasm collection to increase genetic variability and improve genetic merit. *Journal of Dairy Science*, 101(11): 10022–10033. https://doi.org/10.3168/jds.2018-15217

[3] **Doekes, H.P., Veerkamp, R.F., Bijma, P., Hiemstra, S.J. & Windig, JJ.** 2018b. Trends in genome-wide and region-specifc genetic diversity in the Dutch-Flemish Holstein–Friesian breeding program from 1986 to 2015. *Genetics Selection Evolution*, 50(15). https://doi.org/10.1186/s12711-018-0385-y

Although SNP technology is a widespread standard approach and has generated extensive knowledge, it also has disadvantages relative to WGS besides the ascertainment bias. For example, the detectable 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, many breeding programmes in most major livestock species in industrialized countries 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 and may present an opportunity to use genotypic data that has already been generated.

To facilitate genotyping of animals in gene banks, a multispecies SNP array has been developed (see Annex 5.1). By using this array (or any comparable assay) donor animals can be selected on the basis of the real diversity they carry instead of just expectations made from pedigree evaluations. For example, full sibs are expected to have 50 percent of additive genetic variation in common; in reality this will vary around 50 percent. When selecting donors from among a group of full sibs, results from an SNP array can be used to determine the optimal animals to sample to capture the maximum amount of genetic variation. Also, a library of variation in the gene bank will be known, and can be of immense importance in identifying the genetic originality of individuals when samples from the gene bank are subsequently used for breeding in the *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 5.6 about software programs.

It must be noted, however, that in addition to financial aspects, genomic analysis has costs in terms of time, and requires personnel that are capable of performing the required analyses.

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 these data in breeding and conservation decisions. 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 examples of software for management of animal genetic diversity. The first one is the Modular Breeding Program Simulator (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 selection programmes 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 or some combination of traits. 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 thus be minimized, as much as 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 programmes, and thus assist breeders in evaluating and optimizing their breeding programmes (Pook, Schlather and Simianer, 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 today's complex breeding programmes, which may include cohorts of animals with material stored in gene banks. MoBPS includes a variety of pre-implemented functions for common breeding practices such as optimum genetic contribution selection and estimation of genomic breeding values. Although breeds subject to conservation are often small, 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.*, 2020) that includes most of the functionality of the *R*-package.

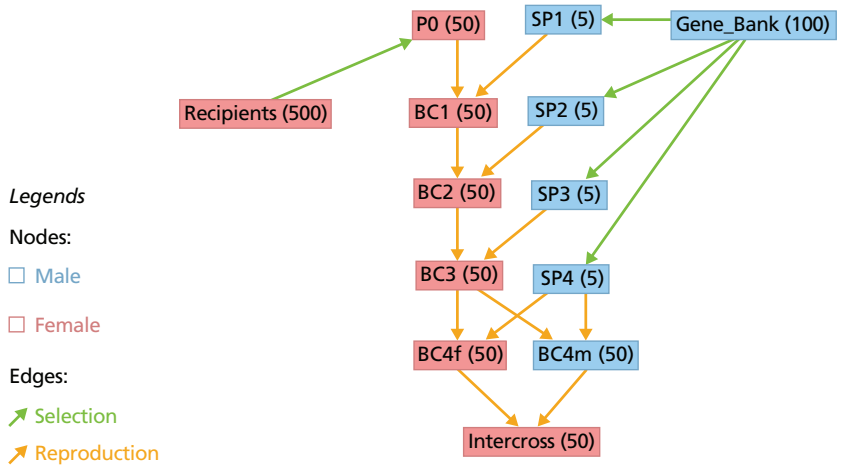
One example for the use of MoBPS is the simulation of a backcross breeding programme to reconstitute an extinct breed from gene bank material, as was described in the previous guidelines (FAO, 2012). In such a breeding programme, only semen has been stored in the gene bank. Females from another breed must thus be inseminated with cryoconserved semen of the breed to be recreated. 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 (see 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 and 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.

Such an exercise may be particularly helpful for the development and utilization of collections consisting of older material and/or material from local breeds, for which knowledge about pregnancy and survival rates with cryopreserved material is typically limited. Although more research is required on such populations to both improve pregnancy and survival rates and to obtain accurate estimates of their values, in the meantime using simulation to perform sensitivity analyses can help a gene bank manager optimize collection procedures to obtain satisfactory rates of breed reconstruction success at a reasonable cost.

Simulations can be executed directly from the web-interface, as the corresponding R-package, which acts as 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 (see Figure 5.3).

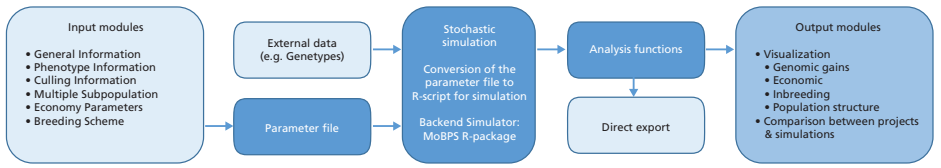
FIGURE 5.2
Set up of the MoBPS program to simulate reconstitution of a breed by backcrossing with semen from a gene bank



Note: 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 is shown.

Source: Author's own elaboration (Torsten Pook).

FIGURE 5.3
Setup of the MoBPS simulation framework



Source: Author's own elaboration (Torsten Pook).

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 throughout, 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 maximized, 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 (see Box 5.5). 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 ROH. Different programs are available for optimum contribution selection, and include the free *R*-package optiSel (Wellmann, 2019), the free software EVA (Berg, Nielsen and Sørensen, 2006), the free software Gencont (Meuwissen, 2002) and the commercial software TGRM (Kinghorn, 2011).

Constraints due 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 eventually leads to the genetic extinction of the local breed because the native alleles are lost. To prevent this, breeders (or other stakeholders) may wish to remove the introgressed "foreign" 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.

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 *in situ* population, as well as the usability and usefulness of the samples in the local gene bank.[1] 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 optimum contribution method was used to develop a hypothetical breeding programme to maximize genetic gain for growth traits while constraining inbreeding in the Blanco Orejinegro breed, and while including the use of semen from the gene bank. Around 50 females and 50 males are registered in the Blanco Orejinegro herdbook 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 optimum contribution selection 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. In the simulated population, both the genetic gain and variability were improved by applying optimum contribution selection and using semen from the gene bank.

[1] **Innovative Management of Animal Genetic Resources Project (IMAGE).** 2020. Project funded by the Horizon 2020 Research and Innovation Programme of the European Union under Grant Agreement Number 677353. France. Cited 26 September 2020. www.imageh2020.eu

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.

In many instances, local breeds that have been subject to crossbreeding will not have material from previous generations stored in a gene bank. Nevertheless, cryoconservation can still play an important role. In such cases, animals within the breed that have remained relatively free from crossbreeding could be targeted for collection for the current gene bank. This process would facilitate the use of the material by today's breeders for matings in the *in situ* population (now and in the future) to accelerate a breeding programme designed to purge foreign haploptes.

5.5 GENE BANKS AS A RESOURCE FOR RESEARCH

In addition to being used to directly support the maintenance and genetic improvement of animal populations, gene banks can be a vehicle to support research. Clearly, studies can be undertaken in gene banks to develop, test and adapt methods for collection, cryopreservation and utilization of germplasm. In addition, gene bank collections can serve as a source of material for genetic and genomic studies of population genetic variation and its 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 IMAGE project, (Passemard *et al.*, 2018), the average onset of sampling for the collections in European cattle gene bank collections was 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).

Some gene bank collections are highly interesting since they are a unique representation of breed evolution over time. Box 5.6 summarizes the results of a survey in which European gene banks reported on the inventory of their collections with respect to the

BOX 5.6

European gene banks as gene archives

A survey was conducted during the Horizon 2020 European Union project Innovative Management of Animal Genetic Resources (IMAGE) study about gene bank collections.[1] 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 was 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 particularly affects rare breeds 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 longer generation interval, the genetic diversity in cattle collections will not change as rapidly as collections for pigs, which have a short generation interval. On the other hand, the selection pressure in dairy cattle is stronger than in horses or sheep, but this may be compensated by a large census size such as in Holstein cattle. Therefore, the only way to accurately determine if a gene bank collection differs radically from a live population is to assess its genetic variability, by using either pedigrees, genotypes, or a combination of the two.

[1] **Innovative Management of Animal Genetic Resources Project (IMAGE).** 2020. Project funded by the Horizon 2020 Research and Innovation Programme of the European Union under Grant Agreement Number 677353. France. Cited 26 September 2020. www.imageh2020.eu

BOX 5.7

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

The aim of this case study was to assess the extent to which 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 1970s to inseminate cows during the years 2004 to 2007 in a regional transboundary dairy cattle breed, the Abondance, was examined.[1] Molecular data (50 000 SNP and high-density 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 a favourable impact for the genetic variability of the breed, as well as some desireable genetic change for specific traits (mostly functional traits) which counterbalanced from an economic perspective the loss of genetic gain in production traits. However, one should bear in mind that this favourable result was obtained in a breed with annual genetic gains that are relatively small in comparison with large international breeds.

[1] **Innovative Management of Animal Genetic Resources Project (IMAGE).** 2020. Project funded by the Horizon 2020 Research and Innovation Programme of the European Union under Grant Agreement Number 677353. France. Cited 26 September 2020. www.imageh2020.eu

species and breeds represented, and the history of collection activities. Box 5.7 reports on a study that examined retrospectively how the past use of semen from a gene bank bull subsequently affected the genetic variation of the *in situ* population. Box 5.8 refers to two studies (from the United States of America and Spain, respectively) where material in gene banks were used as raw material to compare historical changes in breeds, including change that occurred through genetic and geographic isolation and via natural and/or artificial selection. The studies demonstrated that gene bank collections often differ substantially from the live population. Similar results have been previously reported by Danchin-Burge, Hiemstra and Blackburn (2011).

BOX 5.8

Using gene banks samples 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 of Jersey cattle (from Jersey island) and non-island Jersey cattle was analyzed.[1] Samples from Jerseys raised in the United States of America (*n* = 49) and on Jersey (*n* = 34) were obtained from the gene bank of the United States of America. The cross-section of bulls were born from the 1960s to 2000s, and were lowly related to one another. The study revealed that the two Jersey populations had similar degrees of inbreeding, despite their vastly different census sizes. Signatures of past selection were revealed and demonstrated that the two different Jersey populations differed in terms of the genomic locations of key ROH regions. These data provided the first insights into the divergence of two subpopulations of the Jersey breed over decades of isolation between its place of origin and the United States of America.

Cattle samples by breed per birth decade

	Decade of Birth							
Population	**1950**	**1960**	**1970**	**1980**	**1990**	**2000**	**Unknown**	**Total**
Jersey_island		2	8	10	21	8	0	49
Jersey_US	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 across 35 years, from 1980 to 2015.[2]

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 and milk production, immunity and olfaction. The study showed that time series material stored in gene banks served as rich information source in research on breed history and biology.

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[1] **Huson, H.J., Sonstegard, T.S., Godfrey, J., Hambrook, D., Wolfe, C., Wiggans, G, Blackburn, H. & VanTassell C.P.** 2020. A Genetic investigation of Island Jersey Cattle, the foundation of the Jersey breed: Comparing population structure and selection to Guernsey, Holstein, and United States Jersey cattle. Frontiers in Genetics, 11:366. https://doi.org/10.3389/fgene.2020.00366

[2] **Innovative Management of Animal Genetic Resources Project (IMAGE).** 2020. Project funded by the Horizon 2020 Research and Innovation Programme of the European Union under Grant Agreement Number 677353. France. Cited 26 September 2020. www.imageh2020.eu

BOX 5.9

Resampling populations to keep the collection current

Harvey Blackburn

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.[1] This issue can be addressed by executing periodic sampling over time and collecting material from a wide range of animals, including genetically superior donors.[2] 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 of America, but nevertheless maintains an active genetic improvement programme. The gene bank has samples from a time continuum from 1948. Figure 5.4 illustrates how resampling over time has enabled the collection to keep pace with the *in situ* population with respect to the predicted transmitting ability (PTA) for milk yield. The graph also illustrates the diversity collected by comparing individuals from the gene bank to the annualized *in situ* 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, the rate of increase has been moderate, such that the PTA for some bulls collected more 30 years ago have PTA greater than the current breed average. These data suggest that by selecting a semen from a diverse group of bulls, some animals collected years ago remain competitive for use within the current *in situ* population.

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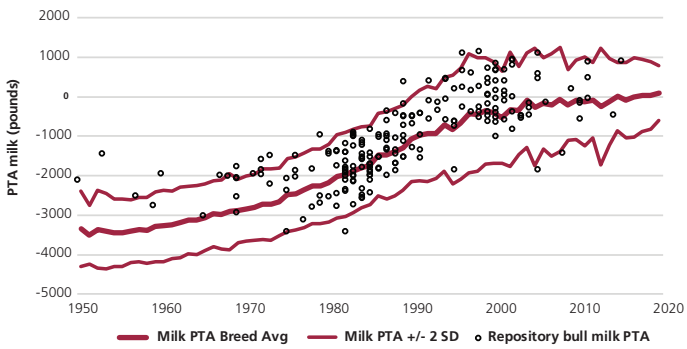
[1] **Leroy, G., Danchin-Burge, C. &Verrier, E.** 2011. Impact of the use of cryobank samples in a selected cattle breed: A simulation study. *Genetic Selection Evolution*, 43(36). https://doi.org/10.1186/1297-9686-43-36

[2] **Blackburn, H.D.** 2018. Biobanking genetic material for agricultural animal species. *Annual Review of Animal Biosciences*, 6: 69–82. https://doi.org/10.1146/annurev-animal-030117-014603

FIGURE 5.4

Distribution of predicted transmitting abilities (PTA) for milk yield of Guernsey bulls stored in the gene bank collection of the United States of America, relative to the distribution (mean and ±2SD) of the general population



Source: Author's own elaboration.

5.6 RECOMMENDATIONS FOR GENE BANK UPDATING

Gene banks are multifunctional. Because cryopreserved material retains its viability for many decades, some gene bank managers (i.e. stakeholder advisory boards) may be tempted to not update the conserved material once a quantity sufficient for reconstitution of an extinct breed has been cryopreserved. However, *in situ* populations are constantly evolving and therefore gene bank managers and breeding organizations should regularly monitor and update their gene bank collections, especially if the collection is being actively used to support breeding in the *in situ* population (see Figure 5.1). As a rule of thumb, the faster the breed develops and changes, the more often the gene bank collection should be evaluated and updated, if necessary. Estimates of how often this should be done have been made, and indicate roughly every 4 to 7 generations (see Box 5.9 and 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 (Leroy *et al.*, 2019). Eventually, establishing breed reconstruction as the exclusive objective can become an obstacle in the utilization of gene bank material, as farmers could then 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 the rate of change in traits subject to selection. For instance, for intensely selected traits such as milk production or stature, Holstein bulls are included in the cryobank if and only if their estimated breeding value is vastly superior to the average (based on the population of bulls in artificial insemination programmes) for the particular trait, on a given year. On the other hand, for traits such as fertility that have a lower genetic progress, the threshold is lowered.

In conclusion, the following recommendations can be used for establishing and maintaining a gene bank:

- Although some prioritization may be necessary, whenever feasible gene bank collections should be established as early as possible for all breeds, regardless of their census size. These collections can then be used to support breeding in the *in situ* population, establish new breeds or subpopulations and to provide a resource for research, in addition to "backing up" populations at risk of extinction.
- Maintenance of an *ex situ* collection will allow breeders and other stakeholders to select the most interesting males to be cryopreserved, while maintaining a sufficient degree of genetic variability in the combined *in situ* and *ex situ* "populations".
- Genomics are a powerful tool for management of gene bank collections. Genomic data provide greater precision of estimates of genetic variation than do pedigrees. Costs of genomic analyses have decreased substantially in recent years, so assessment of all gene bank donors is recommended whenever feasible. Genomic analysis requires particular expertise, however, so specific capacity building is needed (see Section 10).

- Material from individuals in gene bank collections can be used to generate improved offspring which may better meet the recent breeding objectives and farmer expectations. When multiple offspring are available, genomics or evaluation of estimated breeding values can be used to determine the best ones to be susbsequently sampled for the gene bank collection.
- The gene bank collection should be compared to the *in situ* population on a regular basis, and the gene bank collection should be updated with new material based on both genetic variability and genetic merit with respect to the traits in the breeding objectives. Collection activities should continue even if the collection has already met FAO standards for breed reconstitution. Box 5.9 reports on the results achieved by the gene bank of the United States of America through their procedures of continual updating the collection of material for the Guernsey breed of dairy cattle.
- Genetic simulation software such as MoBPS can be used to improve the management of gene bank collections.
- New knowledge and tools for measuring fertility rate in cryoconserved material are needed, since the current knowledge about pregnancy rates obtained with frozen semen is incomplete for many species and breeds, especially for avian species. This knowledge can be used as parameters in simulation studies to better establish collection goals and perform risk analysis.
- For intensively selected breeds, assessment of the collection may also be used to evaluate specific changes in genetics of the breed, for example, depending on: (i) the level of intensity of the selection for a given trait; (ii) the variability and extent of changes applied on selection goals over time; and (iii) the existence of similar collections of the breed in other countries.

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SECTION 6

Collection and cryopreservation of germplasm and tissues

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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 natural biological process *per se,* therefore, most cells and tissues require specific procedures to regain their developmental competence. These procedures vary depending 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 down-stream 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. All cellular processes are ceased during the storage period. The post-thaw viability of the cellular material is ensured by the use of cryoprotectants and controlled rates of temperature changes at freezing and 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 that employ a slow and progressive decrease in temperature to reach freezing. This process reduces ice crystal formation and associated cellular damage. A more rapid increase in temperature at thawing is then used, which helps prevent recrystallization of intracellular water. Alternatively, vitrification consists of an extremely rapid freeze-thaw process that aim to put the frozen cell in an "amorphous" (i.e. without a clear form or structure) state with very little crystal formation, which 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. Also, high concentrations of cryoprotectants are required, which may have negative effects on the viability of cells.

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, Leibo and Seidel, 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 cryoinjury (Mocé *et al.*, 2010). Enhancing cryotolerance by incorporating exogenous cholesterol into the membranes has resulted in improved goat sperm survival after thawing (Salmon, Leclerc and Bailey, 2016).

During freezing and thawing, the formation and melting of ice crystals cause osmotic shifts that induce dramatic effluxes and influxes of cellular water (Sieme *et al.*, 2016). Upon extracellular ice formation, the solute concentration in the extracellular unfrozen medium increases, causing cells to dehydrate and to 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 (Sieme *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 or other cryoprotecting agents, depending on the species, and quite rapid freezing, storage, and rapid thawing. Although sperm are structurally complex, they tend to be cryoresistant 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 the developmental competence of embryos, 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 and 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.

Slow freezing using ethylene glycol as cryoprotectant has been the most widely used cryopreservation method for embryos in cattle, because it allows the direct transfer of embryos from the cryopreservation straw after thawing. The use of vitrification in the dairy cattle industry has increased along with the use of *in vitro* produced ovum pick-up (OPU) embryos and embryo biopsy required for genotyping of embryos. Compared to mammalians, embryo cryopreservation is not possible in avian species, because of the size and structure of eggs. 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 (although vitrification straws are more costly than standard straws for semen and embryos). Vitrification can be carried out under field conditions. Nevertheless, it requires considerable experience to be carried out successfully (Saragusty and Arav, 2011), and its efficacy can depend on the ambient temperature and other environmental conditions.

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 schemes. 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, Hupp and Murphree, 1961), ram (Terril, 1940), buck (Leboeuf, Restall and Salamon, 2000), rabbit (Naughton, Nelson and Thomas, 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.

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 minutes, 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, 2009b; 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 Post-mortem 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 cauda epididymis 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 endophallus, 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, Tarpy and Woyke (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, Herr and Sheppard, 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, 2010b; and 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.

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 not be equivalent.

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,[1] single layer centrifugation,[2] dextran/swim-up[3] and semen filtration with Sephadex [4] 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.

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[1] **Santiago-Moreno, J., Esteso, M.C., Castaño, C., Toledano-Díaz, A., Delgadillo, J.A. & López-Sebastián, A.** 2017. Seminal plasma removal by density-gradient centrifugation is superior for goat sperm preservation compared with classical sperm washing. *Animal Reproduction Science*, 181: 141–150. https://doi.org/10.1016/j.anireprosci.2017.04.002

[2] **Jiménez-Rabadán, P., Morrell, J.M., Johannisson, A., Ramón, M., García-Álvarez, O., Maroto-Morales, A., Alvaro-García, P.J., *et al.*** 2012. Single layer centrifugation (SLC) improves sperm quality of cryopreserved Blanca-Celtibérica buck semen. *Animal Reproduction Science*, 136(1-2): 47–54. https://doi.org/10.1016/j.anireprosci.2012.09.012

[3] **García-López, N., Ollero, M., Muiño-Blanco, T. & Cebrián-Pérez, J.A.** 1996. A dextran swim-up procedure for separation of highly motile and viable ram spermatozoa from seminal plasma. *Theriogenology*, 46(1): 141–151. https://doi.org/10.1016/0093-691X(96)00149-5

[4] **Galarza, D.A., López-Sebastián, A., Woelders, H., Blesbois, E. & Santiago-Moreno, J.** 2018. Sephadex filtration as successful alternative to density-gradient centrifugation procedures for ram sperm selection with improved kinetics. *Animal Reproduction Science*, 192: 261–270. https://doi.org/10.1016/j.anireprosci.2018.03.022

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 and L-carnitine have also been shown to help maintain sperm motility and 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 (see 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 (Thélie *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, Seigneurin and Blesbois, 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 (Váradi *et al.*, 2013). In geese, the use of straws or cryovials were both reported to be efficient (Thai *et al.*, 2001; Lukaszewicz, 2002; Váradi *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 (1979a), 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, Herr and Sheppard, 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, 1979a, 1979b; Hopkins, Herr and Sheppard, 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: (i) block of dry ice to form small pellets then submerged into liquid nitrogen; or (ii) directly into liquid nitrogen to form spherical pellets. The thawing protocols differ between the two pelleting methods. For the first pelleting method, thawing is done in dry glass tubes placed in a 37 °C water bath for 2 minutes. For the second, ultra-rapid thawing is used, for example, by thawing samples on a 60 °C hotplate for 2 to 5 seconds.

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 × 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, Ådnøy and Söderquist, 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 intrauterine (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, Herr and Sheppard, 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 (see 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 (see 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 with welfare concerns, as may laparoscopic insemination. Sedation may alleviate some issues related to animal well-being, although ethical considerations remain.

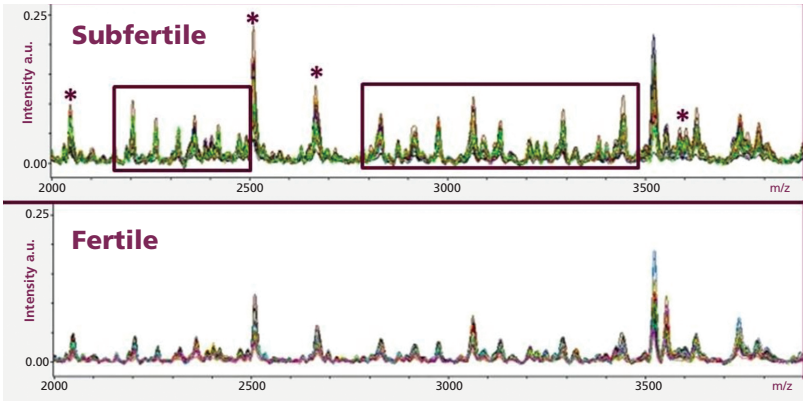
BOX 6.2

New tools for semen quality evaluation

The "omics" revolution of the last decade has led to the emergence of promising preliminary tools for evaluating the intrinsic capacity of sperm to fertilize. One approach is the proteomic analysis of semen, either the seminal plasma and/or the sperm fraction.[1,2,3] 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 1 000 to 30 000 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" (see 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.

FIGURE 6.1

Difference in ICM-MS profiles between subfertile and highly fertile chicken males



Source: Authors' own elaboration.

(Cont.)

[1] **Druart, X. & de Graaf, S.** 2018. Seminal plasma proteomes and sperm fertility. *Animal Reproduction Science*, 194: 33–40. https://doi.org/10.1016/j.anireprosci.2018.04.061

[2] **Labas, V., Grasseau, I., Cahier, K., Gargaros, A., Harichaux, G., Teixeira-Gomes, A.P., Alves S., Bourin, M., Gerard, N. & Blesbois, E.** 2015. Qualitative and quantitative proteomic approaches to phenotyping chicken semen. *Journal of Proteomics*, 112: 313–335. https://doi.org/10.1016/j.jprot.2014.07.024

[3] **Soler, L., Labas, V., Thélie, A., Grasseau, I., Teixeira-Gomes, A.P. & Blesbois, E.** 2016. Intact cell MALDI-TOF MS on sperm: towards a molecular test for male fertility diagnosis. *Molecular and Cellular Proteomics*, 15(6): 1998–2010. https://doi.org/10.1074/mcp.M116.058289

The upper profile is from a subfertile male (40 percent fertility), while 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.[4,5] Researchers now hypothesize that bulls with moderate to high fertility (or low fertility) can be identified by differences in semen miRNA expression.

[4] **Capra, E., Lazzari, B., Turri, F., Cremonesi, P., Rodriguez Portela, A.M., Ajmone-Marsan, P., Stella, A. & Pizzi, F.** 2019. Epigenetic analysis of high and low motile sperm populations reveals methylation variation in satellite regions within the pericentrometric position and in genes functionally related to sperm DNA organization and maintenance in Bos taurus. *BMC Genomics*, 20(1): 940. https://doi.org/10.1186/s12864-019-6317-6

[5] **Capra, E., Turri, F., Lazzari, B., Cremonesi, P., Gliozzi, T.M., Fojadelli, I., Stella, A. & Pizzi, F.** 2017. Small RNA sequencing of cryopreserved semen from single bull revealed altered miRNAs and piRNAs expression between High- and Low-motile sperm populations. *BMC Genomics*, 18(4): 1–12. https://doi.org/10.1186/s12864-016-3394-7

6.3.5.2 Poultry species

The methods of semen collection and artificial insemination are non-invasive, 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. It's also been shown to be successful for honey bees under experimental conditions. In contrast,

embryo cryopreservation is much more difficult for oviparous species due to species-specific egg structures and vitellus storage. Since the early embryo develops from the fertilization of the oocyte, new advancements in oocyte cryopreservation are also under experimentation in mammals paving the way for cryoconserving the female genome.

6.4.1 Potential use of mammalian embryos

Since the birth of normal offspring from cryoconserved mouse embryos was first reported in 1972 (Whittingham, Leibo and Mazur, 1972), similar successes have been achieved in at least 33 mammalian species (see Table A6.3.1 in Annex 6.3 for references), including 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, den Daas and Rall, 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 embryo cryopreservation has been the target of much research in the last decades, resulting in highly efficient standard procedures for which around 60 percent transfers result in pregnancies carried to term with frozen-thawed embryos (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 as fewer resources have been spent on development and refinement of methods, 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 successful (Ulrich and Nowshari, 2002). However, the emergence of vitrification techniques is 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. Ultra-rapid vitrification in thin plastic straws (open pulled straws) provided good 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 ease of collecting and transferring embryos, and whether surgical or non-surgical collection procedures are feasible. Table 6.1 compares surgical and non-surgical embryo collection for major livestock species, while Table 6.2 compares embryo transfer procedures. The information presented in the two tables should be taken into consideration when planning a gene banking programme, both in choosing the type of germplasm to be stored and in deciding upon the quantity needed.

TABLE 6.1
Comparison of non-surgical versus surgical embryo collection in livestock species

Characteristic	Species				
	Cattle	Sheep	Goats	Pigs	Horses
---------- Non-surgical ----------					
ease[a]	1	5	3	3	1
percent of treated females with ≥ 1 embryo per collection	85	> 50	> 70	< 35	80
transferable embryos per collection (*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 ----------					
easea	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]

Note: 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] Ease 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.

Source: Authors' own elaboration.

TABLE 6.2
Comparison of non-surgical versus surgical embryo transfer in livestock species

Characteristic	Species				
	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
easea	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
ease[a]	3	3	3	2	2
recommended for use?	No	Yes	Yes	Yes	No

Note: 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.

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

Source: Authors' own elaboration.

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, Ali and Saleem, 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.

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 (in French; 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, Givens and IETS, 2010), 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

Increasing the number of offspring from a female will help increase the census size, thus helping to prevent breed extinction, and will accelerate breed reconstitution. To increase the number of embryos per collection, donor females are administered various gonadotropin-like hormonal agents to stimulate multiple ovulations. Early reports in cattle, sheep, goats and pigs described the use of pregnant mare serum gonadotropin (PMSG) at various dose levels to cause superovulation by donor females (Elsden *et al.*, 1978). This agent, now termed equine chorionic gonadotropin (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 many countries, although it is still used where other options are not commercially available. PMSG/eCG is still used in small ruminants, although it may be associated with the premature regression of corpora lutea, a common phenomenon in superovulated does and ewes (Pintado, Gutiérrez-Adán and Pérez, 1998).

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

Several of the commonly used superovulation schemes for cattle donors today are presented in Annex 6.5. 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.

Table 6.1 presents the number of transferable embryos that can be obtained after a single superovulation treatment and embryo recovery, on average, as well as estimates of the number of embryos obtainable from one donor female for one year. Animal to animal variability is large, however, because some females simply do not respond well to stimulatory agents, while others may develop physiological conditions that make it difficult to retrieve the embryos. Therefore, 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 procedures. In bovine, the embryos reach the uterine cavity at the late morula, early blastocyst stage at day 4 to 5 post-fertilization.

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 publication by Lindner and Wright (1983) and the IETS manual (Stringfellow, Givens and IETS, 2010).

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). 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 often required, but recent advancements have expanded the application of non-surgical collection (Martinez *et al.*, 2019). In goats, most of the commercial embryo recovery in some countries is non-surgically performed in goats, e.g. Brazil (Fonseca *et al.*, 2013, 2018b).

Non-surgical embryo collection and transfer pose minor risk to the cow, and reduce the time needed for harvesting embryos. 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 from the horn medium containing the embryos. 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 or poor timing when inseminating 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 of Great Britain and Northern Ireland, 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 (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 and Argentina.

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.*, 2013, 2018b). 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). Non-surgically derived embryos lead to acceptable pregnancy rates in goats (Fonseca *et al*., 2018b) and sheep (Figueira *et al*., 2019), 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 than for semen. 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 target species (Fonseca *et al.*, 2018a; De Coster *et al.*, 2020) (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 rapidly cooled (1 °C per min) to a subzero temperature (-5 to -7 °C), a critical step in the freezing process is "seeding", which is the act of inducing ice-crystal formation in the cryoprotectant solution surrounding the embryo. Seeding prevents supercooling, which would result in a liquid storage environment, despite the subzero temperature. After embryos are cooled to approximately -35 °C, the straws are plunged into liquid nitrogen for storage at 196 °C. A summary of the methods and their applications is presented in Leibo (1992) and Rall (1992).

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

1. the quality of the embryo as estimated from its morphology examined under a 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 Section 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 (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 and Kuwayama, 2006; Vajta and Nagy, 2006). For further details on vitrification procedures, see Vajta *et al.* (2005) and Vajta and Kuwayama (2006). Although vitrification is a quick procedure and does not require special equipment, it can be technically more demanding and typically yields pregnancy rates that are 10 to 15 percent lower than those obtained using slow freezing until the technicians have gained enough experience to master the technique.

Success rates with vitrification in cattle (Arshad *et al.*, 2021) and small ruminants (Guignot *et al.,* 2006; Baril *et al.,* 2001) are now similar or superior to the rates achieved with conventional embryo freezing. Commercial kits for vitrification of livestock embryos are available. As vitrification methodologies improve, there are some indications that the approach may have some advantages over standard slow freezing procedures in cattle (Visintin *et al.*, 2002; Arshad *et al.*, 2021), 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, Cueto and Pereyra Bonnet, 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 cavity (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 using polymerase chain reaction (PCR) to detect genes on the Y-chromosomes on one of the two bisected halves. Once the sex is established, the remaining half of the embryo can be transferred to a recipient female.

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; Hirayama *et al.*, 2013) 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 sex diagnosis. The equipment and material 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 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 (see Section 5). 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 studied depends on the precise determination of the developmental stage (Rajamohan, Rinehart and Leopold, 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 sensitivity to cryopreservation. 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 × 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, approximately one 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, Boncristiani and Chen (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 and 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
8. embryo culture

In honey bees, the specific developmental stage must fulfill all the criteria laid out in Leopold and Rinehart (2010) and Rajamohan, Rinehart and Leopold (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 5 000 embryos in one session, only 1–3 honey bee embryos can be cryopreserved per session. Embryos are frozen on a single hair/filament brush (see Figure A6.3.5 in Annex 6.3) or a thin strip of polyvinyl pyrrolidone coated polycarbonate membrane.

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 and 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 for less than 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 trappings 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 allows 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 (see 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 by using the pig as an example.

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 the 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 (Somfai, Kikuchi and Nagai, 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, Somfai and Kikuchi, 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, likely 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 and Goel, 2016; Song and Silversides, 2007c; Rajamohan *et al.,* 2019; Liptói *et al.,* 2020; Liu *et al.,* 2010), making it an effective means of preserving animals of high genetic value. Gonadal tissue cryopreservation is utilized routinely in genetic conservation programs with poultry (see 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 percent 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 and 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, Elsasser and Long, 2017), indicating this window may be longer than 24 hours 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 in Dulbecco's phosphate-buffered saline – fetal bovine serum (DPBS-FBS) solution on ice while awaiting vitrification (see 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 and 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 (Mackensen, 1955). Drone bees are collected from healthy hives in the mid-season of activity when drone production is high in the hive.

BOX 6.3

United States Department of Agriculture (USDA) National Animal Germplasm Program (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 such as 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 labelled with an identification number that corresponds to the chicks, sex, line and 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, that is, 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, 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 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 (Devi and Goel, 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 into open, labelled 1 mL volume cryovials in liquid nitrogen, where they are closed with forceps before long-term storage in nitrogen tanks (see 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, see 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 to 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-FBS for up to 1 hour at 0 °C (Liptói *et al.,* 2020; Barna *et al.,* 2020) (see Annex 6.5).

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 (see 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 and 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, Cheng and Silversides, 2013), 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 (Liptói *et al.,* 2020). In males, the technique is the same as in females, and similar precautions should be exercised (Song and Silversides, 2006, 2007a, 2007b; Liu, 2013; Liptói *et al.,* 2013). In this case, removal of the testes can be performed with fine scissors and forceps (see 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. Anaesthesia 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 and Silversides, 2006, 2007a, 2007b; Liu, 2013; Liptói *et al.,* 2013; Barna *et al.*, 2020) (see Annex 6.5).

The prepared donor ovarian tissue is grafted as close as possible to the anatomical location (Liptói *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 and Silversides, 2006; Song *et al.*, 2012; Liptói *et al.,* 2013; Barna *et al.,* 2020) (see Annex 6.5).

Not all genotypes are suitable recipients among the domestic chicken breeds (Liptói *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 and Silversides, 2006; Liptói *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; Liptói *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 (Liptói *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 labelling (TUNEL) assay (Gastal *et al.,* 2017). Similarly, histological analysis with hematoxylin and 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 (Váradi, 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, Elsasser and Long (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, in order 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, Elsasser and Long, 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 (Liptói *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 colour. However, if the recipient has a similar colour 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 (Liptói *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, the United Kingdom, and the United States of America, 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 (see 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. Embryos have very few PGCs, 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, that is, 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 reamplification *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.*, 2021): 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 and Mitra, 2014; Oatley *et al.*, 2016; Zhang *et al.*, 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 post-mortem or during veterinary castration. The SSCs need to be injected into the mammalian testes to complete 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 *et al.*, 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 *et al.*, 2019; Ballantyne *et al.*, 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 and goats (Wilmut *et al*., 1997; Cibelli *et al*., 1998; Baguisi *et al*., 1999; Polejaeva *et al*., 2000; Galli *et al*., 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 and Yamanaka, 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 *et al*, 2011; Hayashi *et al*., 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 recent 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 for germplasm conservation that have permitted the building of collections, and their efficient use to reintroduce genetic variation into populations *in vivo*. The recent Horizon 2020 European Union project *Innovative Management of Animal Genetic Resources* (IMAGE, 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 country regulations 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

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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 spread of disease, regulations at international, regional or national levels typically require that germinal products collected, produced, processed and stored at specialized facilities are subjected to special animal health and hygiene regimes. At the same time, animals that are admitted into those facilities and classified as donors of germinal products are required to comply with stricter animal health standards than those applied 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 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 (World Organisation for Animal Health (WOAH), 2019) of the World Organization for Animal Health (WOAH). 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, WOAH recommendations regulate the sanitary aspects of collection, processing, storage and transport of germinal products, both at multinational and national levels.

The WOAH 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 WOAH regulations, being the best international standard available. In addition, WOAH 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 WOAH 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

Zhao Xueming

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

- Drastic climate change and human activities, like natural hazards and urbanization, have rapidly deteriorated the habitat of many animals, including endangered livestock breeds.
- 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 of genetic diversity and local genes.
- 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.

(Cont.)

To protect biodiversity, China established the national gene bank for livestock and poultry in 1992. Until 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 more than 1.1 million culled pigs, there were many animals from local breeds with unique 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 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 WOAH 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 WOAH 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 14644-1:2015.[1] 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. Biosafe farms should be used and animal disease testing reports for diseases prohibited the WOAH should be checked when choosing donor animals.

[1] **International Organization for Standardization (ISO).** 2015. *Standard 14644-1:2015 Cleanrooms and associated controlled environments – Part 1: Classification of air cleanliness by particle concentration.* Geneva. 22 September 2020. www.iso.org/standard/53394.html

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 (see 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 European Union (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 429/2022)[1] in 2022, EU regulations were maintained as a reference for the collection and storage of animal germplasm in Spain. However, an exception was included to allow the collection of reproductive material intended to be stored in gene banks (already foreseen in the former Spanish regulation) 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 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 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.

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[1] **Spain.** 2022. Real Decreto 429/2022, de 7 de junio, por el que se establecen normas para la comercialización de los productos reproductivos de las especies ganaderas en el ámbito nacional y se regulan medidas para la aplicación de la normativa europea aplicable a los desplazamientos dentro de la Unión Europea de productos reproductivos de las especies ganaderas. Madrid. Cited 14 November 2022. www.boe.es/diario_boe/txt.php?id=BOE-A-2022-9380

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 direst 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 (EU, 2020) concerning the approval of germinal product establishments and the traceability and animal health requirements for movements of germinal products within the European Union is of great importance. For the 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).

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 WOAH-listed diseases relevant for the species (WOAH, 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.

BOX 7.3

New possibilities for gene banks in the European Animal Health Regulation

In December 2019, the European Union (EU) approved Commission Delegated Regulation (EU) 2020/686, which supplemented Regulation (EU) 2016/429 of the European Parliament and of the Council.[1] 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 authorized or recognized 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.

[1] **European Union (EU).** 2020. Commission Delegated Regulation (EU) 2020/686 of 17 December 2019 supplementing Regulation (EU) 2016/429 of the European Parliament and of the Council as regards the approval of germinal product establishments and the traceability and animal health requirements for movements within the Union of germinal products of certain kept terrestrial animals. Cited 14 November 2020. eur-lex.europa.eu/legal-content/EN/TXT/?uri=CELEX:32020R0686

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 in the legal framework for the country.

Utilizing collection centres with lower sanitary requirements may be a satisfactory solution. The sanitary status of each donor should be documented in the collection centre's or gene bank's database and quarantine measures are recommended.

The following recommendations (most of them derived from the WOAH standard) may be used to establish derogations for collection centres at national level:

- The collection centre should only concurrently 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.
- 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.
- 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 into "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):

- 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.
- Field collection of germplasm requires a mobile collection team with adequate equipment for collecting samples, and preferably for processing and short-term storage.
- 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.
- 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.

- 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.
- 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 avian species), 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 WOAH-listed diseases. In situations 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 WOAH-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.

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 polymerase chain reaction (PCR) testing.

BOX 7.4

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

Sheila Butungi

Uganda, like the rest of Africa, manages livestock in endemic situations for some WOAH-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 multidisciplinary team that includes a veterinarian, laboratory technicians, animal scientists and sometimes social scientists. Limited financial resources impact biosafety and biosecurity, and procedures are optimized 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.

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 authorized laboratories, following the WOAH regulations, or in accordance with the policies and procedures established as part of a derogation established between two countries. 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 standards based 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 authorized 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 sterilized 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 that can ensure high quality processing immediately following collection. All steps of processing germinal products can thus be 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.

Embryos and oocytes are to be washed in sterile media containing antibiotics 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 hood or other suitable environment, where all technical operations associated with specific sterile procedures are conducted. In addition, it is recommended 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 (IETS, 2009). 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 minimize 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):

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

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 stables or 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 (WOAH, 2019) 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 (see 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 Turopolje pig, which has a collection of semen in the Austrian gene bank, which was collected according to the national regulations but cannot be used in transboundary collaboration with neighbouring countries until the new EU regulation enters into force.

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 (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 Viet Nam – Challenges and initiatives

Ngo Thi Kim Cuc

In Viet Nam, more than 70 local breeds are endangered. In 1990, the Vietnamese government initiated a conservation programme 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 cryobank 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 Viet Nam, several WOAH-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 by grouping according to breed or time of collection.

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

BOX 7.6

Recommendations for sanitary measures at the West African regional animal gene bank in Burkina Faso

Isidore Houaga

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 African Union – Interafrican 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 European Union. 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 subregional gene banks throughout Africa, and Centre international de recherche-développement de l'elevage en zone subhumide (CIRDES), a subregional 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 subregional gene bank becomes operational.

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SECTION 8
Databases and documentation

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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 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 be designed considering both present and future needs, and regularly updated as novel 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 which states 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.

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

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

To be Reusable:

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

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 the 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 (Staunton *et al.*, 2019, van der Burg, Wiseman and Krkelja, 2021).

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" (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 as 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 °C. "Frozen, vapor phase" refers to storing samples above liquid nitrogen in the vapor.

Allowed values are:

- ambient temperature
- cut slide
- frozen, -80 °C freezer
- frozen, -20 °C freezer
- frozen, liquid nitrogen
- frozen, vapor phase
- paraffin block
- RNAlater, frozen -20 °C
- TRIzol, frozen
- paraffin block at ambient temperatures (+15 to +30 °C)
- 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 ruleset, 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 allows 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).

8.2.8.3 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.*, 2010).

The massive size of genome data files (genome sequence information of 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.

8.2.8.4 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 list 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

Source: Authors' own elaboration.

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	N
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.

Source: Authors' own elaboration.

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 (see 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 useridpassword or other system to regulate and monitor access to the different parts of the data system is strongly recommended. This system should also enable the differentiation of 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) (FAO, 2017a) 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 on 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 resources, 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 (FAO, 2017b).

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 (CSV) 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 (Germplasm Resources Information Network), in 2001. As time passed, the need for continual evolution and improvement of Animal GRIN, including facilitation of international cooperation, became apparent. In 2006, the programmes for animal genetic resources for food and agriculture in the United States of America, 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 GRINv.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 GRINv.2 was developed in a customized way to meet each country's needs. For example, the National Animal Germplasm Program, or NAGP of the United States of America (USDA, 2021) 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 the Animal

GRINv.2, however, to allow Brazilian Agricultural Research Corporation (Embrapa) of Brazil 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 publicly 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.

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

- United States of America: https://agrin.ars.usda.gov/;
- Brazil: https://an.cenargen.embrapa.br/database_collaboration_page_dev; and
- Canada: https://agriculture.canada.ca/en/agricultural-science-and-innovation/agriculture-and-agri-food-research-centres-and-collections/animal-genetic-resources-canada/about-animal-genetic-resources-collection.

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;
- improve monitoring and assessment of AnGR kept in *ex situ* collections in ERFP member states by sharing information on gene bank collections;
- improve gene bank operations and procedures in ERFP member states by sharing information;
- contribute to the long-term conservation and maintenance of AnGR in *ex situ* collections in ERFP member states;
- use synergies for *ex situ* conservation and sustainable use of AnGR by joint activities of gene banks in ERFP member states;
- increase the efficiency of *ex situ* conservation and sustainable use of the genetic diversity of transboundary breeds;

- promote harmonization of acquisition and access terms for *ex situ* conservation and sustainable use throughout the gene banks in ERFP member states;
- facilitate improved quality management of the gene banks in ERFP member states;
- create an element of European research infrastructure for conservation and sustainable use of AnGR; and
- facilitate a regional European approach for international cooperation and exchange of AnGR in the context of the implementation of the Nagoya Protocol on 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, policymakers 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 European Union 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 Innovative Management of Animal Genetic Resources (IMAGE, 2020a) 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, 2020b).

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:

- a well-defined metadata ruleset ensuring high quality and comparable data across the diverse collections in different storage formats and languages;
- 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 the Europe;
- sustainability by archiving of this data within the BioSamples public archive of EBI; and
- 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) of a reference dataset and a batch of samples of interest (Reich, Price and Patterson, 2008).

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 (Pook, Schlather and Simianer, 2020) (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

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9.1 INTRODUCTION

In the last years, the importance of genetic resources and their sustainable use, development and conservation has been recognized by several international instruments and policies, such as the Convention on Biological Diversity (CBD, 1992), *Global Plan of Action for Animal Genetic Resources* (FAO, 2007), Strategic Plan for Biodiversity 2011–2020 (CBD, 2010a) and the Nagoya Protocol on access to genetic resources and the fair and equitable sharing of benefits arising from their utilization (CBD, 2010b). Many countries that are parties to these international instruments have also adopted domestic legislation on the conservation and sustainable use of genetic resources. This regulatory framework contributes to reinforce the value of genetic resources, but adds complexity to the management of gene banks. Box 9.1 describes some of the commitments from international instruments and policies that concern genetic resources in gene banks.

Many governments, recognizing the importance of national cryoconservation programmes for AnGR, 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 has, in many circumstances, required development of policies and a legal basis for establishment and operation of *ex situ* facilities. Such measures have been in the form of specific national legislation, or through delegation of responsibility and power over management of gene bank related issues to relevant public or private entities.

Generally, legal frameworks of a wider scope related to livestock sector are already in place in many countries, such as animal breeding, animal health and sanitary regulations, and legislation dealing with protection and conservation of genetic diversity including conservation of local endangered breeds, as well as private property and contract laws. Additional specific measures are usually needed for the issues regarding the establishment and management of national gene banks, but in many countries such measures have not been established.

Gene banks must also adhere to other laws and regulations, for instance, veterinary legislation (see Section 7). Animal breeding laws may also have provisions regarding information on the individual donors of reproductive material that must accompany a breeding animal or a sample of its genetic material provided to and distributed 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 operate within such a framework.

BOX 9.1

Commitments from international instruments and policies relevant for genetic resources

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.[1]

The need for establishment of *ex situ* gene banks was also recognized by governments adopting the *Global Plan of Action for Animal Genetic Resources*.[2] 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 (AnGR) (FAO, 2007). Moreover, the *Interlaken Declaration*, through which the *Global Plan of Action* was adopted, recognizes the important role of local and indigenous communities and farmers, pastoralists and animal breeders and their private ownership in the management and conservation of AnGR. Therefore, concurrence with animal owners in the collection and management of germplasm for gene banking purposes is of key importance.[3]

The need to conserve genetic resources of cultivated plants and farm animals was strengthened by the adoption of the Strategic Plan for Biodiversity 2011–2020.[4] 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 maintained in *ex situ* collections.

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[5] recognizes the "importance of genetic resources to food security, public health, biodiversity conservation, and the mitigation of and adaptation to climate change."

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[1] **CBD.** 1992. Convention on Biological Diversity. Montreal. Cited 6 February 2021. www.cbd.int/doc/legal/cbd-en.pdf

[2] **FAO.** 2007. *Global Plan of Action for Animal Genetic Resources and the Interlaken Declaration.* Rome. www.fao.org/3/a1404e/a1404e.pdf

[3] **Blackburn, H.D. & Boettcher P.J.** 2010. Options and legal requirements for national and regional animal genetic resource collections. *Animal Genetic Resources,* 47: 91–100. http://dx.doi.org/10.1017/S2078633610000998

[4] **CBD.** 2010a. Strategic Plan for Biodiversity 2011–2020, including Aichi Biodiversity Targets. Montreal. Cited 8 February 2021. www.cbd.int/sp/

[5] **CBD.** 2010b. 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. Montreal. Cited 8 February 2021. www.cbd.int/abs/doc/protocol/nagoya-protocol-en.pdf

It specifies that its "Parties shall encourage users and providers to direct benefits arising from the utilization of genetic resources towards the conservation of biological diversity and the sustainable use of its components."

Following the adoption of the Nagoya Protocol in 2010, the Commission on Genetic Resources for Food and Agriculture established an Ad Hoc Technical Working Group on Access and Benefit-sharing for Genetic Resources for Food and Agriculture. It was mandated to identify relevant distinctive features of the different subsectors of GRFA requiring distinctive solutions. The Group was also given the task of developing options that would guide and assist countries in developing legislative, administrative and policy measures that accommodate these features. The *Draft Elements to Facilitate Domestic Implementation of Access and Benefit-sharing for Different Subsectors of Genetic Resources for Food and Agriculture*[6] (ABS Elements) were subsequently developed. In 2015, the Commission and the FAO Conference welcomed the ABS Elements and, noting "the complementarity between the work of the Commission and the Nagoya Protocol in regard to access and benefit-sharing for genetic resources," invited countries to consider and, as appropriate, make use of the ABS Elements.[7]

In 2019, the Commission and the FAO Conference welcomed the addition of explanatory notes to the ABS Elements.[8] The ABS Elements with their explanatory notes aim to assist governments considering developing, adapting or implementing ABS measures to take into account the importance of genetic resources for food and agriculture, their special role for food security and the distinctive features and specific practices of the different subsectors of genetic resources for food and agriculture, while complying, as applicable, with international ABS instruments.

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[6] **FAO.** 2014. *Draft Elements to Facilitate Domestic Implementation of Access and Benefit-sharing for Different Subsectors of Genetic Resources for Food and Agriculture CGRFA-15/15/5.* Rome. www.fao.org/3/a-mm521e.pdf

[7] **FAO.** 2015. *Fifteenth Regular Session of the Commission on Genetic Resources for Food and Agriculture.* Rome. www.fao.org/3/mm660e/mm660e.pdf

[8] **FAO.** 2019. *ABS Elements: Elements to facilitate domestic implementation of access and benefit-sharing for different subsectors of genetic resources for food and agriculture with explanatory notes.* Rome. www.fao.org /3/ca5088en/CA5088EN.pdf

Management of gene banks includes entering into 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 and the users of the gene bank's material and, if relevant, obligations and rights of the original owners or donors of the samples. Each country should establish policies and procedures for drawing up such agreements.

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 drawn up 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 by providing examples from different countries.

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

An MAA is an agreement between the gene bank and the provider of material to be stored for conservation, sustainable use, research and development of AnGR. An MTA is a contract between the gene bank and the user of material that accompanies the physical transfer of a genetic material from the gene bank collection to the 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. These two types of agreements will be described more completely later in this section.

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.

Gene banks must contend with a range of situations 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 multistakeholder 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 banking strategy and on policies for acquiring and distributing germplasm. The board would also advise the gene bank on formulation of agreements with other stakeholders.

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 usually private property. As suggested by Blackburn and Boettcher (2010), the gene banks may choose to either have outright ownership over the germplasm entering the collection or develop specific working arrangements to obtain material from individual breeders, companies or breed associations who will themselves maintain rights over the material. The roles 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 United States of America 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 9.2

Examples of national frameworks for establishment of animal gene banking

The French National Cryobank was initiated in 1999 as a project, co-funded by twelve organizations involved in the management of AnGR. They included research institutes, artificial insemination (AI) centres, veterinary laboratories and a federation of domestic breed 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.[1]

In 1990, the **United States Congress** enacted legislation on the National Genetic Resources Program, describing establishment, purpose, administration and functions of the programme (USC, 1990).[2] 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.[3] 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.[4] The programme must implement a number of USC law provisions. For instance, the gene bank has "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." Therefore, 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".[5] The state budget covers operating costs of the gene bank, provides funds for the purchase of reproductive material obtained on a regular basis (mostly semen and embryos), as well as costs for its processing (transport, maintenance and insurance for 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.[6]

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[1] **Duclos, D. & Danchin-Burge, C.,** 2012. The French National Cryobank. Paper presented at the "FAO-EAAP-ERFP Workshop on national legal and institutional arrangements for *ex situ* conservation of animal genetic resources", 23–26 May 2012, Zagreb. www.animalgeneticresources.net/wp-content/uploads/2018/05/ERFP_AdHoc-exsitu_May2012_present-DuclosDanchin.pdf

[2] **United States Congress (USC).** 1990. Public Law 101-624-Nov. 28, 1990. Appendix I, 104 STAT.3744. Title XVI. Subtitle C–*National Genetic Resources Program*. Cited 6 November 2020. www.ars-grin.gov/ngrac/farmbill.htm

[3] **Blackburn, H. D.** 2009. Genebank development for the conservation of livestock genetic resources in the United States of America. *Livestock Science*, 120(3): 196–203. https://doi.org/10.1016/j.livsci.2008.07.004

[4] **United States Department of Agriculture (USDA).** 2017. *Research Project: National Animal Germplasm Program*. Fort Collins, CO. Cited 15 March 2021. www.ars.usda.gov/research/project/?accNo=433404

[5] **CZECHIA.** 2000. *Act No. 154 of 21 June 2000 on breeding and registration of livestock and on the amendment of certain related acts (Breeding Act).* Prague, Czechia. www.zakonyprolidi.cz/cs/2000-154

[6] **CZECHIA.** 2017. *Decree to the Breeding Act on animal genetic resources No. 72 of 8 March 2017.* Prague, Czechia. www.zakonyprolidi.cz/cs/2017-72

BOX 9.3

Examples of diversity in decision-making procedures among gene banks and in the ownership of donor animals or their biological material

In the **French Cryobank**, the decision-making body is the Group Council, representing all partners and the Cryobank Scientific Committee, consisting of 13 technical experts in cryopreservation, population genetics, biodiversity and sanitary rules.[1] This committee served exclusively for the purpose of the gene bank until 2011, but since then its role and functions have been broadened.[2]

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".[3] In this case the decision-making process stays with the national or 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 centre decides on the conditions of its further use. A standard agreement procedure is used for any acquisition or transfer of material.[4]

In the **Netherlands**, a quality management system (QMS) and associated procedures for acquisition and use of biological material have been established. Decisions are taken by gene bank management in consultation with relevant breed societies.[5]

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 becomes property of the government. Distribution of the material is free of charge, after the approval of the National Advisory Board on AnGR.[6]

Contrarily, for the **French** National Cryobank, the governing Group 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 council. If a depositor wants to use his or her 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 replenishing of the stock is obligatory.[1] If commercial use is planned, a financial agreement must be made among the applicant, the material provider and the gene bank.

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[1] **Duclos, D. & Danchin-Burge, C.,** 2012. The French National Cryobank. Paper presented at the "FAO-EAAP-ERFP Workshop on national legal and institutional arrangements for *ex situ* conservation of animal genetic resources", 23–26 May 2012, Zagreb. www.animalgeneticresources.net/wp-content/uploads/2018/05/ERFP_AdHoc-exsitu_May2012_present-DuclosDanchin.pdf

[2] **Tixier-Boichard, M.** 2012. Cryobanks as BRCs: the French CRB-Anim infrastructure project, and prospects for setting up a European network for such BRCs. Paper presented at the "FAO-EAAP-ERFP Workshop on National legal and institutional arrangements for ex situ conservation of animal genetic resources", 23–26 May 2012, Zagreb. www.animalgeneticresources.net/index.php/event/fao-eaap-erfp-workshop-on-national-legal-and-institutional-arrangements-for-ex-situ-conservation-of-animal-genetic-resources-2/

[3] **China.** 2005. *Animal Husbandry Law of the People's Republic of China.* www.npc.gov.cn/zgrdw/englishnpc/Law/2007-12/13/content_1384134.htm

[4] **Matlova, V.** 2012. Legal and instrumental arrangements for genebanks in the Czech Republic. Paper presented at the "FAO-EAAP-ERFP Workshop on National legal and institutional arrangements for ex situ conservation of animal genetic resources", 23–26 May 2012, Zagreb. www.animalgeneticresources.net/index.php/event/fao-eaap-erfp-workshop-on-national-legal-and-institutional-arrangements-for-ex-situ-conservation-of-animal-genetic-resources-2/

[5] **Hiemstra, S.J., Martyniuk, E., Duchev, Z. & Begemann F.** 2014. European Gene Bank Network for Animal Genetic Resources (EUGENA). *Proceedings, 10th World Congress of Genetics Applied to Livestock Production,* Vancouver, 17–22 August 2014.

[6] **Martyniuk, E., Haska, A. & Drobik-Czwarno, W.** 2019. IMAGE – *Innovative Management of Animal Genetic Resources, Deliverable D 1.6: Status, regulations and needs of ABS in genetic collections*. Paris. Cited 3 February 2021. www.imageh2020.eu/deliverable/IMAGE_D1.6_ABS_submitted.pdf

9.3 ACQUIRING SAMPLES

Gene banks should have a set of procedures and agreements in place for acquiring and distributing samples, inasmuch as samples are acquired from various sources that may each require different legal or regulatory approaches and frameworks.

9.3.1 Samples obtained from private entities

In the vast majority of instances, livestock animals have been historically considered private property, 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 usually 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 as well.

Material for gene bank collections 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 once it has been placed 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.

9.3.2 Acquisition of gene bank material from other entities

Acquisition from non-private sources 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 additional 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 the donor is notified 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).

9.3.3 Material acquisition agreements

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.

Before developing MAA, the gene bank should consider:

- national laws and regulations, including regulations on access and benefit sharing (ABS), 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 (ERFP, 2019). The guidelines and elements were developed by the European Regional Focal Point for Animal Genetic Resources to support European Gene bank Network EUGENA (Hiemstra *et al.*, 2014). A summary of these elements is contained in Annex 9.1.

9.4 ACCESS TO GENE BANK COLLECTIONS

The decision to provide germplasm from the gene bank collection will usually depend on the purpose, principles and criteria set by the governing body of the gene bank and be based on conditions initially 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 users and uses. A few examples are in the following.

- **National conservation programmes such as support of *in vivo* conservation or breed re-establishment in case of extinction:** The access conditions will 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 on AnGR (FAO, 2011) or a designated authority to the cooperating entities implementing the programme.

- **Breeders or breed associations and private breeding companies for 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 wish to request that the user re-deposit germplasm from the resulting progeny.
- **Academic organizations for research and development:** Research projects are designed to generate new knowledge, which could ultimately lead to new applications with potential commercial value (such as DNA studies of the genetic background of traits). For these cases, it is recommended to agree upon the conditions for the use of data and information arising from the project and their publication in the gene bank database and/or public domain, in addition to simply the conditions for the use of the material itself.

Some gene banks provide access to information on the scope of their collections, like the French Biological Resource Centre for domestic animals (CRB-Anim, 2020), which is usually extremely useful for users (see Section 8). The IMAGE project (IMAGE, 2020a) included the creation of a European web portal (IMAGE, 2020b) that integrates data from gene banks and collections with genomics data, geographical information system data, and other information.

After the CBD established the ABS principles in 1992 and the Nagoya Protocol entered into force in 2014, many countries introduced national legislation to regulate acquisition, transfer and use of genetic resources and traditional knowledge. Originally, gene banks could only provide access to their own collections, if the material to be released was 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). However, if the country where the gene bank is located is a party to the Nagoya Protocol, gene banks must also comply with domestic ABS measures. Box 9.4 provides key information on the Nagoya Protocol.

9.5 TRANSFER OF MATERIAL

9.5.1 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. However, if the country where the gene bank is located is part of Nagoya Protocol, gene banks must also comply with ABS obligations. As a rule, material acquired for non-commercial purposes cannot be made available to a third party for commercial purposes.

The procedure of transfer of gene bank material begins when 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;

BOX 9.4

The Nagoya Protocol: key information

The Nagoya Protocol[1] 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 for its implementation.

- The first pillar of the Nagoya Protocol is access obligations. Countries can decide to make access subject to their prior informed consent (PIC) or to consent of indigenous and traditional communities involved. National access and benefit-sharing (ABS) legislation or regulatory measures is the most important source of information for potential users of genetic resources from such countries.
- The second pillar is benefit-sharing obligations. Benefits "arising from the utilization of genetic resources as well as subsequent applications and commercialization" must be shared in a fair and equitable way, subject to conditions established by mutually agreed terms (MAT) between the user and the party providing the genetic resources or indigenous and traditional community involved. Benefits may be monetary or non-monetary.
- The third pillar of the Protocol is compliance obligations. Compliance obligations include measures such as monitoring the utilization of genetic resources through establishment of the internationally recognized certificate of compliance (IRCC) and establishment of checkpoints at the national level.

The Nagoya 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 ABS national focal points (ABS-NFP) and competent national authorities (CNA), ABS legislation and administrative measures, as well as permits issued for 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.

[1] **CBD.** 2010. 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. Montreal. Cited 8 February 2021. www.cbd.int/abs/doc/protocol/nagoya-protocol-en.pdf

- type and quantity of genetic material requested:
- purpose for which the material will be used: and
- the import permit, if the requestor's country regulates entry of that material.

For breeding purposes, the requestor should also specify the material use objectives (such as a conservation project, breed improvement or research), which may require additional confirmation from 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 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.

9.5.2 Material Transfer Agreements

The material transfer agreement (MTA) is 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 of MTAs 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 in 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. These terms may include the following:

- the extent to which replication, alteration or utilization of the material is permitted;
- limitations on third party transfer or conditions and procedures that should apply if such a transfer is allowed (assuming that any subsequent transfer should be subject to the same conditions as the initial transfer);
- 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 such as phenotypic and/or genotypic data resulting from the research project (before or after publication); 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.

9.5.3 Intellectual property rights

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, 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).

9.6 GENE BANKING OF MATERIAL FROM FOREIGN SOURCES

National gene banks generally focus on conservation of national AnGR but may also deal with exchange of genetic material between countries, such as operating as a regional gene bank (AU-IBAR, 2020) or participation in and supporting international research projects. 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, especially when a gene bank is taking over another collection.

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 all cases of transboundary movement of material, all veterinary and sanitary requirements for effective management of biosecurity risks associated with the transfer which are valid in the country receiving samples must first be met. In the European Union, for instance, intracommunity trade or transfer of germplasm of purebred animals require also zootechnical certificates (EC, 2017) and traceability and animal health requirements (EC, 2020). However, there may be situations where adherence to all current requirements is not possible (for example, when samples come from historical collections with unknown veterinary status). National veterinary authorities may grant exceptions for such cases, but exceptions must be negotiated, and samples must usually be documented, stored and handled separately from other gene bank materials.

Any samples acquired (physically accessed) from abroad after 12 October 2014 (when the Nagoya Protocol entered into force) are subject to the ABS legislative, administrative and policy measures that have been established by parties to the Nagoya Protocol (see Box 9.4). Therefore, the gene bank must: (i) be able to prove that the samples were accessed/acquired legally from the country of origin and/or legally obtained from other sources; (ii) document the terms and conditions of use and eventual transfer to third parties (PIC and MAT); and (iii) comply with the benefit-sharing obligations associated with the samples, if relevant.

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. All documentation should be stored in the gene bank database.

In situations where genetic material is transferred from one country to another exclusively for storage, and not used for research or breeding in the receiving country, the ABS permits may not be required. Nevertheless, some documentation must accompany the samples when they are registered in the gene bank of the receiving country, including a reference to the arrangements and the contract between the two countries.

The previous cryoconservation 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, in case of transboundary regional breeds, and in all other situations described earlier. In situations where AnGR are acquired from abroad, the new legal landscape established by the Nagoya Protocol becomes relevant. Transfer of material from local breeds is usually in the scope of ABS national access measures (Martyniuk *et al*., 2017; FAO, 2021).

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 the ABS-CH or be obtained directly form ABS NFP or ABS CNA. The gene bank should establish 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.

9.7 GENE BANKING AND ACCESS AND BENEFIT-SHARING

In summary, the gene bank management should consider ABS legislation (national and/or international) in the following situations.

- **Acquisition, when a gene bank is located in a country regulating access to their AnGR:** Gene banks, while developing their own ABS-related protocols and procedures, must balance these with relevant national ABS 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.
- **Acquisition, when a gene bank would like to access AnGR to enhance its own collection from a foreign country that regulates access to its AnGR:** Gene bank managers should become familiar with the legislative, administrative and policy measures on ABS in potential provider countries and understand the associated processes to obtain permits 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 be familiar with these procedures before submitting an access request. The country from which genetic material will be accessed is an important consideration, as countries differ substantially in their national ABS legislation and regulatory frameworks, and especially in practical implementation of ABS measures. This may mean considerable differences in terms of the time needed to complete a formal request process and the costs involved.

- **Acquisition, 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:** Some countries have decided not to regulate access to their genetic resources or to exempt domesticated genetic resources from their access measures (FAO, 2021). However, such counties may require self-registration of access in the database (such as Switzerland). Such registration is of high importance for gene banks, as it will provide a proof of the origin of the samples, when they were accessed, and other information. This is especially important when a gene bank is subject to monitoring of user compliance by the CNA of its own country.
- **Transfer of material obtained from abroad:** Gene banks must 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. Such information is important and must be sought. If the material from the provider country was accessed by the gene bank itself, it is important to confirm whether the original permit for collecting samples allowed transfer to third-party users and, if this is the case, to make the information on the permit available for potential users and to provide it with any material upon transfer. 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 CNA that issued the original permit, so that the potential user can contact that CNA to either seek a new permit for access to the material in the country of origin.
- **Documentation of samples from abroad:** The ABS-related information should be a part of any MAA or 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. Use of MAA and MTA should be integrated into the workflow of the gene bank. 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 and actual users to provide evidence of their legal access and compliance with the national ABS legislation of the provider country.

9.8 RECOMMENDATIONS FOR GENE BANK MANAGERS ON LEGAL ISSUES

Gene bank managers should have a thorough 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, and 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.

The gene bank must maintain a frequent and open communication with their stakeholders and raise awareness about the administrative procedures for its operation.

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SECTION 10
Capacity building and training

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10.1 INTRODUCTION

Knowledge and skills are critical components of smooth and effective operation of cryoconservation programmes. This section will emphasize 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* (FAO, 2007) is to "establish or strengthen national educational and research facilities." Education at academic institutions such as universities and research centres usually involves two distinct programme phases, that is, 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* (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 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* (FAO, 2007) 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 (IMAGE, 2020.) conducted training courses with partner institutions in Argentina, Colombia, Egypt and Morocco (see 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 offering great opportunities for networking.

BOX 10.1

Training courses for non-European partners in the IMAGE project

The European IMAGE project[1] 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 for Animal Genetic Resources*,[2] 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

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[1] **Innovative Management of Animal Genetic Resources Project (IMAGE).** 2020. Project funded by the Horizon 2020 Research and Innovation Programme of the European Union under Grant Agreement Number 677353. Cited 20 October 2020. www.imageh2020.eu

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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

Capacity building for gene banking in Iraq

Sahar Albayatti

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 challenges 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.

Box 10.2 details the approach Iraq has taken during their ongoing process of establishing a gene bank.

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 and 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, genetics, 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 this document, 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 guide on *Genomic characterization of animal genetic resources – Practical guide.* (FAO, 2023).

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, 2018). Practical training in the use of software for population management such as MoBPS (Pook, Schlather and Simianer, 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, that is, 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, 2011), 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.

10.6 REFERENCES

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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.

TABLE A2.1.1
Quality management checklist for animal gene banks

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 of 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?	☐	☐

(Cont.)

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 database restricted?	☐	☐
24. Is the database 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 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?	☐	☐

Source: **Innovative Management of Animal Genetic Resources Project (IMAGE).** 2020. Project funded by the Horizon 2020 Research and Innovation Programme of the European Union under Grant Agreement Number 677353. Cited 31 October 2020. www.imageh2020.eu

Annex 4.1
Application of linear programming to design a material collection strategy

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A4.1.1 BACKGROUND

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. An 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 noncommercial 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

$$\sum_b r_b X_b \leq B \qquad (2)$$
$$\sum_b r_b X_b \leq C \qquad (3)$$
$$\sum_b \leq B \qquad (4)$$

One can note that the 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, that is, sum over X_b cannot be greater than the total cryotank capacity.

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

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Compilation of the following tables will aid in estimation of the costs of gene banking.

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 is 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?

	Material			
Species	**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?

	Material			
Species	**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
Multispecies SNP array

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A5.1.1 BACKGROUND

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 (FAO, 2023). A standardized single nucleotide polymorphism (SNP) tool with information about all stored species can be a valuable decision-making tool. Two open access multispecies 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 (< USD 20/sample). Around 9 000 SNPs per species (see Table A5.1.1 below) were collected from whole genome sequence (WGS) data generated within the IMAGE project (IMAGE, 2020a) 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 major histocompatibility complex (MHC) region, if available; and
6. random SNPs genes located in genes within quantitative trait locus (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 (EMBI-EBI, 2021a) and the genotyped data containing a BioSample identification number can be uploaded to the European Variation Archive (EVA) (EMBI-EBI, 2021b). 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 (IMAGE, 2020b).

A5.1.2 REFERENCES

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TABLE A5.1.1
Numbers of SNP per species and marker characteristic for the two multispecies 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**

Source: Authors' own elaboration.

Annex 6.1
Guidelines for semen cryopreservation

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A6.1.1 INTRODUCTION

Health accreditation of collection, storage and implementation operations. Due to national and international specific regulations and agreements, operators wishing to implement the following procedures are advised to approach the relevant authorities to fully understand the regulations and consider the potential for corresponding constraints.

Know-how. Knowledge and skill are crucial for the success of the procedures and can only be 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 only if the semen quality is sufficiently high. Annex 6.2 describes the basic methods of semen evaluation. Each of the methods must be adapted to the species to which it is being applied.

Breeding factors and welfare. As explained in Sections 3 and 6 of these guidelines, semen cryopreservation must be conducted on males raised under the best conditions, and semen must be collected in the best stage of the donor's reproductive lifespan. For bulls, the website of the National Association of Animal Breeders – Certified Semen Services (NAAB-CSS) of the United States of America presents management guidelines (NAAB-CSS, 2022a) for bulls to help optimize health and welfare and thus semen quality and production.

A6.1.2 CRYOPRESERVATION OF CATTLE SEMEN

A6.1.2.1 Bull (dairy, beef, water buffalo) semen collection, dilution, cooling, and cryopreservation

The NAAB-CSS (2022b) also provides high-quality information for processing and freezing bull semen. Their documentation lists a variety of acceptable cryopreservation media, e.g. Egg yolk-Tris (hydroxymethyl aminomethane), milk and other acceptable freezing diluents, for use with bull semen as well as appropriate antibiotic treatments, and hygiene standards that must be used for international exchange. If holding/shipping the samples is necessary, then the Egg yolk-Tris media (method listed below) is recommended, whereas the milk-based media and other diluents are discouraged because the post-thaw quality is less. In addition, the sperm concentration specified in the protocol below is meant to be used with bulls of unknown fertility. If the fertility of a bull is known to be high, as determined with frozen-thawed semen and artificial insemination (AI), then the sperm concentration prior to freezing can be adjusted lower, resulting in the production of more straws/doses semen straws per ejaculate.

A6.1.2.2 Recommended protocol for semen collection

1. Collect semen from sexually mature bulls and ensure the sample is free of urine and other contaminants.
2. Determine the sperm concentration, ensure the sample has acceptable motility, and maintain samples at 37 °C.
3. Add antibiotics to the semen and cryopreservation media per the NAAB-CSS standards (NAAB-CSS, 2022b). Current recommendations for antibiotics include the use of Gentamicin sulfate, Tylosin and Linco-Spectin.
4. Dilute the samples in 15 or 50 ml tubes to 120 × 106 sperm/ml with 37 °C Tris-egg yolk A (TCA; see recipe below).
5. Place the samples in a 37 °C water bath and cool to 5 °C in 2 hours.
6. If the samples will be transported overnight, then, after cooling, the samples should be placed in an insulated shipping container with enough cold packs to maintain the samples at 5 °C for at least 24 hours.
7. Once the samples reach 5 °C, or when the samples arrive at the laboratory for freezing, samples are to be 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 × 106 sperm/ml.
8. Load the samples into 0.25 or 0.5 ml CBS or wick and powder (French) semen straws.

A6.1.2.3 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 minutes. 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.

A6.1.2.4 Recommendation for thawing and insemination

- Thaw samples for 30 seconds in a 37 °C 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 natural or synchronized estrus using single semen straws per insemination. However, either single or double (2 inseminations per estrus) inseminations may be performed.

A6.1.2.5 Recipes

The preferred semen cryopreservation media recipe is from Purdy and 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.

A6.1.3 CRYOPRESERVATION OF BOAR SEMEN

A6.1.3.1 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. The methods are described below as a supplement to the methods described in the previous guidelines (FAO, 2012).

1. 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.
2. 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.
3. Determine the volume, sperm concentration and sperm count of the sperm-rich ejaculate, and the volume of the total ejaculate (i.e. sperm-rich plus gel).
4. Dilute the required sperm-rich fraction 1:1 (volume to volume) with 37 °C semen extender in a 37 °C beaker.
5. 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.
6. Cool the sample to 23 °C in 1 hour and shield it from light during this time.
7. Cool the sample to 15 °C in 1.5 hours and maintain it at this temperature during any transportation. If the samples will be transported overnight, then, after cooling, place the samples in an insulated shipping container with sufficient cold packs to maintain the samples at 15 °C for at least 24 hours.
8. Upon receipt of the samples, centrifuge the diluted ejaculates for 10 minutes at 800 × g and 15 °C.
9. Remove the supernatant, combine the pelleted sperm by boar, determine the sperm concentration and ensure the sample has acceptable motility.

10. Dilute the samples to 600 × 106 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.
11. 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 × 106 sperm/ml.
12. Load the samples into 0.5 ml CBS or wick and powder (French) straws and freeze them using:
    - 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
    - a programmable freezer and the following curve: 5 °C to -8 °C at -20 °C/min; -8 °C to 120°C at 60 °C/min; -120°C to -140°C at -20°C/min.
13. Plunge the samples into liquid nitrogen for storage.

A6.1.3.2 Thawing and artificial insemination

Thaw samples for 20 seconds in a 50 °C water bath, and evaluate the motility 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 × 109 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 (one insemination per sow or gilt) (Martinez *et al.*, 2001, Roca *et al.*, 2003) with 1 × 109 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.

A6.1.3.3 Recipes

BF5 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 × 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

A6.1.4 CRYOPRESERVATION OF GOAT SEMEN

A6.1.4.1 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.

1. Collect semen from sexually mature bucks and inspect samples to ensure it is free of urine and other contaminants.
2. Determine the sperm concentration, ensure the sample has acceptable motility and maintain samples at 37 °C.
3. Add antibiotics to the neat semen and the cryopreservation media per the NAAB-CSS (2022b) standards. Current recommendations for antibiotics include the use of Gentamicin sulfate, Tylosin and Linco-Spectin.
4. Dilute the samples in a 15 or 50 ml tube to 400 × 106 sperm/ml with 37 °C Tris-egg yolk glycerol medium (TEYG) (Mook and Wildeus, 2008). See recipe below.
5. 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 the seminal plasma.
6. Dilute the sample 4 to 5 times with egg yolk and glycerol free TEYG medium, and centrifuge at 800 × g for 10 min. Determine the sperm concentration and dilute with TEYG as described previously.
7. Place the samples in a 37 °C water bath and cool to 5 °C over 2 hours.
8. 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.
9. Load the samples into 0.5 ml CBS or wick and powder (French) semen straws, and freeze them using:
   - 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
   - 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.
10. Plunge samples into liquid nitrogen for storage.

A6.1.4.2 Thawing an insemination

Thaw samples 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.

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).

A6.1.4.3 Recipe

Tris-egg yolk-glycerol (TEYG) diluent, for a 100 ml volume (pH to 6.8–7.0):

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

A6.1.5 CRYOPRESERVATION OF RAM SEMEN

Many factors contribute to success/fertility with these techniques. It is recommended that rams used for semen collections be at least 1-year-old and ewes be 4 to 6 years of age, although, 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 following removal of the controlled internal drug release (CIDR) synchronization device. 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 by testing multiple insemination times to identify an effective protocol that will result in the highest fertility possible.

A6.1.5.1 Semen collection and processing

1. Collect semen from sexually mature rams and inspect samples to ensure they are free of urine and other contaminants.
2. Determine the sperm concentration, ensure the sample has acceptable motility and maintain samples at 37 °C.
3. 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) 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.

A6.1.5.2 Semen cryopreservation using the SMEY diluent

1. Dilute ram semen samples to 1200 × 106 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 within a period of 45 to 60 min.
2. 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 × 106 sperm/ml.

3. Load the samples into 0.5 ml CBS or wick and powder (French) semen straws and freeze.

A6.1.5.3 Semen cryopreservation using the TEYG diluent

1. Dilute the ram semen samples drop-wise, in one step, with the TEYG freezing medium to 400 × 106 sperm/ml.
2. Cool the sample to 5 °C over 90–120 minutes.
3. 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.
4. Load the samples into 0.5 ml CBS or wick and powder (French) semen straws and freeze.
5. Samples are frozen by:
   - 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
   - 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.
6. Plunge the samples into liquid nitrogen for storage.

A6.1.5.4 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; (available from Pfizer Animal Health, New York, NY) followed by pregnant mare serum gonadotropin (PMSG) administration (400 IU, intramuscularly; total volume = 4 ml using an 18 gauge needle) 24 hours prior to, or at sponge removal;
- Artificial insemination: inseminations of 100 × 10^6 motile sperm are performed at 53 and again at 57 hours post-CIDR removal. To help in identification of an optimal insemination time for a flock/breed, a low dose insemination of 70 × 106 motile sperm in a single dose can be used over a range of insemination times and ewe groups. However, this approach will not result in maximal fertility when applied for the first time, but will rather 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.
- 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 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.

A6.1.5.5 Recipes

Skim milk-egg yolk (SMEY) cryopreservation medium (Paulenz, Ådnøy and Söderquist, 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 minutes.
- Cool the solution to room temperature and add 5 ml of egg yolk, at a minimum 1mg/ml streptomycin sulphate (or antibiotics according to NAAB-CSS standards (NAAB-CSS, 2020b) 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.

A6.1.5.4	12.112 g
Citric acid	6.8 g
Glucose	5.0 g
Glycerol	25 ml

Fill to 400 ml with distilled, deionized water and add the following:

- Egg yolk 100 ml (20% by volume); and
- Antibiotics (either NAAB-CSS standards (NAAB-CSS, 2020b) or at least 500 mg streptomycin sulfate).

A6.1.6 CRYOPRESERVATION OF HORSE SEMEN

Since the seventies, different methods of cryopreservation of stallion sperm have been developed (Pickett *et al*., 1975; Martin, Klug and Günzel, 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 is mostly used in France is described below.

A6.1.6.1 Collection

Stallions are subject 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.

A6.1.6.2 Freezing procedure

1. Dilute sperm in 50 ml Falcon tubes at 50 × 106 spermatozoa per ml in the first extender (INRA96) in the water bath at 37 °C.
2. Transfer the diluted sperm to a water bath at 22 °C for 10 minutes before centrifugation at 600g for 10 minutes.
3. Discard the supernatant and resuspend the pellet in INRA FreezeTM extender to obtain a concentration of 100 × 106 spermatozoa per mL.
4. Cool the diluted sperm at 4 °C for 75 minutes before packaging and sealing in polyvinyl chloride 0.5mL straws.
5. Freeze straws with a programmable freezer from 4 °C to -140 °C at a rate of -60 °C per minute, and then plunge into liquid nitrogen and store at -196 °C until use.

A6.1.6.3 Extenders

INRA96 and INRA Freeze™ extenders were patented by INRAE of France and licensed to IMV-Technologies (L'Aigle, France). The two extenders are produced and commercialized throughout the world by IMV-Technologies.

A6.1.6.4 Thawing

The thawing process consists of:

1. Plunge straws into a water bath at 37 °C for 30 seconds.
2. Wipe the straws before transferring the content into a tube:
   - containing INRA96 extender when quality parameters of sperm cells are evaluated; or
   - without an extender if thawed sperm is inseminated.

A routine insemination dose is composed of 8 straws (400 × 10^6 total sperm cells).

A6.1.6.5 Sperm evaluation

Sperm evaluation at thawing is usually based on microscopic evaluation of motility. However, an increasing number of laboratories are using an automated system, such as computer-assisted sperm analysis (CASA). For a recent review, see Amann and Waberski (2014).

This technology allows the measurement of other parameters such as sperm membrane integrity and organization, viability, acrosome integrity, oxidative level, DNA integrity, mitochondrial activity. Some of these parameters have been shown to be related to fertility (Barrier-Battut *et al.*, 2017).

A6.1.7 CRYOPRESERVATION OF CHICKEN SEMEN

As indicated in Section 6, there are different methods to cryopreserve chicken semen. One of them, the glycerol method (Tselutin, Seigneurin and Blesbois, 1999; Blesbois *et al.*, 2007; Thélie *et al.*, 2019; Lin, Blesbois and Carvalho, 2021) is standardized for males with different fertility levels, while the others (Woelders *et al.*, 2006; Sasaki *et al.*, 2010; Blesbois, 2011, Abouelezz, Sayed and Santiago-Moreno, 2017; Thananurak *et al.*, 2020) are recommended for highly fertile males. This annex describes the standardized glycerol method and one other simple method updated in 2020, the "dimethyl formamide (DMF) method". For both methods, the instructions are based on eventual storage in straws, as this is the most practical approach for cryoconservation. Irrespective of the method used, great care must be taken, and all steps must be undertaken as rapidly as possible, and involve a limited number of samples to ensure efficiency.

A6.1.7.1 Glycerol method of chicken semen cryopreservation

Semen collection, treatment and freezing

1. Collect semen from each male (Burrows and Quinn, 1937) in plastic tubes containing 200 µl LPC diluent at 20–25 °C (Lake and Stewart, 1978; see recipe below). This is the Dilution 1. The time of collection must be of a maximum 10 minutes 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 10minutes.
3. Mix each sample with a volume of LPC (at 4–5 °C) diluent equivalent to twice the initial semen volume containing 22 percent glycerol (Dilution 3). So, the final semen dilution will be 1:3, and the final glycerol amount 11 percent.
4. Equilibrate for 10 minutes at 4–5 °C with gentle shaking.
5. Put the diluted semen in 0.5 ml straws previously identified per male, then make a bubble at the top of the straws, seal the straws, and transfer them in a programmable freezer.
6. Freeze the straws according to the following programme: -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 the liquid nitrogen tank.
8. Record the placement of the samples in the tank, and register in the gene bank database.

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 minutes in a 1 litre beaker 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 into separate beakers which are identified according to the male and containing an amount of LC diluent (Lake and Stewart, 1978; see recipe below) corresponding to dilution step a in Table A6.1.1. The volume of diluent required depends on the number of 0.5 ml straws thawed per male.
3. Undertake the dilution steps b to f (in Table A6.1.1), waiting 2 minutes between each step. This permits slow removal of the intracellular glycerol.
4. Centrifuge each sample for 15 minutes at 500 g, 4–5 °C.
5. Discard the supernatant (which now contains 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 intravaginal insemination of a mean of 200 to 400 million sperm/female. Great caution must be used, since frozen-thawed semen is more sensitive to all variations than fresh semen, and because 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 to avoid opposite vaginal peristalsis.

Recipes

LPC diluent

Add the following to distilled/de-inonized water to obtain a final volume of 1 L:

Magnesium acetate	0.7 g
Sodium glutamate	5.0 g
Potassium acetate	5.0 g
D-Fructose	8.0 g
BES	1.0 g
Polyvinylpirrolidone 10 000	3.0 g
NaOH 1N	4 ml

The final diluent will have pH 7.1 and osmotic pressure 340 mOsm.

In the case of semen with low expected fertility, add 10 mM Valine (Bernal-Juarez *et al.*, 2020).

LC diluent

Add the following to distilled/deionized water to obtain a final volume of 1 L:

Magnesium acetate tetrahydrated	0.8 g
Potassium citrate monohydrate	1.28 g
Sodium glutamate	19.2 g
D-fructose	6.0 g
Sodium acetate	5.1 g
TES	5.0 g
NaOH 1N	5.2 ml

TABLE A6.1.1
Dilution volumes (ml) before centrifugation and pellet resuspension after centrifugation

	Number of straws (0.5 ml)								
Dilution step	**2**	**4**	**6**	**10**	**12**	**20**	**24**	**30**	**40**
A	0.07	0.13	0.20	0.33	0.39	0.65	0.72	1.00	1.30
B	0.18	0.36	0.54	0.90	1.10	1.80	2.00	2.70	3.60
C	0.33	0.66	0.99	1.65	2.00	3.30	3.66	2×2.48	2×3.30
D	0.60	1.20	1.80	3.00	3.60	2×3.00	2×3.32	2×4.50	4×3.00
E	1.24	2.50	3.71	6.25	2×3.75	3×4.12	4×3.40	4×4.70	6×4.12
F	1.58	3.20	4.73	8.00	2×4.80	4×4.00	4×4.33	6×4.00	7×4.50
Total diluent vol (ml)	4.00	8.05	11.97	20.13	24.00	40.00	43.94	60.30	80.00
Straw vol (ml)	1.00	2.00	3.00	5.00	6.00	10.00	12.00	15.00	20.00
Total vol (ml)	5.00	10.00	14.97	25.13	30.00	50.00	55.94	75.30	100.00
15 ml tubes (N)	2	3	4	8	10	12	14	20	32
Vol/tube (ml)	2.50	3.35	3.74	3.13	3.00	4.16	4.00	3.75	3.13

Source: Authors' own elaboration.

Table A6.1.1 shows the partial and total diluent volumes required according to the number of 0.5 ml straws to be filled. 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.

A6.1.7.2 DMF method of chicken semen cryopreservation

The second method of chicken semen cryopreservation was designed to be as simple as possible. It does not need a programmable freezer, and the internal cryoprotectant (dimethyl formamide, 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 semen from each male according to recommended massaging procedures (Burrows and Quinn, 1937) at room temperature (mean of 25 °C).
2. At room temperature, dilute very rapidly the semen with BHSV-based diluent (see recipe below) at a 1:2 ratio (semen to diluent).
3. Slowly cool the semen to 5 °C by placing it in a temperature-controlled refrigerator.
4. When the semen has reached 5 °C, add another fraction of diluent prepared at 5 °C (i.e. equivalent to initial semen volume and containing DMF 24 percent). The final semen dilution will thus be 1.3 and the final percentage of DMF in the diluted semen will be 6 percent.
5. Load the diluted semen in 0.5 ml plastic straws sealed with PVP powder, and equilibrate for 15 minutes at 5 °C.
6. After equilibration, lay the filled straws horizontally onto a freezing rack, place them 11 cm above the surface of the LN (-35 oC) for 12 minutes, followed by 3 cm above liquid nitrogen vapor (135 oC) for 5 minutes, and subsequently immersed in LN.

7. Transfer the straws to a LN tank, once again recording the location in the database.

Thawing and insemination

1. Thaw the straws in a water bath at 5 °C for 5 minutes.
2. Wipe the outside of the straws, empty the semen into separate beakers for each donor male.
3. Conduct the inseminations intervaginally without delay, following the same precautions as for the glycerol method. The insemination must be conducted a minimum of 3 hours before or after the lay, to limit the semen rejection due to the lay peristalsis

Recipe

BHSV-based diluent (Schramm, 1991),

The following are dissolved in 1L of double-distilled water:

Glucose	5.0g
Inositol	2.5g
Sodium glutamate	28.5g
Magnesium acetate tetrahydrate	0.7g
Potassium acetate	5.0g

Supplement with Serine (4 mM) and Sucrose (1 mM) (Thananurak *et al*., 2019; 2020).

A6.1.8 CRYOPRESERVATION OF GUINEA FOWL SEMEN

Two common methods are used for guinea fowl semen cryopreservation, one that uses the cryoprotectant DMF and straw packaging (Seigneurin *et al.,* 2013), and the other using ethylene glycol and a packaging in glass ampoules (Váradi *et al*., 2013). The method of using straws is described here, because straws are the most popular storage vessel for animal cryobanking.

A6.1.8.1 Semen collection, treatment and freezing

1. Collect semen by using the massage technique (Burrows and Quinn, 1937).
2. Dilute semen 1:1 in BHSV diluent (see recipe above) at room temperature (20–25 °C).
3. Cool diluted semen to 4 °C for 15 minutes (must be checked).
4. Add a second volume of BHSV diluent at 4 °C, containing 18 percent DMF. The final semen dilution is thus 1:2, with a final percentage of DMF of 6 percent.
5. Equilibrate for 4 minutes at 4 °C and then load the diluted semen into 0.5 ml plastic straws sealed with PVP powder.
6. Freeze the straws in a programmable freezer with the freezing rate of 30 °C/min from +4 °C to -40 °C.
7. Transfer the straws into the liquid nitrogen storage tank, recording the storage location.

A6.1.8.2 Thawing and insemination

1. Straws are thawed in a water bath at 40 °C for 45 seconds.
2. Wipe the outside of the straws, then empty the semen into identified beakers.
3. Intravaginal inseminations are then conducted rapidly with the same precautions as for chicken sperm methods.

A6.1.9 CRYOPRESERVATION OF GANDER SEMEN

Methods used for cryopreservation of gander semen (male geese) depend on species. Different methods have been proposed for Asian or European ganders (Tai *et al*., 2001; Lukaszewicz, 2002), which use different volumes of the same cryoprotectant, that is, dimethyl acetamide (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) for Asian ganders.

A6.1.9.1 Semen collection, treatment and freezing

1. Collect semen by massage of the gander (Burrows and Quinn, 1937).
2. Dilute 1:1 in Tai diluent containing 15 percent egg yolk (Tai *et al.*, 2001) at room temperature (20–25 °C).
3. After 15 minutes at room temperature, add 2 new volumes of diluent containing 18 percent DMA. The final semen dilution is thus 1:3 and the final percentage of DMA is 9 percent.
4. Equilibrate for 10 minutes, and then load the diluted semen in 0.5 ml plastic straws sealed with PVP powder.
5. Place straws on dry ice for 30 minutes or in nitrogen vapor.
6. Transfer the straws into a LN storage tank and record storage location.

A6.1.9.2 Thawing and insemination

1. Straws are thawed in a water bath at 40 °C for 45 seconds.
2. Wipe the outside of the straws, empty the semen in separate beakers for each male.
3. Intravaginal inseminations are then conducted rapidly following the same precautions as for chicken insemination methods.

A6.1.10 SEMEN OF OTHER DOMESTIC BIRD SPECIES

As described in Section 6, routine protocols for cryopreservation of turkey semen are not yet fully available, but work by Di Iorio *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.

A6.1.11 CRYOPRESERVATION OF HONEY BEE SEMEN

Some of the most widely used honey bee 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, because the sterility and quality of the raw yolk

derived from chicken eggs reared under different 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, Herr and Sheppard (2012).

A6.1.11.1 Cryopreservation with egg yolk-free diluent

The following protocol describes a method to cryopreserve ~50 µl of semen (25 µl of semen plus 25 µl of cryoprotective diluent) in a 0.25 ml PETG sperm straw (e.g. Cryo Bio System™, model 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 fragile, move in an amoebic undulatory process. Therefore, it is prudent to avoid rapid pipetting, vortex mixing or centrifuging at unprescribed rates. Mixing is preferably done by using a capillary tube with a blunt end made by melting it. 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.

Prerequisites

- Solution A – Semen extender (see Table A6.1.2) without dimethyl sulfoxide (DMSO).
- Solution B – A 2 × stock solution of semen extender containing 20 percent DMSO (see Table A6.1.2) (keep chilled in a refrigerator).
- Solution C – Simple saline comprising of the 0.2M Tris buffer pH 7.2 with 1.12 percent potassium chloride and 0.88 percent 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.

TABLE A6.1.2
The content of an extender used for semen collection, storage and shipment

Constituent	Concentration	Milligrams/100 ml of the extender
penicillin	10 k Units	N/A
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

Source: **Rajamohan, A., Danka, R.G., Hopkins, B.K. & Rinehart, J.P.** 2020. A non-activating diluent to prolong in vitro viability of Apis mellifera spermatozoa: Effects on cryopreservation and on egg fertilization. *Cryobiology*, 92: 124–129. https://doi: 10.1016/j.cryobiol.2019.11.045

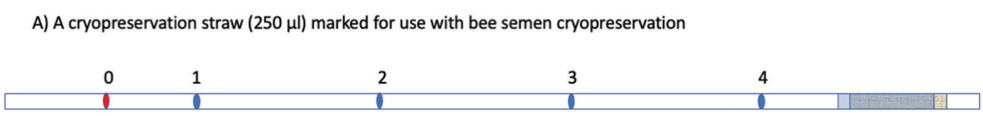
From the open-end tip, using a permanent marker, mark a spot every 25 mm, and also mark another spot at 15 mm from the open tip in a different colour. There should thus be 5 spots on the straw as shown in Figure A6.1.1 below.

- A 1 ml syringe barrel with appropriate silicone connectors to connect the straw (see Figure A6.1.2)
- Polyvinyl alcohol straw sealing powder.

Semen collection, dilution, cooling and freezing

1. Collect semen from the eversion of the endophallus. 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 coloured 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, keep the tip of the capillary immersed in an Eppendorf tube 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 can 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 (see Figure A6.1.2). 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 its 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.

FIGURE A6.1.1
Straw markers for drone semen freezing



Source: Authors' own elaboration.

FIGURE A6.1.2
Syringe to load bee semen

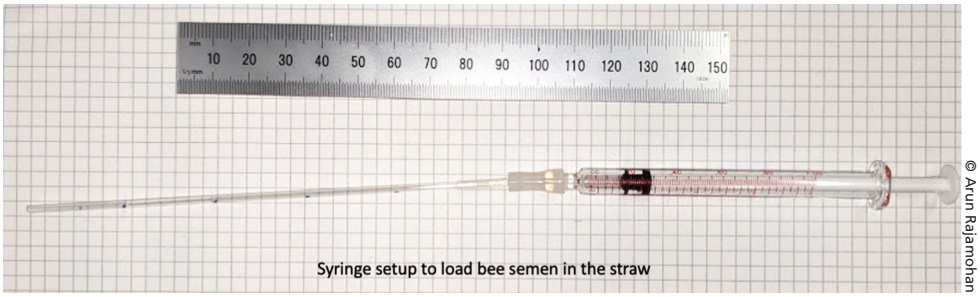
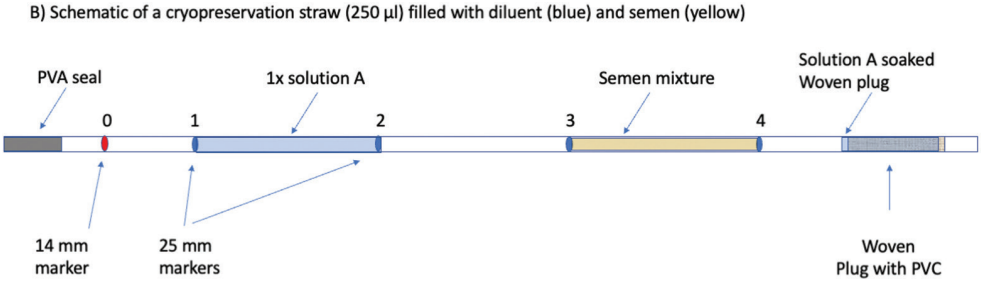


FIGURE A6.1.3
Straws filled with semen



Source: Authors' own elaboration.

10. 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 A6.1.3.
11. 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.
12. Transfer the straw to the programmable freezer set at 4 °C and begin the programmed freezing to -45 °C at 3 °C/min.
13. When at -45 °C, the straw is removed from the freezer and plunged into liquid nitrogen.
14. The straw is thereafter transferred to visotube which is placed in a daisy goblet (e.g.Cryo Bio Systems™) and transferred as fast as feasible into a LN storage canister and container.

Thawing and insemination

1. Rapidly retrieve the straw from the LN, plunge it into a 37 °C water bath and agitate until the frozen contents have visibly thawed.
2. Empty the contents into a sterile 0.2 ml PCR vial by cutting both ends of the straw. The vial is then 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 × g for 10 minutes. Most of the supernatant can then be removed, and an equal volume of replacement solution 1 is then added. Mix the contents very gently using a sealed tip capillary for not more than 20 seconds. The reconstituted semen sample can then be stored at 14 °C in the dark.
4. Artificial insemination is performed as in the following procedure "Conventional cryopreservation using egg yolk cryoprotectant diluent."

A6.1.11.2 Conventional cryopreservation using egg yolk cryoprotectant diluent

Semen collection is carried out as described for the previous method by the eversion of the endophallus of the drone. The cryopreservation diluent includes three components: 50 percent Harbo's buffer, 25 percent DMSO cryoprotectant, and 25 percent chicken egg yolk.

- Harbo's buffer consists 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 with a 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 (see Figure A6.1.3). The ends of the straws are then heat sealed or sealed using commercially available ultrasonic straw sealer. Straws are then labelled appropriately.

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 °C /min. Once the samples reach -40 °C, straws are removed one at a time, and rapidly plunged into liquid nitrogen and packed in a goblet for storage. The storage location should be recorded in the gene bank database.

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 (Schley, 2021) 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's syringe (Harbo, 1985; Cobey, Tarpy and Woyke, 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 CO2 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
Evaluation of sperm number and quality

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A6.2.1 INTRODUCTION

Semen cryopreservation is an important tool in ensuring the long-term survival of a given animal genetic resource. 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 to assess 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 sperm 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.

A6.2.2 SPERM CONCENTRATION

A6.2.2.1 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.

A6.2.2.2 Evaluation by haemocytometry

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.

A6.2.3 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, that is, (i) gross motility and (ii) individual motility.

A6.2.3.1 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.

A6.2.3.2 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, such as 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

A6.2.3.3 Sperm motility variables objectively evaluated by computer-assisted semen analysis

The percentage of total motile spermatozoa, the percentage of spermatozoa showing non-progressive motility, and the percentage showing progressive motility are recorded by the CASA system. Sperm movement characteristics such as curvilinear velocity (VCL), straight-line velocity (VSL), average path velocity (VAP), amplitude of lateral head displacement, beat-cross frequency, are also analysed by CASA. Three progression ratios, expressed as percentages, are calculated from the three velocity measurements described above: linearity (calculated as VSL/VCL × 100), straightness (calculated as VSL/VAP × 100), and wobble (calculated as VAP/VCL × 100). A minimum of 500 sperm tracks should be evaluated.

A6.2.4 VIABILITY/PLASMA MEMBRANE INTEGRITY

The sperm viability is evaluated by dyes related to the evaluation of plasma membrane integrity/permeability/elasticity, and may be analysed by vital staining (e.g. eosin-nigrosin), the hypo-osmotic swelling test (Jeyendran *et al.*, 1984), or fluorescence microscopy (e.g. using the fluorochromes propidium iodide and SYBR14 dye). During sample staining with eosin-nigrosin, eosin Y dye enters dead sperm cells (i.e. cells with damaged plasma membrane) and stains them red.

A6.2.4.1 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 coloured spermatozoa are deemed to be alive (Chalah and Brillard, 1998; Chalah *et al.*, 1999).

A6.2.4.2 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. 25 µl 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. The semen is incubated for 30 minutes at 37 °C.
3. Fix the sample in buffered 2 percent glutaraldehyde solution at 37 °C.
4. Count 200 sperm and calculate the proportion of sperm with coiled tails (i.e. with a functional membrane) under a phase-contrast microscope.

A6.2.5 SPERM MORPHOLOGY

Abnormally shaped or damaged sperm are less likely to be capable of fertilization than normal sperm (Berndtson, Olar and Pickett, 1981). Fixing samples in 2 percent glutaraldehyde buffered solution at 37 °C 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: (i) primary abnormalities which are assumed to have occurred in the testes; and (ii) secondary abnormalities

which arise later, in the epididymis or in the ejaculate. For most species, the proportion of sperm considered as normal should be more than 70 percent. However, this may vary with the standard of the species.

A6.2.6 MIXED EVALUATION VIABILITY-SPERM MORPHOLOGY

Combined analysis of viability and morphology can be done by using Eosin-nigrosin staining and counting of viable sperm (not red), and of normal and abnormal sperm. The protocol for Eosin-nigrosin staining is as follows:

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 1 000 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 in percentage.
7. Discard semen if the proportion of abnormalities is too high (e.g. more than 30 percent).

A6.2.7 ACROSOME INTEGRITY

A6.2.7.1 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. Mix 25 µl of semen sample with 500 µl of buffered 2 percent glutaraldehyde solution at 37 °C.
   - Buffered solution (BL-1):
   - Glucose 2.9 g
   - Sodium citrate $2H_2O$ 1 g
   - Sodium bicarbonate 0.2 g
   - Distilled water: 100 mL

2. Count 200 sperm and calculate the proportion of sperm with alterations in the apical ridge, such as 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).

A6.2.7.2 In birds

In birds, the percentage of sperm with full acrosomes may be determined under a phase contrast microscope by examining 200 sperm previously fixed with glutaraldehyde and stained with aniline blue (1 000x 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 1 000x magnification. 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).

A6.2.8 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 analyse 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, Evenson and Saacke, 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.

A6.2.8.1 TUNEL assay for mammals and birds

1. Semen samples are fixed in 4 percent formaldehyde (volume to volume) in PBS solution at a concentration of 5×10^6 sperm/mL for 30 minutes.
2. Place 10 µl of the sample onto a glass slide previously demarcated by a liquid-repellent slide marker pen for staining procedures and left to dry on a thermic plate set at 37 °C.
3. The slides are permeabilized with 0.1 percent Triton X-100 (volume to volume) for 5 minutes in a humidified chamber at room temperature and washed with PBS.

4. The slides are then incubated for 1 hour in a humidified chamber in the dark at 37 °C with the TUNEL reaction mixture, which contains terminal deoxynucleotidyl transferase (TdT) plus a TMR-Red label.
5. Wash the slides with PBS, stain with Hoechst 33342 (1 mg/mL) and mount them with fluoromount aqueous mounting medium.
6. Count 200 sperm and calculate the proportion of sperm with damaged DNA (TUNEL positive; i.e. sperm with red fluorescence) under an epifluorescence microscope.

A6.2.8.2 TUNEL assay for bee sperm

(modified from Wegener *et al.*, 2012):

1. Diluted raw semen 1:500 in "simple saline" (0.2 M Tris buffer pH 7.2 containing 1.12 percent potassium chloride and 0.88 percent sodium chloride).
2. Smear a thin layer of 20 µl of the above sample on a charged slide (e.g. Globe Scientific) and air dried.
3. A positive control is treated with DNase I. A negative control is prepared with no treatments.
4. Treat the slides with 4 percent paraformaldehyde in PBS with pH adjusted to 7.4 for 1 hour, and then gently rinsed in PBS at least three times.
5. Permeabilization of the cells is done by immersing the slides for 2 minutes in a 0.1 percent solution of sodium citrate containing 0.1 percent triton X-100 on ice or in a refrigerator.
6. After the slides are washed 2–3 times in PBS and air dried, add 50 µl of TUNEL reagent (e.g. Roche/Sigma-Aldrich) to each coverslip and incubate the slides at 37 °C in the dark in a Petri plate with a moist filter paper.
7. The slides 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. Photograph the slide on a 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 oocyte and embryo cryopreservation

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A6.3.1 INTRODUCTION

Know-how is essential for successful implementation of the procedures. Table A6.3.1 shows key literature for embryo cryopreservation for both domesticated and wild animals, which will provide an introduction to methods, but true know-how is acquired with time through experience and relevant training. We therefore recommend contacting the authors of the guidelines, if necessary.

To comply with national and international specific regulations and agreements, operators wishing to implement the following procedures are advised to approach the relevant authorities.

TABLE A6.3.1
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, Leibo and Mazur,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, Niwa and Iritani,1982
baboon	Live birth following cryopreservation and transfer of a baboon embryo	Pope, Pope and Beck,1984
human	Two pregnancies following transfer of intact frozen-thawed embryos	Zeilmaker *et al.*, 1984

(Cont.)

Species	Paper	Author(s), year
African Eland antelope (*Tragelaphus oryx*)	Cryopreservation followed by successful transfer of African Eland antelope (*Tragelaphus oryx*) embryos.	Dresser, Kramer and Dahlhausen, 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. silenus*)]	Macaque monkey birth following transfer of *in vitro* fertilized, frozen-thawed embryos to a surrogate mother	Cranfield *et al.*, 1992
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 (*ustela putorius*)	Lindeberg *et al.*, 2003
camel (*Dromedary camel*)	Offspring resulting from transfer of cryopreserved embryos in camel (*Camelus dromedarius*)	Nowshari, Ali and Saleem, 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, Gomez and Dresser, 2006
pig-tailed 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

Source: Authors' own elaboration.

A6.3.2 CRYOPRESERVATION OF BOVINE EMBRYOS

The method described here is a summary of the method described for bovine embryos in Appendix G of the previous guidelines on Cryoconservation of animal genetic resources (FAO, 2012).

A6.3.2.1 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 at around room temperature (20 to 30 °C). Freezing should be done as soon as possible after collection, that is, 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 percent 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 seconds in total) to remove or deactivate any viruses. Trypsin wash is sterile porcine-origin trypsin (1:250) in Hank's balanced salt solution at a concentration of 0.25 percent.
6. Wash the embryos an additional five times in PBS – antibiotic solution with 2 percent BSA.
7. Equilibrate the embryos at room temperature for ten minutes in PBS with 10 percent fetal calf serum and 10 percent glycerol.

A6.3.2.2 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 contacting 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.

A6.3.2.3 Thawing

1. Select the appropriate straw from the liquid nitrogen storage tank. *Important!* Do not bring the straws up above the frost line of the liquid nitrogen tank (the neck of the tank) until the correct straw is identified for embryo transfer.
2. Thaw the straw rapidly in a water bath at 20 °C for 30 seconds, or 39 °C for 8 to 25 seconds, depending on the initial embryo-freezing rate. Then, cut the ends of the straw and remove the embryo. Rehydrate the embryo in 1 M (molar) sucrose solution for 10 minutes; then reduce the sucrose concentration in a stepwise procedure.

A6.3.2.4 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.

A6.3.3 CRYOPRESERVATION OF PIG EMBRYOS

In pigs, due to a large amount of lipids inside the embryonic cells and to high cold-sensitivity, it is recommended to apply an ultra-rapid vitrification method to cryopreserve embryos. The vitrification procedure described here is currently being practiced in several laboratories (Cuello *et al.*, 2010), and was also validated in the IMAGE European project (Guignot *et al.*, 2019).

A6.3.3.1 *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 hours after the onset of estrus with 3×10^9 spermatozoa per insemination per female.

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 anaesthesia is maintained by inhalation of 3 percent 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 $g.L^{-1}$ polyvinyl-alcohol (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 an intact zona pellucida should be selected for cryopreservation.

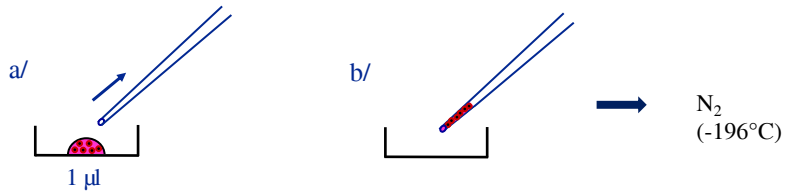
A6.3.3.2 Embryo cryopreservation

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 to 7 embryos are vitrified together using a four-well multidish.

Vitrification

Embryos are washed twice at least for 5 minutes twice in Talp-Hepes containing 0.1 $g.L^{-1}$ PVA. They are then transferred in the same medium complemented with 5 percent ethylene glycol (EG) and 7.5 percent dimethyl sulfoxide (DMSO) (equilibration solution: ES) for 3 minutes. Finally, they are incubated in vitrification solution (VS), i.e. Talp-Hepes containing 0.1 $g.L^{-1}$ PVA, 16 percent EG, 16 percent DMSO and 0.4 M sucrose, for 1 minute. During the last step, embryos are placed in a 1-µl droplet, and then loaded together by capillary action into the narrow end of superfine open pulled straws (SOPS). Straws with embryos are then plunged horizontally into liquid nitrogen (see Figure A6.3.1).

FIGURE A6.3.1
Vitrification of embryos in a superfine open pulled straw (SOPS)



Note: The embryos are in the red "liquid".
Source: Authors' own elaboration.

Thawing

At thawing, straws are held in the air for 2 seconds, and the narrow end is immerged in a well of four-well multidish containing 1 000 µL Talp-Hepes with 0.1 $g.L^{-1}$ PVA and 0.13 M sucrose. The embryos are pulled out of the straw by capillary action, rinsed in this medium for 5 minutes, and then in Talp-Hepes 0.1 $g.L^{-1}$ PVA without sucrose for 5 minutes. After this, the embryos are ready for transfer.

A6.3.3.3 Embryo transfer

Before transfer, host females should be synchronized. 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 anaesthesia is maintained by inhalation of 4 percent isoflurane. After a mid-ventral laparotomy, 30 blastocysts are transferred to the upper end of one uterine horn. Pregnancy can be assessed by ultrasonography at around 25 days post estrus. Farrowing is expected around 115 days after estrus, that is, 3 months, 3 weeks and 3 days.

A6.3.4 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 still 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* produced embryos. The slow freezing method described here 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 percent for both NSER and laparotomy techniques, and the number of viable embryos recovered after superovulation varies considerably, on average 4 to 6 per female.

A6.3.4.1 *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.

A6.3.4.2 Embryo collection and quality of the cryopreserved embryo

Prior to embryo recovery, the ovaries may be evaluated by either ultrasonography or laparoscopy 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 anaesthesia is maintained by inhalation of 1.5 percent isoflurane. Lidocaine (5 $mg.kg^{-1}$) diluted up to 1 percent 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 catheter firmly into a 90-mm Petri dish or 50 mL Falcon tube.
- At the utero-tubal junction, place a catheter through a small incision made into the lumen. Inject at least 30 mL/uterine horn (totalling 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.

Nonsurgical embryo recovery

- Donors do not need to be fasted before nonsurgical embryo recovery (NSER).
- A hormonal relaxation treatment is needed. In sheep, 1 mg estradiol benzoate i.m. and 37.5 µg cloprostenol i.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 i.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 percent acepromazine maleate (0.1 $mg.kg^{-1}$ bodyweight, i.m.) 20 minutes before flushing followed by 2 percent lidocaine (2 mL/animal) via the epidural (S5-C1) route 5 minutes before flushing. Right after specula introduction, a 2 percent 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, totalling 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.

A6.3.4.3 Embryo cryopreservation (slow freezing)

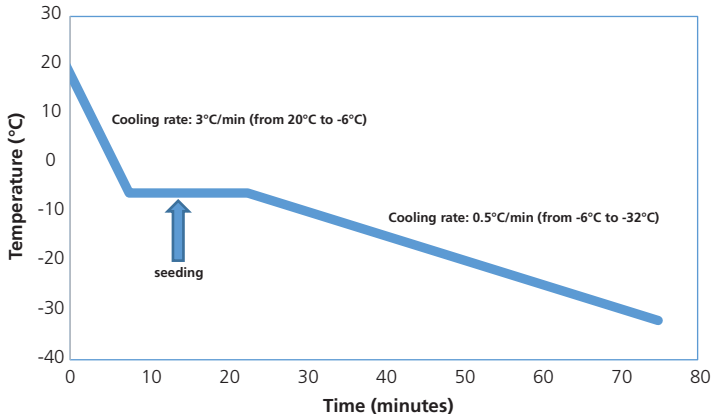
Freezing

- Embryos collected from a donor female must be kept at room temperature for 10 minutes in PBS with either 0.4 percent of BSA or 10 percent of fetal calf serum.
- Equilibrate the embryos using 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 percent 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 (see Figure A6.3.2).
- 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.

Thawing

- Select the appropriate straw from the 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.

FIGURE A6.3.2
Cooling rates during slow freezing of small ruminant embryos



Source: Authors' own elaboration.

- Embryos can be either evaluated at this stage or directly transferred to the prepared recipient.

A6.3.4.4 Embryo transfer

Embryo transfer by a semi-laparoscopic procedure is used worldwide in small ruminants. At Day 5.5 or 6.5 after ovulation, either ultrasonography or laparoscopy is performed to detect the presence, number and viability of corpus lutea. For the recipients, 24 hours of fasting is recommended. Through a tiny abdominal incision, the anterior portion of the uterine horn corresponding 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 at 25 to 30 days post estrus. Progeny are expected around 150 days after estrus.

A6.3.5 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 percent can be obtained (90 percent for young maiden or fertile mares, but only 10 to 20 percent 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) requires 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.

A6.3.5.1 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.

A6.3.5.2 Embryo cryopreservation

Blastocoel fluid aspiration

The fluid aspiration can be done with micromanipulator equipment under a microscope (see Figure A6.3.3). 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 bevelled 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 percent to 90 percent (Choi *et al.*, 2011; Guignot *et al.*, 2014, 2015; Diaz *et al.*, 2016; Sanchez *et al.*, 2017). The micropipette is pulled out of the embryo and the embryo is ready for vitrification procedure.

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 (see Figure A6.3.4) and the embryo is placed in a medium without protein (for example, Ringer's lactate solution), so it will be attached to the bottom of the dish.

FIGURE A6.3.3
Fluid aspiration under microscope

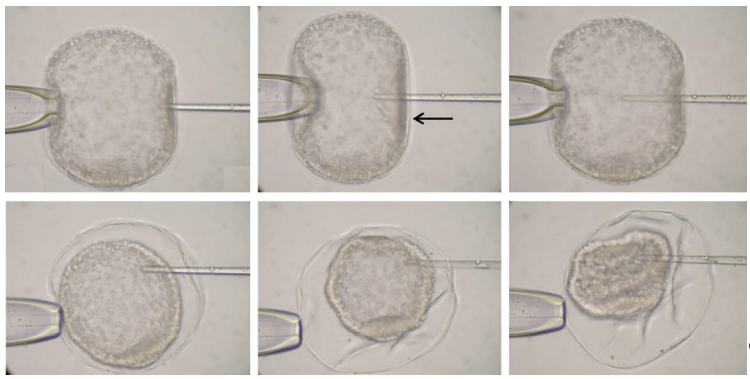
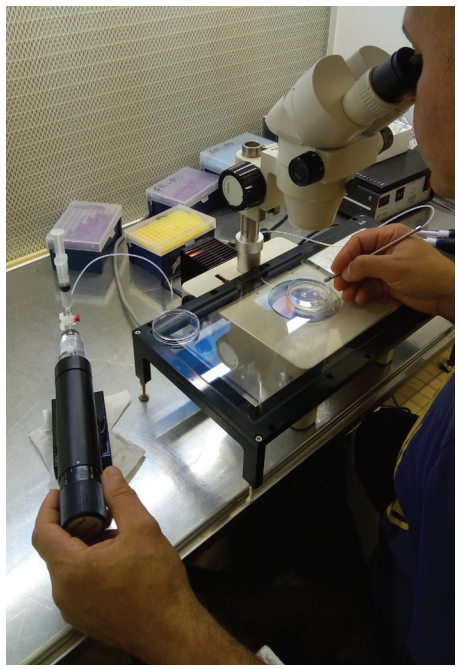


FIGURE A6.3.4
Fluid aspiration under stereomicroscope



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Vitrification

The embryo is vitrified immediately after fluid aspiration using the OPS procedure, with one embryo per straw. Three steps are performed before vitrification; all are done at 38.5 °C.

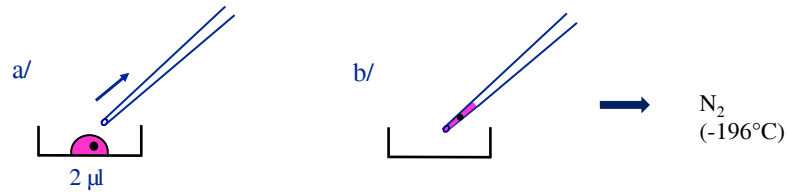
1. The embryo is placed in EHM supplemented with 20 percent of fetal calf serum (EHM serum) for 2 × 5 minutes;
2. the embryo is placed in EHM serum containing 1.5 M ethylene glycol (EG) for 5 minutes; and
3. the embryo is placed in EHM serum containing 7 M EG supplemented with 0.6 M galactose for 30 seconds.

During this last step, the embryo is loaded by capillary action as a 2-µL droplet into the narrow end of one open pulled straw (OPS). The OPS with the embryo is then plunged horizontally into liquid nitrogen (see Figure A6.3.5). As OPS is not a closed straw, it is possible to use another device, such as high security straw, which is sealed before plunging it into liquid nitrogen.

Thawing

At thawing, straws are held in the air for 2 seconds and the narrow end is immerged in a well of four-well multidish with 1200 µl of EHM serum containing 0.22M sucrose. Then, the embryo is pulled out of the straw by capillary action, rinsed first in this medium for 3 minutes, and then, in EHM serum with 0.13 M sucrose for 3 minutes, and finally placed

FIGURE A6.3.5
Vitrification in an open pulled straw



Source: Authors' own elaboration.

in EHM serum without sucrose. In the case of closed straws, the sealed ends of the straw must be cut before immersion in medium. The embryo is then ready for transfer.

Future improvement of this described technique of vitrification may be the possibility, to use two cryoprotectants in the third step of the procedure to reduce the amount of cryoprotectant necessary to vitrify the embryo.

A6.3.5.3 Embryo transfer

Embryo transfer is a commonly used procedure in equine breeding worldwide. The embryos are transcervically transferred to host females at Day 5 or 6 after ovulation, one blastocyst per female. The embryos are loaded into an embryo transfer gun and transferred within 5 minutes 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.

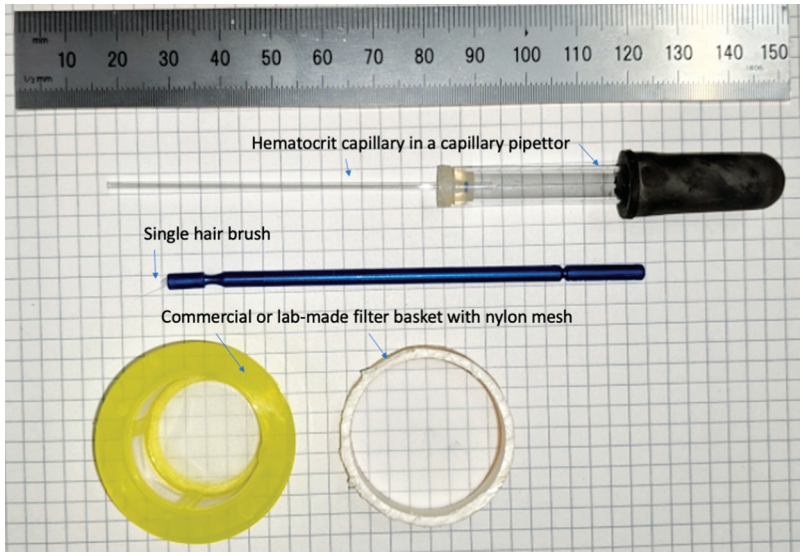
A6.3.6 CRYOPRESERVATION OF HONEY BEE EMBRYOS

As mentioned in Section 6, the method to cryopreserve honey bee embryos is technically demanding and uses non-standardized components to assist in the process. A protocol for this process is given below. While this method is still in the experimental stage, it is currently being routinely used to obtain viable honey bee embryos and larvae for grafting into hives or reared *in vitro* (Rajamohan, Danka and Reinhart, 2020).

A6.3.6.1 Prerequisites

- 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 ones as shown in Figure A6.3.6
- Single filament brush to handle the eggs
- Capillary tubes with internal diameter of 1 or 2 mm and capillary tube fillers (Fisher microhematocrit capillaries are perfect)
- Nuclepore polycarbonate membrane (e.g. Millipore, 0.2 µm, PVP coated)

FIGURE A6.3.6
Equipment for honey bee embryo preparation for freezing

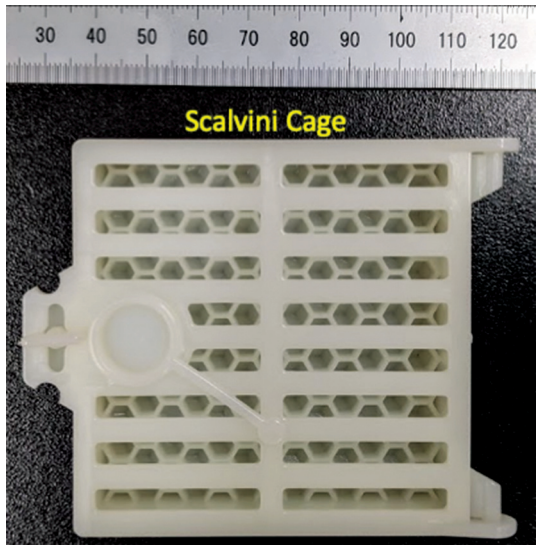


- **Chemicals:** Sodium hypochlorite (10 percent active; Sigma-Aldrich), 2-propanol (< 99 percent water), heptane, trehalose dihydrate, ethane-1,2-diol (ethylene glycol), Schneider's Cell Culture Medium (SCCM) with calcium chloride and sodium bicarbonate, antibiotic-antimycotic mixture (e.g. Sigma-Aldrich)
- **Wash/culture medium:** Prepare Schneider's CCM with 50 µl of antibiotic-antimycotic solution per litre
- **Thawing medium:** As above with 0.5M trehalose (18.5 g/100 ml)
- Cryoprotectant pretreatment: Schneider's CCM with 10 percent ethane-1,2-diol
- **Vitrification solution:** Schneider's CCM with 0.5 M trehalose and 36.5 percent ethane-1,2-diol.
- Suitable foam container to hold liquid nitrogen with an undisturbed layer of vapor at least 10 cm above the surface of the liquid nitrogen. The liquid nitrogen must not boil vigorously during use.

A6.3.6.2 embryo collection

Embryo collection has been described elsewhere in Section 6. Briefly, the Scalvini cages as shown in Figure A6.3.7 are left overnight 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 to 120 minutes. Following this, the queen is 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 dislodge the embryos from the cage onto the Petri plate. Using a dissecting microscope, the eggs are checked for damage, and the

FIGURE A6.3.7
A Scalvani cage



damaged eggs being either removed or marked. The plate is incubated in the dark at 34 °C and 60 percent humidity for 62 to 65 hours before assessment and treatment.

A6.3.6.3 Embryo cryopreservation

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 should appear as shown in Figure A6.3.8. 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.

A6.3.6.4 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 percent diluted sodium hypochlorite (in tap water) in the hematocrit capillary to about 1 cm. Place it under a dissection microscope with the capillary tip clearly visible as shown in Figure A6.3.9 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 (see Figure A6.3.9) while observing the introduction under a microscope.

FIGURE A6.3.8
Microscopy of the anterior embryonic morphology at 68 hours of development

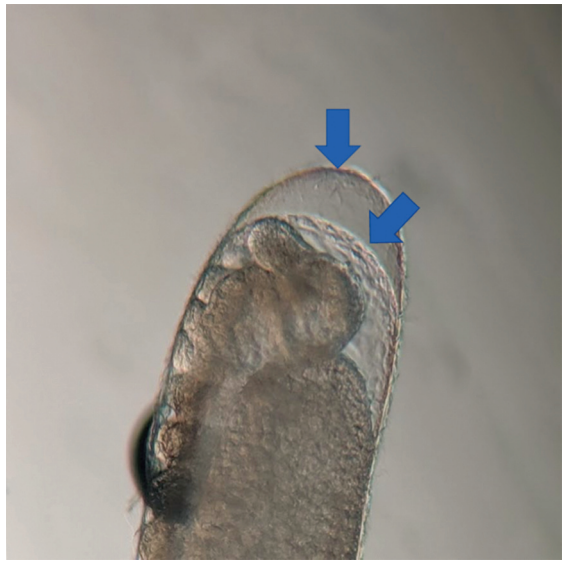
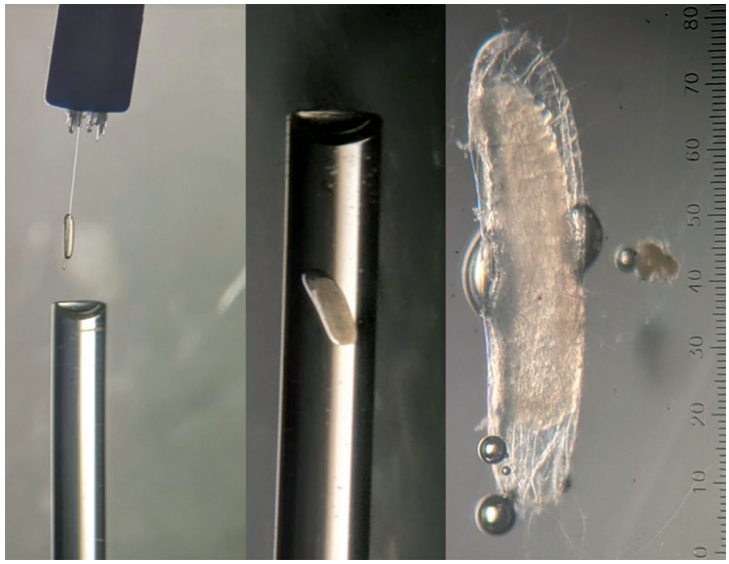


FIGURE A6.3.9
Dechorionation and observation of the embryo when the chorion begins to peel



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 hairbrush bristle. 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 seconds, expel the embryo onto a 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 dry the isopropanol from the embryo.
8. Fill a shallow glass Petri plate with heptane, and a sterile 3 cm disposable Petri plate with Schneider's CCM at room temperature.
9. Permeabilization: Slowly place the basket with the embryo into the heptane, ensuring that the embryo does not move from its position on the mesh. Remove the basket after 15 seconds and blow air to dry for 1 minute. Quickly place the basket 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, thus 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 percent 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 excess medium and place the basket into 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, 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. 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, thus 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 in the vapor phase (about 1 cm above the liquid nitrogen) for 1 minute. Place a dry mesh basket in the liquid nitrogen and drop the membrane with the embryo into the basket, ensuring that the membrane is not lost in the liquid. If long-term storage is intended, a histological cassette is used to trap the membrane containing the embryo and is clasped shut under the liquid nitrogen.
15. Thawing: Fill a 3 cm Petri plate with thawing medium (0.5 M trehalose in Schneider's CCM). Keep it close to the freezing container. Remove the embryo from the

liquid nitrogen and hold it for one minute in the vapor phase 1 cm above the surface. Note the side where the embryo is stuck on the membrane. Rapidly transfer the embryo side of the membrane onto 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, use a pipette to drain most of the solution and replace it with Schneider's CCM. Repeat the process every 7 minutes for 3 more times. Then let the embryo float on the SCCM for 24 hours in the incubator until the larva hatches.

16. Larval rearing: The viable larva is scooped from the surface of the Schneider's CCM and placed in a 3 cm Petri plate on a 100 µl drop of a mixture of royal jelly, glucose, fructose, yeast extract and water as described by Kaftanoglu, Linksvayer and Page (2011), which will provide nourishment for the larva. In brief, this 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 percent humidified chamber at 34 °C until emergence. It should be noted that the diet shown here only yields worker bees.

A6.3.7 VITRIFICATION OF PORCINE IMMATURE OOCYTES

The method described herein for vitrification of porcine oocytes was recently developed in the studies by Somfai *et al*. (2014); Appeltant, Somfai and Kikuchi (2018); and Nguyen *et al.*, (2018). It should be stressed that this method is still at the experimental stage.

A6.3.7.1 Oocyte and zygote collection

Porcine ovaries are collected from pre-pubertal gilts placed in Dulbecco's phosphate buffered saline at 35 °C and transported within one hour to the laboratory for further processing. In the laboratory, 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).

A6.3.7.2 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 percent (v/v) ethylene glycol and 2 percent (v/v) propylene glycol at room temperature for 13–15 minutes.
3. Wash oocytes/zygotes in 3 drops (50 µl) of a vitrification solution within 40 seconds 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 percent (v/v) ethylene glycol + 17.5 percent (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.

A6.3.7.3 Thawing

1. From liquid nitrogen, place microdrops/Cryotop devices immediately into 2.5 ml of a warming solution in a 35 mm Petri dish at 42 °C and keep them there for 2 minutes. The warming solution is 0.4 M sucrose in BM.
2. Gather oocytes/zygotes in a group and transfer them stepwise into solutions with gradually reduced concentrations of sucrose (0.2 M, 0.1 M and 0.05 M for 1 minute each) at 38 °C. Finally, keep oocytes/zygotes in BM for 3 minutes.

A6.3.7.4 Use of frozen/thawed immature oocytes and zygotes

To utilize vitrified immature oocytes, *in vitro* oocyte maturation and fertilization (or alternatively ICSI) would be required. These methods are still at the experimental stage (Grupen, 2014; Romar, Funahashi and Coy, 2016).

A6.3.8 VITRIFICATION OF MATURE BUFFALO OOCYTES

The method described here has been developed following the studies of Liang *et al.* (2011, 2012, 2020); Liang and Parnpai (2018); Parnpai *et al*. (2016). It assumes that oocytes are collected from ovaries obtained during slaughter. Figure A6.3.10 shows the bitrification, thawing and viability evaluation steps.

A6.3.8.1 Oocyte collection and *in vitro* maturation

Buffalo ovaries obtained from a slaughterhouse are transported within 4 hours to the laboratory in physiological saline solution (0.9 percent 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 percent polyvinyl pyrrolidone (PVP). A group of up to 20 COCs is cultured in 100 µL IVM medium at 38.5 °C under a humidified atmosphere of 5 percent CO_2 for 23 hours. The IVM medium consists of TCM199 supplemented with 10 percent fetal bovine serum, 0.02 AU/mLFSH, 50 IU/mL hCG and 1 µg/mL estradiol-17β.

A6.3.8.2 Vitrification of matured oocytes

1. After dilution in IVM, COCs are partially denuded by gentle pipetting with a pulled-pipette using 0.1 percent (v/v) hyaluronidase.
2. Place a group of five oocytes in base medium (BM: TCM199-Hepes + 20 percent FBS) supplemented with 10 percent (v/v) dimethyl sulfoxide (DMSO) and 10 percent (v/v) ethylene glycol (EG) for 1 minute.
3. Expose oocytes to vitrification solution (BM supplemented with 20 percent (v/v) DMSO, 20 percent (v/v) EG, and 0.5 M sucrose) at 22–24 °C for 30 seconds.
4. Place oocytes onto the end tip of a Cryotop (Kitazato Supply Co., Tokyo, Japan) in a small volume of vitrification solution, and within 30 seconds, directly plunge into liquid nitrogen.

A6.3.8.3 Thawing vitrified matured oocytes

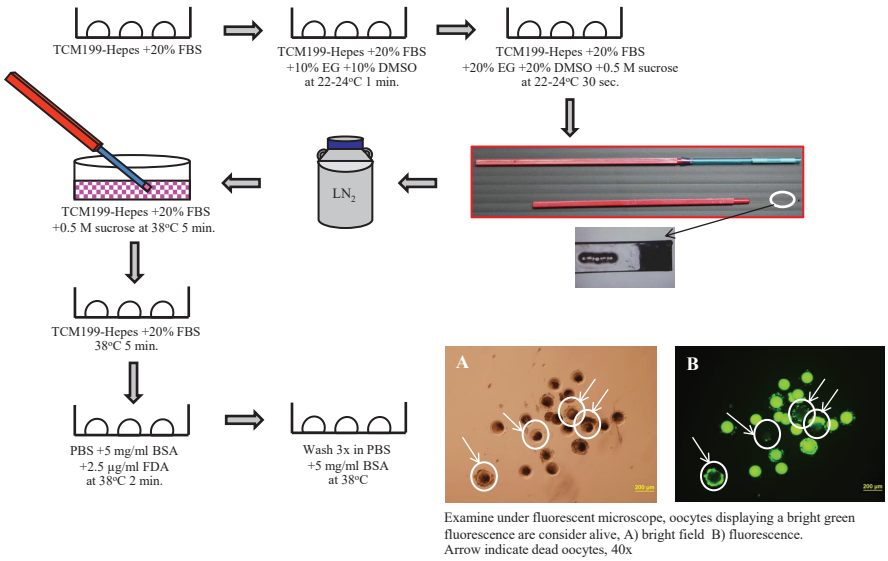
1. Remove stored oocytes from liquid nitrogen and immerse the tip of the Cryotop in 3 mL of 0.5 M sucrose in BM at 38.5 °C for 5 minutes.
2. Transfer the oocytes into BM without sucrose for 5 minutes.
3. Place vitrified-warmed oocytes in BM under a humidified atmosphere of 5 percent CO2 at 38.5°C for 1 hour.

A6.3.8.4 Evaluation of oocyte viability

Viability of the oocytes can be examined using fluorescein diacetate (FDA) staining as described by Mohr and Trounson (1980).

1. Treat the oocytes with 2.5 µg/mL FDA in phosphate buffered saline (PBS) supplemented with 5 mg/mL bovine serum albumin (BSA) at 38.5 °C for 2 minutes in a dark room.
2. Wash the oocytes three times in PBS supplemented with 5 mg/mL BSA.
3. Examine the oocytes under a fluorescent microscope with UV irradiation with an excitation wavelength of 460–495 nm and emission of 510 nm. Oocytes displaying a bright green fluorescence are considered to be alive and can be used in subsequent experiments.

FIGURE A6.3.10
Vitrification, thawing and viability evaluation of mature oocytes



Examine under fluorescent microscope, oocytes displaying a bright green fluorescence are consider alive, A) bright field B) fluorescence. Arrow indicate dead oocytes, 40x

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A6.3.8.5 Use of vitrified/thawed matured oocytes

To optimally utilize vitrified/thawed matured buffalo oocytes, procedures for *in vitro* fertilization, ICSI and SCNT must be further developed. These methods are still in the experimental stage (Suresh, Nandi and Mondal, 2009; Marin *et al.*, 2019). As an insurance against breed loss, oocytes from at risk breeds can be stored now, in the anticipation that the methods will be improved when or if the materials are need in the future.

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Annex 6.4
Guidelines for gonadic and peri-gonadic tissue cryopreservation

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A6.4.1 INTRODUCTION

Health accreditation of collection, storage and implementation operations. 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. Knowledge and skill are very important for the success of the procedures. This know-how is acquired with experience and time. Training is often required. Users of these guidelines may thus contact the authors of the procedures before applying them.

A6.4.2 CRYOPRESERVATION AND USE OF GONADAL TISSUE IN POULTRY

Vitrification has been proven to be a fast and successful method for freezing of newly hatched chick, Japanese quail and turkey gonadal tissues (Liu *et al.,* 2010; Liu, 2013; Liu, Elsasser and Long, 2017; Barna *et al.,* 2020; Liptói *et al.*, 2020). This procedure 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.

A6.4.2.1 Chicken

Preparation and vitrification of donor tissues

1. Prepare the vitrification solutions prior to the collection of gonads as follows:
   a. Basal solution: Dulbecco's phosphate-buffered saline (DPBS) with 20 percent fetal bovine serum (FBS). (Can be stored for up to 1 hour at 0 °C, if necessary)
   b. Vitrification solution 1: Basal solution with 7.5 percent dimethyl sulfoxide (DMSO) and 7.5 percent ethylene glycol (EG)

   c. Vitrification solution 2: Basal solution with 15 percent DMSO and 15 percent EG and 0.5 M sucrose
2. Euthanize 1-day-old chicks via cervical dislocation.
3. Remove feathers from the abdomen and clean the abdomen with 70 percent ethanol.
4. Perform the following under sterile conditions in a laminar flow hood:
   a. make a 2 to 3 cm transverse incision on the left side of the chick and remove the yolk sac;
   b. open the incision further to facilitate the removal of the gastrointestinal tract from the cavity;
   c. remove the air sacs and serous membranes;
   d. remove the ovary or testes in the caudocranial direction with a fine forceps or microsurgical scissors (e.g. iris scissors); and
   e. place the donor organs into Basal solution, in antiseptic Petri dishes on ice; maintain the organs in this solution for no longer than 30 minutes.
5. Once removed, and depending on the size, organs can be halved, and three of these gonads (halved or whole) are then placed onto a human acupuncture needle (e.g. 0.18 × 38 mm – Dongbang Acupuncture Inc, Korea).
6. 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 (Liptói *et al.*, 2020). Note: Wang *et al.* (2008) treated the donor organs in two solutions for 10 and 2 minutes, respectively.
7. Vitrify the samples by plunging the needles into liquid nitrogen and placing them in labelled 1 mL cryovials (e.g. Labsystem Comp., Hungary).
8. 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 to be performed immediately prior to implantation

1. All media should be prepared, warmed and maintained on a fixed-temperature heating plate in sterile, closed and labelled vials in a laminar hood.
2. Prepare the following solutions, sterilize using a 0.2 µm filter:
   a. Basal solution: DPBS with 20 percent FBS. (Can be stored for up to 1 hour at 0 °C, if necessary)
   b. Thawing solution 1: Basal solution with 1.0 M sucrose
   c. Thawing solution 2: Basal solution with 0.5 M sucrose
   d. Thawing solution 3: Basal solution with 0.25 M sucrose
3. 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 (Liptói *et al.*, 2020).
4. 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).
5. Following thawing, cut each organ into 2–4 pieces under a stereomicroscope using an iris scissor.

Preparation of the recipient animals

1. If possible, determine the sex of the chicks prior to surgery, which makes the interventions more efficient.
2. Maintain chicks in a heated room, under infrared heating lamps for pre-, peri- and post-operative care.
3. Administer local anaesthesia, 0.1 mg xylazine (Narcoxyl 2) and 0.5 mg ketamine (CP Ketamin 10 percent), i.m., per chick.
4. Place a chick in a recumbent position on a heating pad and administer general anaesthesia (isoflurane: Forane) using a mask during the procedure.
5. Remove feathers from the abdomen and wash the surgical site with 70 percent ethanol.
6. Open the abdomen using a 2 cm transverse incision, and a 1 cm incision to the last rib.
7. Tie off the yolk sac and remove it from the chick.
8. 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, the ovary is a triangular organ, 5–6 mm long, 1.5–2 mm wide, and yellowish-white in colour.
9. 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 (Liptói *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.
10. Note: Testes removal is performed in a similar manner (Song and Silversides, 2006, 2007a, 2007b; Liu, 2013; Liptói *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 of the recipient chick.
- 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

- Inject 0.05 mg dexamethasone intramuscularly, immediately after surgery to reduce edema and inhibit an immune response
- Administer Mycophenolate mofetil (i.e. CellCept; Hoffmann-LaRoche Ltd., Mississauga, ON, Canada) at a rate of 100 mg/kg bodyweight orally and daily for 2 weeks,

then, once per week for 6 weeks to support gametogenesis and inhibit B and T cell activation, thus facilitating implantation of donor tissue (Song and Silversides, 2006, 2007a, 2007b; Song *et al*., 2012; Liptói *et al.,* 2013; Barna *et al*., 2020).

- *Optional antibiotic*: Ceftiofur, Excenel subcutaneously, 2.55 mg, although this may not be necessary, as it does not influence the mortality or the adherence of the grafted organs (Song and Silversides, 2006, 2008; Song *et al.* 2012; Liptói *et al*., 2013, 2020).

A6.4.2.2 Japanese quail

Live progeny are 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 the chicken, with a few differences that are highlighted below.

Preparation and vitrification of donor tissues

1. After euthanasia, ovaries are immersed in Dulbecco's modified Eagle medium (DMEM) with 10 percent FBS on ice up to 4 hours. Note: Testes are placed into Basal solution on ice (Liu, 2013).
2. Place four or five tissue pieces on a single acupuncture needle (Cloud & Dragon Medical Device Co. Ltd. Jiangsu, China).
3. Submerge the needles successively into Vitrification solution 1, then, Vitrification solution 2 for 10 and 2 minutes, respectively, at room temperature.
4. Remove the vitrification solution from the organs by blotting with a piece of gauze.
5. Place the needles with gonads into cryovials under liquid nitrogen and close with precooled caps.

Thawing of donor organs

1. Remove the vials from storage.
2. Wash the needles with vitrified gonads through the three thawing solutions at room temperature, in order, 1 to 3 for 5 minutes each. For testes, increase the temperature of the solutions to 40 °C instead of room temperature.
3. Place the ovaries into DMEM and the testes into Basal solution on ice. The tissues can be maintained in this manner for up to 4 hours.

Preparation of the recipient animals and transplantation

1. Administer local anaesthesia, 0.1 mg of ketamine (Ayers Laboratories, Guelph, ON, Canada) and 0.05 mg of xylazine (Bayer Inc., Toronto, ON, Canada), subcutaneously per chick.
2. Administer isoflurane gas.
3. Place the chick on a heating pad, and open the left side the abdominal cavity distal to the last rib.
4. Ovarian transplantation:
   a. 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.
   b. Remove the host ovary with fine forceps.

   c. Place the donor ovary in the anatomical site and cover with the parietal layer of the abdominal air sac.
5. Testes transplantation:
   a. Make a 1.5 cm incision 1 cm left of the medial plane.
   b. Displace the abdominal organs to expose the testes.
   c. 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).
6. Close the skin with 4–5 interrupted sutures.

Postoperative treatments

Administer Mycophenolate mofetil (i.e. CellCept) orally at a rate of 100 mg/kg bodyweight daily for 2 weeks.

A6.4.2.3 Turkey

In turkeys, only the vitrification and warming methods of ovarian tissue have been published (Liu, Elsasser and Long, 2017).

Preparation and vitrification of the donor ovarian tissues

1. Prepare the vitrification solutions prior to beginning the collection of gonads. The content of the Basal and Vitrification solutions is the same as for the chicken.
2. Immediately after euthanasia, recover the ovaries with a fine forceps (Fine Science Tools, Foster City, California, United States of America) and place them in DPBS.
3. Remove the surrounding connective tissue.
4. Submerge the ovaries in Basal solution.
5. Impale the organs on an acupuncture needle (J-type, Size No. 2 (0.18) × 30 mm, SEIRIN-America, Weymouth, MA, United States of America). Note: Utilizing a dissecting microscope may help to minimize handling damage.
6. Place the needles into Vitrification solution 1 for 10 minutes, then, into Vitrification solution 2 for 2 minutes at room temperature.
7. Blot the ovaries briefly with a piece of gauze.
8. Place the needles with vitrified gonads into cryovials under liquid nitrogen and close with precooled caps.

Thawing of donor organs

1. 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.
2. Remove the needles and wash the organs in fresh Basal solution. Gonads can be stored in Basal solution on ice until use (no more than 4 hours).

A6.4.3 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.

A6.4.3.1 Materials required

Tissue collection and culture

- Leibovitz L15 cell culture medium modified with 0.25 percent glucose, 0.25 percent trehalose and 10 percent fetal bovine serum
- sterile filtered simple saline (0.2 M Tris buffer pH 7.2 with 1.12 percent potassium chloride and 0.88 percent sodium chloride)
- sterile pipettes
- sterile 3 cm Petri plates
- dissection tools

For cryopreservation

- 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, which then are also marked. The testis will regress progressively after day 3, and will 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 a source of tissue for cryopreservation.

A6.4.3.2 Tissue harvesting

1. Drones of specific ages are brought to the lab and anaesthetized using carbon dioxide. The wings and the limbs are clipped, and the drone is quickly rinsed in chilled 5 percent dilute Clorox™ followed by immersion in chilled 70 percent 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 + SV 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 days old, the testis is separated from the SV and transferred to another drop of modified L15 medium for testicular cryopreservation. In bees older than 3 days, the (shrunken) testis is left intact on the SV.

A6.4.3.3 Cryopreservation

1. The existing medium is replaced with ice cold modified L15 including 12 percent DMSO. After 5 minutes, the medium is again replaced with ~200 µl of the modified 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 (e.g. Cryo Bio System, France). After leaving a gap of the same length as the loaded suspension, aspirate 0.5 ml of modified L15 medium without DMSO. Centre 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 and 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. Cool the contents from 4 °C to -10 °C with a cooling rate of 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 by using sterile forceps.
5. After seeding, the straw and vials are further chilled, following a programme that 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 final storage container. A cryovials are placed on the prelabelled cryocanes and directly transferred to a marked canister in the storage container. Storage locations are then recorded in the gene bank database.

A6.4.3.4 Thawing and assessment

1. Thawing is performed by swiftly transferring the straw or vial to a 37 °C water bath. The entire content (tissue suspension plus L15 medium) is expelled into an Eppendorf tube. Another 250 µl of L15 medium is added to the suspension. The contents are centrifuged at 1200 × g for 10 minutes, and the supernatant is replaced with fresh modified L15 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 by using GFP and RFP or Texas red filters.

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Annex 6.5

Guidelines for conservation of diploid germ cells: Isolation, establishment, cryopreservation and use of *in vitro* propagated chicken primordial germ cells

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A6.5.1 INTRODUCTION

Health accreditation of collection, storage and implementation operations preamble. Due to national and international specific regulations and agreements, persons wishing to implement the procedures described herein are advised to approach the relevant authorities to take into account the corresponding regulatory constraints.

Know-how. The appropriate knowledge and skills are very important for the success of the procedures. This know-how is acquired with the experience and the time. Training is often required. We thus suggest contacting the authors of the Annex and/or the papers cited before any use.

A6.5.2 COLLECTION AND *IN VITRO* CULTURE OF PRIMORDIAL GERM CELLS

Fertile eggs of the breed to be preserved must be transported to a laboratory equipped with cell culture facilities and an egg incubator. The eggs are placed in the incubator and incubated under standard conditions (see below) until collection of the primordial germ cells (PCG).

The PGCs can be collected from multiple sites within the avian embryo (Barna *et al.*, 2020, Nakamura, Kagami and Tagami, 2013, Song *et al.*, 2014). However, collection from the following two embryonic sites is preferred (Nakamura, 2016):

1. The blood at the stage when PGCs circulate and migrate towards the forming gonads (HH stages 13–17) (Hamburger and Hamilton, 1951). At this stage, there are approximately 100 to 200 circulating PGCs (cPGC) in the vascular system, which represent about 0.02 percent of blood cells.
2. The embryonic gonads colonized by PGCs (HH stages 27-31). Gonadal PGCs (gPGCs) represent 2 percent of gonadal cells.

Embryonic blood can be used directly for subsequent culture of PGCs (Naito, Harumi and Kuwana, 2015). The number of PGCs and their proportion in the gonads are higher

than in the blood, but 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 and is used for PGCs in blood. Protocol 2 may be applied in specific circumstances when the embryo is older and PGCs are taken from the gonads.

A6.5.2.1 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
- pipettes

Renewable supplies

- sterile phosphate buffered saline (PBS) solution
- PGC culture medium (Whyte *et al.*, 2015), which consists of a custom basal medium that is a modification of knockout DMEM (250 mosmol/L, 12.0 mM glucose, and $CaCl_2$-free; ThermoFisher Scientific) supplemented with the following:
  - 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 percent ovalbumin (Sigma);
  - 0.01 percent 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); and
  - 0.2 percent chicken serum (Biosera) or 10 µg/mL ovotransferrin (Sigma).

Collection of PGCs from the blood

The following procedures require good technical expertise. Blood is collected from individual embryos at HH stages 13–17 (52–58h of incubation; Hamburger and Hamilton, 1951).

1. Fertilized chicken eggs are incubated for 52–58 hours at 37.7°C and 45–51 percent humidity until the embryo reaches the embryonic stage (HH 14–16). Wipe the egg surface with 70 percent ethanol to sterilize. The incubation period may vary slightly 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, and 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 of the embryo, and extract 1–3 µL of blood using a mouth aspirating device.
4. Immediately place the blood into a single well of a 48-well cell culture plate containing 300 µl PGC culture medium. (Whyte *et al.*, 2015).
5. Collect tissue samples for sex-determination from each isolated embryo, and store them at -20 °C until further use (Lázár *et al.*, 2021.

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 eliminates other cell types. Established homogeneous PGC culture can be used for cryopreservation. The isolated blood from single embryos, which contains circulating PGCs, is cultured in PGCs culture medium at 37–37.7 °C with 5 percent 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, about 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 minutes. 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 x 10^5/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 × 105 cells in 3–4 weeks, the establishment of the permanent culture is considered successful (Lázár *et al.*, 2021).

A6.5.2.2 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
- 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 for 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 PBS [-].
4. The centrifuge tubes are placed in an incubator maintained at 37.8 °C for 1 to 1.5 hours.
5. Gonads are pipetted gently from the centrifuge tube and 100 µl of cell suspension is placed into wells on 48-well plate, each 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.

A6.5.3 FREEZING OF CULTURED PRIMORDIAL GERM CELLS

The cryopreservation strategy and protocol are essentially as described by Nandi *et al*. (2016).

A6.5.3.1 Required equipment

- cell culture 24-well plates
- heating block
- freezer
- laboratory centrifuge
- pipette
- Eppendorf tubes

- Nunc cryotubes
- freezer box
- 80 °C freezer

A6.5.3.2 Renewable supplies

- Avian DMEM containing 4 percent DMSO and 5 percent chicken serum (freezing medium) or Stemcell Banker commercial freezing media
- liquid nitrogen storage

A6.5.3.3 Freezing

1. PGCs are suspended carefully from the bottom of the culturing well, and then pipetted into 1.5 mL Eppendorf tubes.
2. Centrifuge (1 000 × g, 3 minutes) and remove the supernatant.
3. Carefully resuspend 5 to 6x104 cells in 250 µl of DMSO-free freezing medium for PGCs and pipette them into labelled cryotubes.
4. Slowly (drop-wise) add 250 µl of freezing medium for PGCs to each tube, and then place them in a freezing box and into the freezer at -80 °C.
5. Alternatively, resuspend the cell pellet in 250 µl of Stemcell Banker before transfer to the freezing box.
6. After one night, the samples are moved into the liquid nitrogen storage tank, recording their location in the gene bank database.

A6.5.4 THAWING OF CRYOPRESERVED PRIMORDIAL GERM CELLS

A6.5.4.1 Required equipment

- water bath
- heating block
- laboratory centrifuge
- non-adhesive 24-wells cell culture plates
- pipettes

A6.5.4.2 Renewable supplies

- culture medium for PGCs described in Protocol 1

A6.5.4.3 Thawing procedure

1. Remove the cryotube(s) from storage and place them in a water bath at 37 °C.
2. Slowly pipette 2 mL of PCG culture medium into each of the thawed tubes.
3. Centrifuge the samples at 1 000 × g for 10 minutes and remove the supernatant.
4. Finally, the cells are resuspended in fresh culture medium for PGCs, placed into cells of a 24-well cell culture plate and cultured as described in Protocol 1.

A6.5.5 TRANSFER INTO HOST EMBRYOS

A6.5.5.1 Required equipment

- egg incubator
- chick hatcher

- laminar box
- binocular magnifier or stereomicroscope
- fibre optic light source
- dry block heater with thermostat
- sterile pulled glass microcapillary
- mouth aspirating device
- Eppendorf tubes
- pipettes

A6.5.5.2 Renewable supplies

- 70 percent alcohol
- laboratory parafilm
- sterile phosphate buffered saline solution (PBS)
- DMEM with high glucose, no glutamine and no calcium (Gibco) and sterile water (ratio 2:1).

A6.5.5.3 Transfer procedure

The following procedure requires high technical expertise, obtained through training and practice.

1. Fertilized recipient eggs are incubated for 52–58 hours (HH 14–16, Hamburger and Hamilton, 1951) at 37.7 °C.
2. After centrifugation in a mixture of DMEM and sterile water (ratio 2:1), the PGC pellet is resuspended in DMEM. Optionally, 1 or 2 µL of 1 percent 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. Disinfect the recipient eggs.
4. Start the transfer by opening a 10 mm diameter window at approximately the one-third position of the egg, closer to the pointed end.
5. Inject 1 µl (approximately 3-5 000 PG cells) of the prepared cell suspension into the heart or the dorsal aorta of the 52–58h recipient embryos.
6. Following the injection, drip 50 µl of sterile prewarmed 1 × PBS onto the embryo.
7. The hole is sealed with two layers of melted laboratory parafilm, or sealed with 1 cm of leukosilk tape. Alternatively, the hole can be covered with egg shell membrane and sealed with melted parafilm.
8. Place the egg into a stationary egg incubator for 24 hours. After 24 hours, the incubator is rocked at 45 until day 18 of incubation. The egg is then transferred to a hatcher until hatching occurs after 21 days. (Barna *et al.*, 2020).

A6.5.6 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.

A6.5.6.1 Protocol to maintain 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. As appropriate for species, poultry parent stock should be vaccinated for the following diseases:

Viral

- Marek's disease
- Newcastle disease
- infectious bursitis
- infectious bronchitis
- Avian encephalomyelitis
- fowl pox

Bacterial

- Salmonella enteritidis infection
- Salmonella typhimurium infection

Mycoplasma infection is prevented through antibiotic treatment of the laying hen or of the recipient eggs in the last third of hatching.

A6.5.6.2 Protocol for treatment of eggs with antibiotics

1. Disinfect eggs with iodine solution prior to the injection.
2. Prepare the treatment solution by mixing 5 ml of 10 percent Baytril solution in 200 ml of physiological saline.
3. Inject 0.1 ml of the Baytril solution into each egg (use of an automatic syringe is recommended). Injection should be targeted above the air chamber at a maximum depth of 5 mm.
4. After the injection, seal the hole with glue.

A6.5.7 REFERENCES

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Annex 9.1
Potential set of elements in material acquisition agreements

A9.1.1 BACKGROUND

The European Regional Focal Point for Animal Genetic Resources (ERFP), which is a regional platform for the support of management, conservation and sustainable use of animal genetic resources for food and agriculture, has developed various resources for stakeholders in its member countries. Among these resources are *Guidelines for the development of material acquisition agreements (MAA)*, which can be found on the ERFP website (ERFP, 2019).

The guidelines provide a potential set of elements which could be incorporated in an 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)

A9.1.2 REFERENCE

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Annex 9.2
Potential set of elements in material transfer agreements

A9.2.1 BACKGROUND

The European Regional Focal Point for Animal Genetic Resources (ERFP), which is a regional platform for the support of management, conservation and sustainable use of animal genetic resources for food and agriculture, has developed various resources for stakeholders in its member countries. Among these resources are *Guidelines for the development of material acquisition agreement (MTA) for conservation and breeding*, which can be found on the ERFP website (ERFP, 2019).

The guidelines provide a potential set of elements that could be incorporated in an 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

A9.2.2 REFERENCE

ERFP. 2019. *Guidelines for the development of material transfer agreement (MTA) for conservation and breeding*. Paris, France. Cited 15 March 2021. www.animalgeneticresources.net/wp-content/uploads/2019/11/MTA-Conservation-and-Breeding-final-version.-04_11_19.pdf

FAO ANIMAL PRODUCTION AND HEALTH GUIDELINES

1. Collection of entomological baseline data for tsetse area-wide integrated pest management programmes, 2009 (En)
2. Preparation of national strategies and action plans for animal genetic resources, 2009 (En, Fr, Es, Ru, Zh)
3. Breeding strategies for sustainable management of animal genetic resources, 2010 (En, Fr, Es, Ru, Ar, Zh)
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Innovations in cryoconservation of animal genetic resources
Practical guide

Corrigendum

15 February 2023

The following corrections were made to the PDF of the report after it went to print.

Page	Location	Text in printed PDF	Text in corrected PDF/ Notes
59	Middle of page	(none)	The following text was added before " 3.10 References": **The National Livestock Cryobank of the Philippines** *Lilian P. Villamor* The National Livestock Cryobank (NLC) was established by the Department of Agriculture in 2012 and is located at the Philippine Carabao Center in the Science City of Muñoz. The Korean International Cooperation Agency provided financial support. The NLC supports the existing genetic improvement programmes and underlies the livestock sector's response to future threats posed by climate change. The NLC's strategy includes (i) the collection and preservation of genetic material, (ii) data banking, (iii) provision of access to stored samples, (iv) and dissemination of information. The NLC aims to preserve the diversity of native breeds, and oversee the introduction of exotic breeds that may be economically beneficial while still exhibiting resilience towards endemic diseases and the local environmental conditions. The collection currently consists of semen and oocytes and emphasizes buffaloes (91 percent), but also includes cattle (8 percent), goats and swine (<1 percent). Whole blood cells and DNA from various species are also preserved for research purposes. The NLC envisions establishing a national repository of samples from the diverse range of livestock breeds and species, as well as threatened and wild animals in the Philippines.

Contact: publishing-submissions@fao.org

Cryoconservation (the deep freezing of semen, embryos and other cells in a gene bank) is a powerful tool for managing the diversity of animal genetic resources, but is technically demanding. This publication constitutes a practical tool to guide gene bank managers and other stakeholders in the main activities of cryoconservation.

This document is part of a series of guidelines published by FAO to support implementation of the *Global Plan of Action for Animal Genetic Resources*, which was adopted in 2007, and remains the internationally agreed framework for the management of biodiversity in the livestock sector.

These guidelines on *Innovations in cryoconservation of animal genetic resources* complement and update the previous edition of guidelines on *Cryoconservation of animal genetic resources*, published in 2012. They describe the key processes of operating a programme for cryoconservation of animal genetic resources, starting with developing a gene banking strategy. Subsequent sections address quality management of gene banks, the choice of material to be stored, financial aspects of gene banking, development and use of collections, collection and storage of genetic material, sanitary issues in gene banking, data management, legal issues, and capacity building and outreach. Appendices provide detailed protocols for collection and cryopreservation of various tissues for common livestock species.

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