联合国粮食及农业组织 | Food and Agriculture Organization of the United Nations | Organisation des Nations Unies pour l'alimentation et l'agriculture | Продовольственная и сельскохозяйственная организация Объединенных Наций | Organización de las Naciones Unidas para la Alimentación y la Agricultura | منظمة الأغذية والزراعة للأمم المتحدة

COMMISSION ON GENETIC RESOURCES FOR FOOD AND AGRICULTURE

Item 6.2 of the Provisional Agenda

Twentieth Regular Session

Rome, 24–28 March 2025

EX SITU IN VITRO GENE BANKING OF AQUATIC GENETIC RESOURCES FOR FOOD AND AGRICULTURE – DRAFT PRACTICAL GUIDE –

At its Second Session, the Intergovernmental Technical Working Group on Aquatic Genetic Resources for Food and Agriculture (Working Group) recommended the development of a number of voluntary guidelines and frameworks, including[1] on gene banking of aquatic genetic resources for food and agriculture (AqGR).

In 2022, FAO started a process towards the preparation of guidelines focusing on *ex situ in vitro* gene banking of AqGR. In line with the recommendation of the Working Group,[2] the Commission, at its last session welcomed the ongoing development of such a document and recommended that FAO finalize it for review by the Working Group at its next session.[3]

This document contains the draft *Practical guide for ex situ in vitro gene banking of aquatic genetic resources for food and agriculture.* The guide draws on contributions from nearly 40 experts. The first part of the document falls into ten authored chapters, addressing the complexity and multiple aspects of *ex situ in vitro* gene banking, the broad diversity of AqGR and relevant methods and requirements for their *in vitro* conservation. The second part includes additional information material, including laboratory protocols for the *ex situ in vitro* gene banking of some key aquaculture species and examples of existing gene banks.

The draft Practical Guide has been reviewed by three external reviewers and by Members of the Working Group which recommended its finalization for consideration by the Commission at its Twentieth Session.[4]

[1] CGRFA/WG-AqGR-2/18/Report, paragraph 25.
[2] CGRFA/WG-AqGR-4/23/Report, paragraph 17.
[3] CGRFA-19/23/Report, paragraph 116.
[4] CGRFA/WG-AqGR-5/24/Report, paragraph 14.

Ex situ in vitro gene banking of aquatic genetic resources for food and agriculture
Practical Guide

Table of contents

Objectives and structure of the Practical Guide

This Practical Guide aims to provide essential technical background for the establishment and maintenance of *ex situ in vitro* gene banks of aquatic genetic resources, particularly those used for aquaculture. The Practical Guide was prepared considering the wide taxonomic diversity of species that characterizes the aquaculture sector, which includes freshwater and marine finfish, crustaceans, molluscs, and micro- and macroalgae. The main body of the document (Chapters 5–7) presents overviews of current options for the *ex situ in vitro* conservation of different life stages of these major taxonomic groups.

The Practical Guide try to capture the multiple aspects of a gene banking process, and consequently target different stakeholders intervening and operating at different steps. The primary target stakeholders are policy makers, resource managers and producers, but the sections with more technical information are expected to be of significant interest also to practitioners and researchers from academia.

The Practical Guide are organized in ten chapters, whose contents are briefly summarized below.

Chapter 1

This chapter defines the scope and the target audience of the Practical Guide, briefly informs readers on the status of development of *ex situ in vitro* gene banking of aquatic genetic resources and its challenges, also compared to genetic resources used in terrestrial agriculture. It then identifies the main applications of *in vitro* conservation of aquatic species.

Chapter 2

The chapter discusses the main *ex situ in vitro* conservation methods for aquatic species: cryoconservation, desiccation**,** chilled storage and tissue culture. It highlights cryoconservation as the most widely utilized method and presents the basic principles of cryobiology.

Chapter 3

This chapter discusses the role of gene banks as repositories of genetic resources and illustrates their organizational structure which consists of three main hierarchical levels: preservation and quality management pathways; community interactions, represented by all the processes happening at the repository; and the establishment of networks.

Chapter 4

The chapter describes in detail the critical steps of an *ex situ in vitro* preservation pathway, from cells and tissues collections and shipping to their freezing and thawing up to quality assessments of the preservation methods. The major focus and examples provided are on cryopreservation of finfish sperm, but the steps and indications provided are applicable also to other taxonomic groups for which specific pathways are illustrated in later chapters.

Chapter 5

This chapter provides a brief overview on the status and scope of gene banking in freshwater and marine finfish related to finfish reproduction strategies. It discusses the finfish cells and tissues that can be conserved *in vitro* and related progress in the successful preservation of these different genetic resources. Finally, it discusses future directions and challenges for finfish cryopreservation.

Chapter 6

This chapter provides a brief overview on the status and scope of gene banking in shellfish (crustaceans, molluscs and echinoderms) and on shellfish reproduction strategies. It discusses the shellfish cells and tissues that can be conserved *in vitro* and related progress in the successful preservation of these different genetic resources. Finally, it discusses future directions and challenges for shellfish cryopreservation.

Chapter 7

This chapter provides highlights the broad diversity of forms and life-cycles of microalgae and macroalgae, and their different uses in an aquaculture context. It discusses the available methodologies for algae preservation, particularly: culture in agar slants and plates or liquid media; lyophilization; and cryopreservation. Finally, it discusses future directions and challenges for algae *ex situ in vitro* conservation.

Chapter 8

This chapter discusses aspects related to access and benefit-sharing (ABS) in gene banking of aquatic genetic resources and the impact of ABS measures and mechanisms on how a gene bank can legally obtain, use and distribute genetic resources and their associated information, with consequences in case of non-compliance. The chapter discusses practical considerations (e.g. administrative and contractual procedures; determining which ABS laws apply to a country etc.) for creating ABS protocols in gene banking, and their possible integration into the collection and preservation protocols. It further discusses ABS actors in a gene bank (providers, users and intermediaries) and aspects of traceability and reporting requirements linked to the exchange of gene banked samples.

Chapter 9

This chapter helps to identify the optimal gene bank strategy and associated infrastructure based on the purposes for which a gene bank is established, and the rate of samples processed, with principles and approaches applicable across all taxa. The chapter also discusses how the different gene banking steps influence the planning, management and cost of the gene bank. It finally identifies key requirements for each step of the gene banking process.

Chapter 10

The last chapter discusses the challenges undermining a broader application of *ex situ in vitro* gene banking to the conservation and management of aquatic biodiversity and identifies solutions and future directions.

The second part of the Practical Guide provides laboratory protocols for the *ex situ in vitro* gene banking of some key aquaculture species; and a second part containing examples of existing gene banks and additional technical information supporting the information presented in the chapters.

Acknowledgements

The preparation of the Practical Guide was possible thanks to the contribution of several experts working daily in the field of *ex situ in vitro* gene banking of aquatic genetic resources. Professor Terrence R. Tiersch and his research group's experience and contribution were critical in the early stages of development of the document as well as in the preparation of a significant part of the technical contents. The other authors which contributed with drafting and/or reviewing specific chapters are here reported in alphabetical order: Ajithkumar, T.T., Amanda Pereira dos Santos Silva, Amber H. Scholz, Anastazia T. Banaszak, Brian Harvey, Cecilia Rad-Menendez, Elsa Cabrita, Fran Humphries, Francisca Felix, Gabriella Braga Carvalho, George Shigueki Yasui, Gonzalo Gajardo, Harvey D. Blackburn, Huiping Yang, J K Jena, Jack C. Koch, Joachim Carosfeld, Jonathan Daly, José Augusto Senhorini, José Beirão, Julien Vignier, Kuldeep K. Lal, Lucia Suarez Lopez, Luciana Cristine Vasques Villela, Mary Hagedorn, Maureen Ritter, Michael Ross, Michael W. Lomas, Patrick Sorgeloos, Rajeev K. Singh, Samuel Rezende Paiva, Sarah Bodenstein, Serean L. Adams, Terrence R. Tiersch, Valentin Ananiev, Vindhya Mohindra, Virginia M. Weis, and Yue Liu. The authors' names and their affiliations are also reported at the beginning of each individual chapter, annex and/or box.

The document was reviewed by three reviewers external to FAO, particularly Brian Harvey, Michael Ross and Maureen Ritter, and by FAO staff, particularly Graham Mair, Senior Fishery Officer of the FAO Fisheries and Aquaculture Division.

The preparation of the Practical Guide was coordinated by Daniela Lucente and Joachim Carolsfeld, who had editorial oversight of each section.

FAO is grateful to all those that have dedicated their time and have shared their knowledge for the preparation of the document.

Abbreviations and acronyms

3-D	three-dimensional
ABS	access and benefit-sharing
AGGRC	Aquatic Germplasm and Genetic Resources Center
A-GRIN	Animal Germplasm Resources Information Network
AqGR	aquatic genetic resources for food and agriculture
AqGRISI	Aquatic Genetic Resource Information System
ARARC	Asian Regional Artemia Reference Center
BAC	bacterial artificial chromosome
BBNJ	Agreement under the United Nations Convention on the Law of the Sea on the Conservation and Sustainable Use of Marine Biological Diversity of Areas Beyond National Jurisdiction
BSA	bovine serum albumin
CAD	computer-aided design
CARMABI	Caribbean Research and Management of Biodiversity Foundation
CASA	Computer-assisted sperm analysis
CBD	Convention on Biological Diversity
CCAP	Culture Collection of Algae and Protozoa
CEPTA	Centro Nacional de Pesquisa e Conservação da Biodiversidade Aquática Continenta (Brazil)
CGRFA	Commission on Genetic Resources for Food and Agriculture
CIDA	Canadian International Development Agency
CPA	Cryoprotective agents
DES	discrete-event simulation
DIC	Dairy Improvement Center
DMA	dimethylacetamide
DMSO	dimethyl sulfoxide
DSI	digital sequence information
EMPRABA	Brazilian Agricultural Research Corporation
FAO	Food and Agriculture Organization of the United Nations
GSC	germinal stem cells
HBSS	Hanks' balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
ICAR	Indian Council of Agricultural Research
ICMBio	Chico Mendez Institute for Conservation of Biodiversity
IDRC	International Development Research Centre
IFGB	International Fisheries Genebank
IPLCs	indigenous people and local communities
ISO	International Organization for Standardization
ITPGRFA	International Treaty on Plant Genetic Resources for Food and Agriculture
IVF	*in vitro* fertilization
KAUST	King Abdullah University for Science and Technology
LSU	Louisiana State University
Nagoya Protocol	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
NAGP	National Animal Germplasm Program
NBFGR	National Bureau of Fish Genetic Resources
NCMA	National Center for Marine Algae and Microbiota
NERC	Natural Environment Research Council
NOAA	National Oceanographic and Atmospheric Association

MLS	Multilateral System
OSC	oogonial stem cell
OsHV-1	Pacific oyster herpes virus
PGC	primordial germ cells
PGR	plant genetic resources
PVC	polyvinyl chloride
QC	quality control
SIDS	Small Island Developing States
SDG	Sustainable Development Goal
SNP	single-nucleotide polymorphism
SSC	spermatogonial stem cell
UNESCO	United Nations Educational, Scientific and Cultural Organization
USDA	United States Department of Agriculture
ZIRC	Zebrafish International Resource Center
WFT	World Fisheries Trust

Chapter 1. *Ex situ in vitro* conservation of aquatic genetic resources: importance and applications

Daniela Lucente, Food and Agriculture Organization of the United Nations, Rome, Italy
Joachim Carolsfeld, World Fisheries Trust, Victoria, Canada

Introduction

Genetic diversity is the diversity within and among individuals and populations of wild and domesticated species that allows species to evolve, adapt and survive over time. It is the legacy of millions of years of evolution and, for those species used for food and agriculture, it can also be the result of artificial selection through domestication and genetic improvement.

The maintenance and restoration of genetic diversity within and between populations of wild and domesticated species, in order to maintain their adaptive potential, is among the targets of the Kunming-Montreal Biodiversity Framework (Convention on Biological Diversity, 2022). Genetic diversity forms the basis on which farmers can maintain, diversify and improve livestock breeds, crops and aquatic farmed types,[5] and it is a source of other societal needs.

Several approaches exist to preserve genetic resources. Ideally, these should be applied in a synergistic and standardized fashion in any conservation strategy. One of these approaches is *in situ* conservation which is defined by the Convention on Biological Diversity (CBD) (United Nations Environment Programme, 1992) as *the conservation of ecosystems and natural habitats and the maintenance and recovery of viable populations of species in their natural surroundings and, in the case of domesticated or cultivated species, in the surroundings where they have developed their distinctive properties*. A second approach is *ex situ* conservation, defined by the CBD as the *"conservation of components of biological diversity outside of their natural habitats"* (CBD, 1992). Several *ex situ* conservation strategies and methods exist and can be broadly classified as *in vivo* and *in vitro*. *Ex situ in vivo* conservation includes aquaria, zoos and botanical gardens and live gene banks in captive-breeding programs, where whole, live animals or plants are maintained outside of their natural habitat or farm environment. *Ex situ in vitro* conservation consists of maintaining cells, tissues and life stages of animals and plants alive or in suspended animation with the capacity to be reconstituted as or into live organisms at a later time.

Ex situ in vitro conservation of aquatic genetic resources for food and agriculture (AqGR) is the focus of this guide.

Scope of the Practical Guide and target audience

The Practical Guide focus on *ex situ in vitro* conservation of AqGR. The promotion of *ex situ* conservation for AqGR is one of the strategic priorities of the FAO *Global Plan of Action for the Conservation, Sustainable Use and Development of Aquatic Genetic Resources for Food and Agriculture* (Global Plan of

[5] The term "farmed type" is a descriptor applied to farmed aquatic organisms at a level below species, including strain, variety, hybrid, triploid, monosex group, other genetically altered forms or wild-sourced.

Action) (FAO, 2022) which stresses how *ex situ* conservation (including *in vitro* conservation) should be integrated with *in situ* management efforts and should consider the future genetic status of both wild relatives and farmed types of aquaculture species.

Compared to the crop and livestock sector, *in vitro* conservation of AqGR is not well developed. Preservation methods and the processes for establishing *in vitro* gene banks are hindered by the lack of standardized procedures and terminology, the lack of effective reporting guidelines, and limited gene banking knowledge resources available to users and stakeholders. Furthermore, the aquaculture sector is characterized by a particularly wide range of farmed species and taxonomic groups. These diverse species have many different characteristics and life stages, which increases the complexity of developing successful and generally applicable preservation protocols.

The target audience of the Practical Guide primarily includes policy makers, resource managers, conservationists, aquaculture producers and researchers. The overall aim of this guide is to aid countries and target stakeholders to make greater use of *in vitro* gene banking. The document provides, among other things, practical background information on: i) the variety of applications and existing methods, with a main focus on cryopreservation, the most common *ex situ in vitro* conservation method; ii) the establishment and organization of gene banks; iii) regulating access to and sharing of gene banked samples; and iv) cost implications and strategies for establishing and long-term maintenance of a gene bank. This guide also provides a framework for further, future synergized and standardized development of the field.

The guide also covers technical elements, including examples of laboratory protocols (Annex 1, 2 and 3), that provide basic knowledge on the existing *in vitro* conservation methods for AqGR, which are particularly useful for both practitioners and researchers. However, the Practical Guide is not meant to be an exhaustive literature review of protocols and basic scientific research, therefore, researchers are directed to relevant literature for more detailed information. Rather, this document is meant to serve as a starting point for method development and optimization, when this is required.

The principal applications for gene banking considered here are for aquaculture and for the conservation of aquatic species (not necessarily only those directly used in aquaculture). Applications limited to research are not covered specifically, though relevant advances derived from this research are reported.

The guide covers the wide range of aquatic taxonomic groups (particularly finfishes, invertebrates, seaweeds, and microalgae), and describes the *ex situ in vitro* conservation technologies currently available for each group.

Current status of *ex situ in vitro* gene banking

Since the 1950s, hundreds of scientists have studied the cryopreservation of sperm of terrestrial livestock, its application in breeding and its commercialization. This technology development was spurred by large investments from public and private sectors, and it took years to develop the repositories and platforms currently serving the billion-dollar global markets for livestock germplasm.

Over the last 65 years, *ex situ in vitro* preservation protocols have also been developed for many aquatic species (Cabrita *et al.*, 2008; Martinez-Paramo *et al.*, 2017; Tiersch and Green, 2011). However, most efforts have been at the research level, often with a focus on empirical protocols. A lack of standardized tested approaches, terminology, and reporting guidelines (e.g. standardized data sheets for sample collection, checklist of critical information to report on scientific publications to facilitate the

reproducibility of studies) has made comparisons difficult (Torres an Tiersch, 2018). Some recent published protocols are not significantly different from those developed 30 years ago. More development approaches based on known principles are needed to build reliable protocols that can be scaled up for gene banking.

Numerous AqGR gene banks have been created over the last decades with different scopes, such as: research or in-house support to breeding programs; national (the National Marine Fishery Genetic Resources Bank, in China, is the largest and most comprehensive national AqGR gene bank in the world) and regional conservation; and aquaculture production. Collections of AqGR are often components of gene banks and repositories with a more general taxonomic coverage. There are also some species-specific gene banks, for example, gene banks of the Pacific cupped oyster (*Magallana gigas*) in France, New Zealand and in the United States of America (see Chapter 6) and *Artemia* gene banks in China, Belgium, and the United States of America. No doubt there are numerous other small AqGR gene banks, public and private, that support research activities and in-house aquaculture breeding programs. A full list of existing AqGR banks is not publicly available but non-exhaustive lists are provided in this guide for the the main taxonomic groups of aquaculture species (Chapter 5, 6 and 7). The FAO database for AqGR, AquaGRIS,[6] will also record, over time, links to gene banks were individuals of farmed and wild stocks of aquaculture species are kept worldwide.

Applications of *ex situ in vitro* gene banking of aquatic genetic resources for food and agriculture

In vitro gene banking of AqGR has a variety of applications, as described below.

1. Long-term conservation of genetic diversity

This is usually the most common objective of existing AqGR gene banks (FAO, 2019), and can apply to both wild populations and farmed types[7] of aquatic species. This application is premised on holding genetic materials and associated information (e.g., genotypic and phenotypic information) in a safe repository for use when it is needed to reconstitute a wild population or a farmed type. This application requires long-term investment and, in the case of wild populations, can be part of a conservation strategy that considers multiple integrated components, including habitat conservation and restoration. In aquaculture, this can be a supplement to live gene banks.

2. Support of breeding programs for aquaculture

Cryopreservation and other *in vitro* conservation methods are useful tools to address several issues related to captive breeding. Examples of applications are the use of conserved material to resolve problems of asynchronous maturation of sexes, and for increasing genetic diversity in selective breeding programs. In the context of genetic improvement, *in vitro* conservation of AqGR can also be used to maintain a baseline stock against which genetic gains can be measured, and as back up in case of lost of genetic material.

3. Scientific research

In vitro conservation of genetic material can also be used for a variety of research purposes, including development of laboratory protocols, pharmaceutical uses, and biofuel development. Biomedical

[6] www.fao.org/fishery/aquagris/en

[7] The term "farmed type", recently introduced by FAO and coined by an expert group, is a descriptor applied to farmed aquatic organisms at a level below species, including strain, variety, hybrid, triploid, monosex groups, other genetically altered or wild-sourced forms.

application is also among the main destination uses of genetic material conserved *in vitro*, but is not included in the scope of the Practical Guide which is focused on gene banking in connection with food supply.

Different applications require different planning and process designs. For example, conservation applications require long-term funding support and collection of sufficient samples that adequately represent the genetic diversity and geographic distribution of the wild populations that are the object of the conservation initiative, whereas mitigating asynchronous maturation in aquaculture requires higher volumes of specific material (e.g., sperm) but features shorter-term goals. Public repositories generally can serve multiple purposes such as conservation, community access, information management, education training, and funding of collection activities.

Effective gene banking requires greater effort than simply collecting and accumulating samples. Developers of public gene banking programs must consider the need to demonstrate the impact of the work to general audiences, and to remain accountable to users and sources of funding. This means that the preserved material must be identifiable and linked to relevant information available through an accessible database. Gene banked samples and their associated information should be collected and maintained with the assumption that new users will need to be served in potentially novel ways, sometimes many years in the future.

References

Cabrita, E., Robles, V. & Herráez, P. (eds.) 2008. *Methods in reproductive aquaculture: marine and freshwater species*. Boca Raton, Florida, CRC Press.

CBD. 2022. *The Kunming-Montreal global biodiversity framework*. Canada, Secretariat of the Convention on Biological Diversity. www.cbd.int/doc/c/e6d3/cd1d/daf663719a03902a9b116c34/cop-15-l-25-en.pdf

FAO. 2019. *The state of the world's aquatic genetic resources for food and agriculture*. FAO Commission on Genetic Resources for Food and Agriculture Assessments. Rome, FAO.

FAO. 2022. *Global plan of action for the conservation, sustainable use and development of aquatic genetic resources for food and agriculture*. Commission on Genetic Resources for Food and Agriculture. Rome, FAO. https://doi.org/10.4060/cb9905en.

Martínez-Páramo, S., Horváth, Á., Labbé, C., Zhang, T., Robles, V., Herráez, P., Suquet, M., Adams, S., Viveiros, A., Tiersch, T.R. & Cabrita, E. 2017. Cryobanking of aquatic species. *Aquaculture*. 472: 156–177. doi: 10.1016/j.aquaculture.2016.05.042. Epub 2016 Jun 1. PMID: 29276317; PMCID: PMC5737826.

Tiersch, T.R. & Green, C.C. (eds.) 2011. *Cryopreservation in aquatic species: a comprehensive overview of current practices, programmatic development and future directions for cryopreservation of gametes, embryos, and larvae of aquatic species*. 2nd edition. Advances in World Aquaculture, World Aquaculture Society, Baton Rouge, Louisiana, 1003 pages.

Torres, L. & Tiersch, T.R. 2018. Addressing reproducibility in cryopreservation, and considerations necessary for commercialization and community development in support of genetic resources of aquatic species. *Journal of the World Aquaculture Society*, 49(4): 644–663. [Cited 10 January 2025]. doi: 10.1111/jwas.12541. PMID: 30467453; PMCID: PMC6241537.

United Nations Environment Programme. 1992. *Convention on biological diversity*, Nairobi, UNEP. June 1992. https://wedocs.unep.org/20.500.11822/8340.

Chapter 2. Gene banking methods: a technical overview

Terrence Tiersch, Aquaculture Germplasm and Genetic Resources Center (AGGRC), Louisiana State University Agricultural Center, Baton Rouge, Louisiana, United States of America

Introduction

In vitro preservation of aquatic genetic resources for food and agriculture (AqGR) includes cryopreserved cells (particularly gametes) and tissues, desiccation of embryos and sporophytic material of algae, chilled (non-frozen) storage, and cell and tissue culture. This chapter starts introducing the main methods for *ex situ in vitro* conservation of AqGR and provides readers with basic principles of cryopreservation.

Main *ex situ in vitro* conservation methods

The main existing methods for *ex situ in vitro* conservation of AqGR include cryopreservation, desiccation**,** chilled storage, tissue culture. Cryopreservation is the most widely used technology of *in vitro* conservation whilst the other three are considered minor technologies.

Cryopreservation or *cryoconservation* is the main technology for *in vitro* conservation of AqGR and it is used, at various scales, in all taxonomic groups of AqGR. It relies on the near cessation of metabolic processes at cryogenic temperatures, typically that of liquid nitrogen (-196 ^{o}C). The fundamentals of low-temperature biology, upon which cryopreservation is based, are described in Box 1 and help readers to understand the methodologies and laboratory protocols presented in later chapters and annexes for the different taxonomic groups (Chapter 5,6 and 7; Annex 1, 2 and 3).

Desiccation is used in the gene banking of brine shrimp *Artemia* spp., one of the few animals to evolve the capacity to survive in highly dry conditions by entering a quiescent state. Thanks to such desiccation resistance, Artemia naturally-produced cysts, containing embryos, can be stored for many years (Annex 4). Some reproductive tissues of macroalgae can also be dried and preserved through desiccation (see Chapter 7), as also can cyanobacteria. Otherwise, other dried storage, including freeze-drying, has not proven generally effective in preserving viable AqGR.

Chilled storage at temperatures just above freezing (4–8 ^{o}C) of finfish gametes, is useful for short term storage of about a week (depending on the species), to facilitate breeding programs or transport material between hatcheries or to cryopreservation facilities. It is not an effective long-term method for these materials, although tissue culture (described below) may be carried out at these temperatures.

Tissue culture is the *in vitro* culture of cells, tissues, organs, and their components under defined physical and chemical conditions (Thorpe, 2007). Itis used in AqGR gene banking primarily for algal tissues of microalgae and macroalgae. This maintains the genetic material alive in the environment of culture chambers (Chapter 7). More recently, finfish cells culture is also gained popularity thanks to progress made in this field, particularly in finfish, and in response to pressures to find alternative ways to reduce and/or replace animal use in scientific research (Aarattuthodi et al., 2021). It consists in the raising of living cells in an artificial culture medium (Khurshid et al., 2024) and have several potential applications in aquaculture such as to reconstituting an organism as an alternative approach to reconstitution from frozen milt (Annex 11). However, most of these potential applications are still under research.

In vitro conservation methods for aquatic animals and algae should reflect the reasons for collection in the first place. As indicated in Chapter 1, these reasons may include:

1. Preservation of genetic material for the reconstitution of wild populations and farmed types.
2. Preservation of genetic material from captive populations to protect selected farmed types from potential loss, and for distribution or continued broodstock development; and
3. Scientific research for the development or improvement of laboratory protocols, pharmaceutical applications, and biofuel development.

The location and facilities available for *in vitro* conservation of genetic material in the wild or from wild organisms are different from those for in vitro conservation of tissues from organisms held in a laboratory, a hatchery, or farm. Procedures that are appropriate for a temperature-controlled facility with electrical power and water may be impossible to implement in the field or their implementation may take so long that, in the meantime, individuals from elusive species have left the area and cannot be sampled. In such cases, complicated approaches may therefore be counterproductive. Also, biosecurity measures are harder to implement in field conditions, but strict sterility is unlikely to be an issue for many of the users of field-collected genetic material. Again, the end-use should help decide what would be deemed as appropriate approaches.

It is important to set the criteria for success at a comprehensive practical level rather than focusing too much on optimizing individual features. For example, a simplified field procedure may reduce fertility by a small amount but could be desirable overall because it can enable collection of material from wild populations. This is a typical trade-off. Once the natural history of the species and the physiology of the life stage to be preserved (e.g., finfish sperm cells) are well understood, tailoring a procedure to different end-purposes can be done with some confidence. This is where some parts of a cryopreservation protocol can benefit from "informed simplification" for field collection, or to make procedures more teachable and widely applied. The steps for the development of cryopreservation protocols for a species or different scenarios are described in detail in the following chapters.

BOX 1
CRYOBIOLOGY: A PRIMER

Brian Harvey

Introduction

The temperature of nitrogen in its liquid state is -196 °C. A drop of liquid nitrogen can instantly destroys a layer hundreds of cells thick. How is it possible then that a cryobiologist can retrieve a vial of oyster larvae from the vat of liquid nitrogen where it has spent decades, plunge it into warm water, put a droplet under a low power microscope and watch microscopic organisms zoom back to life?

This primer on cryobiology explains how living cells can survive the seemingly impossible journey to -196 °C and back to normal viability. For readers who are wondering why most of the methods involve freezing to the temperature of liquid nitrogen, the answer is a practical one: although the temperature of dry ice (-79 °C) is low enough to complete the process of cryopreservation, liquid nitrogen at -196ºC offers longer storage viability (decades, at a minimum). At this temperature most biochemical reactions are suspended. Liquid nitrogen is also relatively easy to obtain and store and can be used to create temperature differentials for cooling and freezing.

The scope of cryobiology

Cryobiology is a large scientific discipline and related research is split between studies aimed at understanding the biophysics of the cellular response to low temperature, and studies with the goal of developing practical methods for cryopreserving specific cell types in service of medicine, animal husbandry, agriculture or conservation. It is important to stress that "practical" researchers, especially those in aquaculture, do not usually interact with the biophysicists and biochemists doing basic science on cryobiology. However, a good understanding of the freezing and thawing processes would help eliminating the repetitions and unwarranted steps which are common in empirically worked-out methods. At the very least, it is critical for applied scientists and users of guides like this one to know the basics.

What happens when a cell is frozen?

The most important effect of cooling is the inevitable freezing of intracellular water, which makes up a high percentage of the cell. The ice that forms *outside* the cell is not usually a problem, unless one is freezing an assemblage or tissue where ice can mechanically disrupt the connections between cells. The intracellular ice concentrates what is dissolved in the remaining liquid. The formation of internal ice crystals is normally lethal (unless they are very small), although it is still not entirely clear why (Jang *et al.*, 2017). Mechanical damage is probably less important than the "solute effect" that results from the higher concentrations of water-soluble cell components that inevitably follow the removal of water from the cell in the form of ice (Figure 1).

How can intracellular ice formation be prevented?

An important achievement in the history of cryopreservation was the discovery that the use of glycerol, as a medium for cell cryopreservation, increased the survivability of spermatozoa after thawing, as it prevented the formation of ice crystals (Polge *et al.*, 1949; Whaley *et al.*, 2021). In this context, glycerol acts as a *cryoprotectant*, a compound with the property to permeate the cell membrane displacing intracellular water and lowering the freezing temperature, consequently delaying the formation of intracellular ice. Glycerol (also called glycerin) is a common natural compound used in foods, medicine, industry, and

cryopreservation. As of 2024, at least 100 cryoprotectants are now known, with dimethyl sulfoxide (DMSO), methanol, glycerol ethylene glycol, and propanediol being among the most used for aquatic species. To act as an effective cryoprotectant, other than increasing the porosity of cellular membranes, a compound must be highly soluble in water at low temperature in order to facilitate its mitigation of osmotic imbalances and have low toxicity (Whaley *et al.*, 2021).

Cells are exposed to the cryoprotectant at the beginning of the cryopreservation process, before being frozen, and then plunged into liquid nitrogen. The freezing rate affects how the fluids move and offsets the toxicity of any solutes that are concentrated by freezing (see below). The cryoprotectant must then be removed after thawing, which is usually done by successive washes in a cryoprotectant-free medium during reconstitution or fertilization. However, the cells tend to swell during this process as water enters the cells more rapidly than the cryoprotectant leaves. Cell expansion is less tolerated than shrinking, so the thawing process can sometimes damage the cells more than the freezing. The concentration of the cryoprotectant utilized is critical to optimize the cryoprotectant effect. The cryoprotectant needs to be concentrated enough to ensure it passes through membranes quickly enough to minimize damage of osmotic imbalances, but it can also be toxic at higher concentrations.

Cryoprotectants thus make freezing possible, but there is a cost, necessitating trade-offs. To understand the importance of shrinking and thawing, it helps to think of the process independently from freezing. When cells are put in a solution of cryoprotectant, for example glycerol, they first shrink because the water leaves the cell (by osmosis) faster than glycerol can enter (by diffusion). When the glycerol finally enters, the cells regain their shape. Once cells are placed back in their normal fluid surroundings, the reverse happens: the water rushes in and dilutes the glycerol inside the cell, the cell expands, the glycerol makes its way back out through the membrane, and the cell shrinks back to normal. The complication added by cooling is that the cells keep losing water after the ice forms outside the cell, because they are trying to equilibrate to a changing external environment. This is a good thing though and most conventional cryopreservation methods count on progressive dehydration during cooling, because it pulls water out of the cell, reducing ice crystal formation.

Cryoprotecting reagent is not enough

Adding a cryoprotectant is only part of the solution to minimize the likelihood of intracellular ice formation. Another key factor is the rate at which the protected cells are frozen – and thawed. In the 1960s it was discovered that how fast the cell is frozen determines whether ice crystals form inside the cell (Mazur, 1963). With slow enough freezing, water can leave the cells enough that ice crystals will primarily form outside the cell. If the cooling is too slow, however, the cell will be exposed to high solute concentrations long enough to suffer toxic effects. Thus, for a given set of conditions, each kind of cell can have an optimal cooling rate, and the range is large, from less than one degree/minute to 50 or more. The warming rate is important too: if too slow, harmless microcrystals can grow into damaging ones; whether if too fast, some cells will not have enough time to offload their cryoprotectant and will potentially tumesce and burst as water rushes in. Optimal cooling and warming rates depend on how permeable the membrane is to water and how large the cell is (small cells have relatively more surface area in relation to volume, so they can transport water and cryoprotectants faster than larger cells).

It is probably clear by now that a lot happens when cells are protected, cooled, thawed and recovered. Water and cryoprotectants move back and forth across cell membranes in ways that are acutely sensitive to the rate of cooling and warming. Somewhere there is usually an acceptable balance between ice crystal formation and toxic concentration effects, but the widely varying methods for aquatic organisms – with different containers, different cooling devices, different cryoprotectants and rates – reflect how elusive that

balance can be. An *extender solution*, sometimes called a "diluent", combined with the cryoprotectant, helps to improve the freezing process, as well as improving the handling of milt or other tissues. Undiluted semen is not suitable for freezing. An *extender* may be added before adding a cryoprotectant to dilute and stabilize the milt, followed by the addition of cryoprotectant shortly before freezing, or a one-step method may be suitable. The relative effectiveness may be species-specific and depend on the chosen preservation strategy. The solutions containing cryoprotectant are also referred to as cryoprotective agents (CPA).

The extender is usually similar in internal physiological characteristics to the organism of interest. It functions as a medium for cryoprotectants, minimizing injuries to the cell from potential intracellular ice crystals at the time of freezing and thawing and the effect of concentrated cell solutes. Determining the cryoprotectant type and its appropriate concentration in an extender medium is an important step of developing cryopreservation protocols. In the case of spermatozoa, the extender also inhibits the activation of spermatozoa. Spermatozoa only have a limited amount of stored energy, so in general have a very limited life after activation. In the case of sperm of aquatic animals, activation is generally triggered by exposure to water of lower salinity than in the organism. Cryopreservation protocols aim to postpone activation until after thawing.

An extender is typically as solution of balanced salts and sugars that inhibits the activation of motility. A good extender for the preservation of semen of a species matches with the properties of the seminal fluid of that species and inhibits the activation of sperm.

What makes some cells easier to cryopreserve?

Several factors contribute to make a cell easier or more difficult to cryopreserve:

- **Cell permeability**
  As already mentioned, the more a cell is permeable to water and cryoprotectants, the less the freeze-thaw process will damage it.
  In terms of permeability, another factor to consider is the presence of a cell wall in addition to a semi-permeable cell membrane (like in the case of plant cells) because it can reduce the cell permeability.
- **Cell type**
  Among cell types, spermatozoa and red blood cells are quite permeable and relatively easy to freeze. Small size cells are also generally easier to freeze.
- **Cell content**
  The cell content counts too: the most extreme example is the high fat content of the unfertilized eggs of animals that provide a yolk supply for developing embryos. For example, salmon sperm cells are easy to freeze, but their so far eggs have proved impossible to effectively freeze.
- **Presence of complex cell structures**
  Cells characterized by the presence of complex structures may also be more difficult (e.g. flagellated algae).
- **Cell condition**
  A much more subtle example is the cell condition: cells from a well-fed, unstressed organism may look the same as those from an unhealthy one, but they will survive freezing and thawing much better. The same can be said for the reduction in quality that comes from refrigerated storage and shipping of otherwise robust cells. Contamination by urine or other contaminating fluids will also reduce the success of cryopreservation, particularly if they trigger motility in spermatozoa.

Freeze-thaw success is also influenced by how closely cells are packed together (Pegg *et al.*, 1984). Even in red blood cells, which must be concentrated to make freezing and storage practical, there is a limit to how tightly they can be packed. The reason is probably physical damage from extracellular ice (Pegg, 1987).

How good is "good"?

Cells are damaged by freezing and thawing (Figure 1). Electron micrographs reveal punctured membranes and other disruptions, and it is common that thawed finfish spermatozoa, which are expected to swim vigorously long enough to encounter an egg, may cover less distance and swim for less time than fresh cells. This should not be surprising, because the activation of spermatozoa is a membrane effect. Fortunately, damaged cells can recover, such that post-thaw function is largely normal. In many cases, the simple mitigation for such damage is to freeze more cells. To return to the case of finfish sperm, doubling the amount of frozen and thawed sperm per egg will often approach normal fertilization levels.

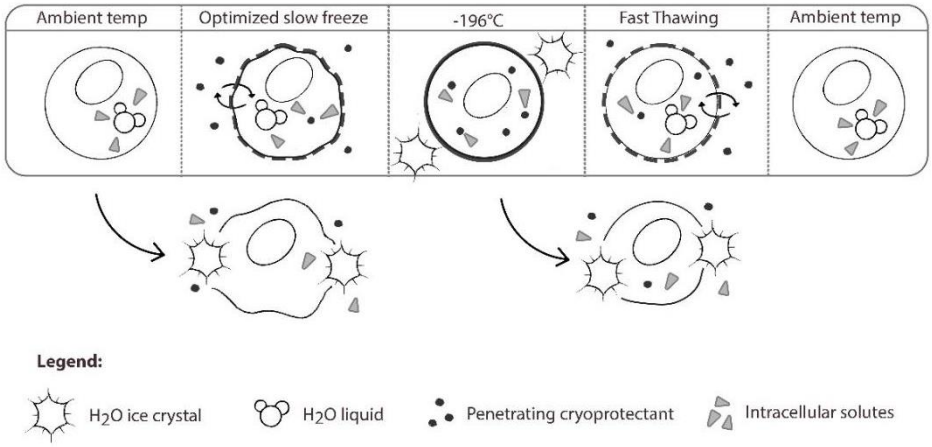
Why are multicellular systems harder to freeze than individual cells?

Tissues, organs, embryos, and larvae are made up of many kinds of cells that can potentially react differently to any given procedure (Jang *et al.*, 2017). Furthermore, for a multicellular structure it takes longer for water and cryoprotectants to move in and out of the cell interior. One way around this dilemma is to increase the dose of cryoprotectant. A larger problem can be the formation of extracellular ice, which is normal for single-cell situations but, in the case of tissues, extracellular ice may be inside the system being preserved, and can exert forces that damage cells (for example, ice between muscle cells, or rupture of blood vessels within a tissue). However, in several cases, multicellular systems can still be preserved successfully. For example, the trochophore larvae of oysters and mussels, which are about 60–80 µm, oval in shape and equipped with a row of cilia and a flagellum that make them able to swim (FAO, 2004), can survive freezing and thawing.

Vitrification: when freezing does not produce crystals

What if ice were allowed to form during freezing, but not in the crystalline state? Since the beginning of cryobiology, researchers have searched for ways to vitrify cells, that is, to make the cellular environment so internally viscous ("glassy") that the freezing produces a solid state without the formation of internal ice crystals. For the vitrification to work, the freezing rate must be extremely fast, the opposite of what is needed in the "conventional" cryopreservation process. If it is not, the relatively high concentrations of cryoprotectants needed (often combined in "cocktails") will be toxic. The subsequent warming also needs to be rapid. This approach can work where slow-freezing methods fail, with one well-known early example being the embryo of the fruit fly (Mazur *et al.*, 1992). So far, cell vitrification has not yet been successful in aquatic species, with a few exceptions mainly represented by coral cells (Annex 5).

Figure 1. Freezing process of cells in optimized freezing protocol and damages of water crystallization during freezing and recrystallization during thawing resulting from too slow freezing (1) and thawing (2). During slow freezing, internal and external ice crystals break membranes, during slow thawing, large crystals also form and disrupt membranes. Penetrating cryprotectants help replace water to minimize internal ice development and mitigate toxicity of internal solutes, whereas non-penetrating cryoprotectants strengthen membranes.



References

Helm, M.M. , Bourne, N., & Lovatelli, A. 2004. *Hatchery culture of Bivalves – a practical nanual*. FAO Fisheries Technical Paper 471, FAO, Rome.

Jang, T.H., Park, S.C., Yang, J.H., Kim, J.Y., Seok, J.H., Park, U.S., Choi, C.W., Lee, S.R., & Han, J. 2017. *Cryopreservation and its clinical applications*. Integrative Medicine Research, **6** (1):12-18.

Mazur, P. 1963. *Kinetics of water loss from cells at subzero temperatures and the likelihood of intracellular freezing*. J. Gen. Physiol. **, 47: 347–369.**

Mazur, P., Cole, K.W., Hall, J.W., Schreuders, P.D., &Mahowald, A.P. 1992. *Cryobiological preservation of Drosophila embryos*. Science 258: 1932–1935.

Pegg, D.E., Diaper, M.P., Skaer, H. le B., & Hunt, C.J. 1984. *The effect of cooling rate and warming rate on the packing effect in human erythrocytes frozen and thawed in the presence of 2M glycerol*. Cryobiology 21: 491–502.

Pegg, D.E. 1987. *Mechanisms of Freezing Damage. In: Temperature and Animal Cells, Symposia XXXXI of the Society for Experimental Biology* (Bowler, K. & Fuller, B.J. eds), The Company of Biologists Ltd., Cambridge, UK, pp. 363–378.

Polge, C., Smith, A.U., and Parkes, A.S. 1949. *Revival of spermatozoa after vitrification and dehydration at low temperatures*. Nature 164**:** 666.

Thorpe, T. A., & Thorpe, T. A. (2007). *History of Plant Tissue Culture*. *Molecular Biotechnology*, 37(2), 169–180. https://doi.org/10.1007/s12033-007-0031-3

Whaley, D., Damyar, K., Witek, R.P., Mendoza, A., Alexander, M. & Lakey, J.R. 2021. *Cryopreservation: an overview of principles and cell-specific considerations*. Cell Transplantation, 30. https://doi.org/10.1177/0963689721999617

Chapter 3. Frameworks for gene banking

Terrence Tiersch, Aquaculture Germplasm and Genetic Resources Center (AGGRC), Louisiana State University Agricultural Center, Baton Rouge, Louisiana, United States of America

Introduction

Gene banks are repositories for the collection, preservation, and management of genetic resources. However, a large integrated central repository is responsible not only for storage but also for strategy development, sample receipt, curation of associated information, and distribution to community members (Liu *et al.*, 2021; Torres and Tiersch, 2018). The term "repository" is used here specifically to mean a fully integrated gene bank, a gene bank model that has good stability and reliable results. This applies in some form to most governmental and non-governmental AqGR repositories. Smaller gene banks may have different approaches but can be organized to be able to deal with the same issues. These approaches are considered "Frameworks".

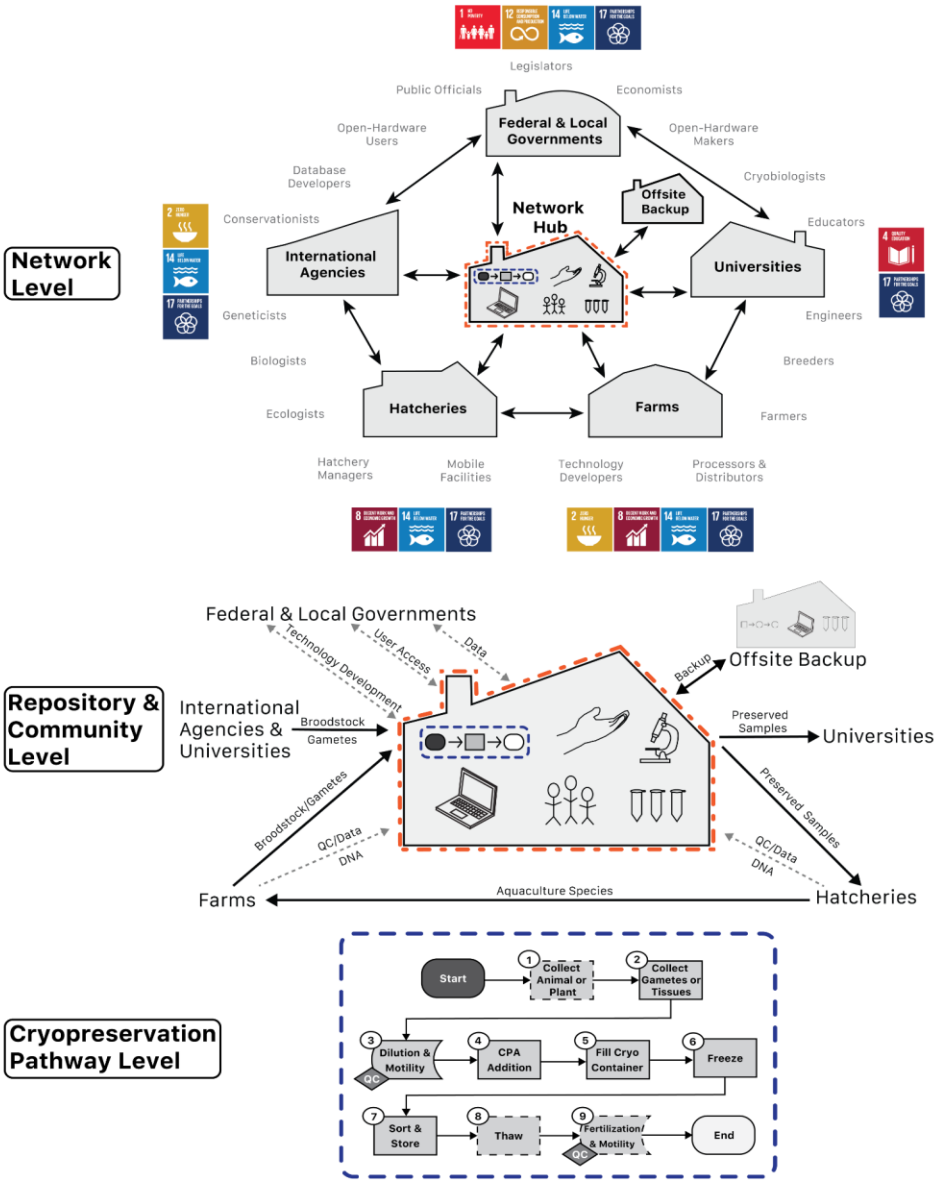
The organizational structure of successful central repositories can be viewed at three levels:

1) preservation, storage, and use protocols and quality management pathways (placing protocols into robust applied frameworks);
2) community interactions; and
3) formation of networks.

The three levels can be hierarchically related as illustrated in Figure 2. While Figure 2 is based on cryopreservation, similar structure and processes can apply to other AqGR preservation methods.

This chapter explains in detail: the organizational structure of a repository; the requirements for the development of standardized pathways for effective samples processing, long-term storage and exchange; and the interaction of the gene-bank with its community members and with other repositories and entities.

Figure 2. Germplasm repository activities and relationships presented in process mapping format.[8] *The three diagrams represent three levels of organization. The foundation of a repository activities is provided by preservation pathways (based on applied research) that are standardized to reliably process samples with quality management and record-keeping. With protocol pathways in place, the repository can interact with its community members and networks to develop strong, flexible working relationships.*



[8] Process mapping can be used for planning and design of frameworks. With sufficient data collection including features such as cost, quality, labor, and time overlayed on the process maps, simulation modelling can be employed to predict and improve operational efficiency (see Annex 6).

Preservation pathway levels

A preservation pathway relies on proven protocols that have transitioned from research to applied practice and are scalable and robust (Tiersch, 2011). These may vary in detail, depending on the preservation strategy chosen. For example, rapid preservation in the field may use methods in a pathway slightly differently than remote collection, transport of fresh material, and preservation in more sophisticated facilities. Nevertheless, equipment and protocol harmonization among repositories would ensure improved collaboration, quality assurance, and reliability over time. Protocols may initially be based on single species or species groups but can be adapted to other species with research that takes into account known characteristics of the process and the species (Annex 1, 2 and 3).

Quality management (QM) is a key element of repository pathways. Quality management in repositories comprises *quality control* (QC), *quality assurance* (QA), and other relevant elements. The investment of effort and money in repository development needs to be supported by informed estimates of sample quality, especially if storage is intended for several decades. A QM pathway should be developed, integrated, and documented in parallel with the preservation pathway (Torres *et al.*, 2016), and allow personnel at other facilities to produce samples of similar quality. The QM pathway should include training to standardize processes across facilities and allow personnel to communicate using shared terminology.

QC focuses on monitoring materials throughout the process (Torres *et al.*, 2016). An example of a QC activity is the recording of sperm motility after its collection, just before freezing, and just after thawing to ensure it meets minimum requirements. Poor initial motility, for example, indicates the sperm should not be banked, and the pre-freezing baseline allows identification of problems in freezing or storage. QA is process-oriented, making sure the process minimizes biological limitations, and human, or machine error (Torres *et al.*, 2016). Examples of QA include a checklist for maintenance and calibration of all equipment to be used in the freezing process, a systematic plan for QC testing, adequate training of personnel, and sourcing of reliable equipment and materials. A single-point quality evaluation check such as post-thaw motility is not a QC or QM activity because it comes too late in the process to prevent defects in product quality and losses in time and supplies.

Equipment harmonization makes samples and measurements comparable among institutions and over time (Cuevas-Uribe and Tiersch, 2011). Harmonization in this context can involve testing how different equipment and materials influence the quality of results. Laboratories or facilities may not all have access to the same equipment, but when employing a standardized preservation pathway, the samples produced can all be of similar quality, with comparable data. Similarly, protocol harmonization ensures comparability among different protocols by identifying standardizable elements. Some research has focused on these comparisons, but further work is needed, especially generating agreement on comparable quality measures and terminology. If adequately robust, these can be scaled to operate at small and large levels of production (Varga and Tiersch, 2012). Chapter 9 provides a list of equipment needed for the gene banking of AqGR (with a focus on cryopreservation) whilst Annex 6 provides an overview of process mapping.

Most of the components of preservation pathways are generalizable, so those developed for one species can be used as a starting point when attempting to preserve material of another similar species (Liu *et al.*, 2018). The extensive research literature often provides clues to how particular protocols may be applied across species and can provide shortcuts for adaptive research, even if it does not yet provide detailed systematic

comparisons. For example, the cryoprotectant DMSO may not be available in some regions, but a suitable alternate cryoprotectant and starting protocol may be identifiable from the literature on similar target species. In this regard, it is particularly important to note that there are no *sensu stricto* optimal cryopreservation conditions, because numerous factors interact, and changing any single factor can affect others (e.g., a change in the cryoprotectant may involve a change in the cooling rate). Also, many of the steps in preservation pathways are similar even if the specifics of the actions differ. For example, once sperm are collected and combined with the cryoprotectant they must be packaged in a container before freezing. While the container and method of aliquoting may change from species to species, the "fill cryo-container" step (Step 5, Figure 2) is always included in the pathway, making the pathway generalizable and useful for planning. Research on developing protocols ideally takes this eventual generalization into account. Studies on novel cryoprotectants, for example, would include comparisons with established protocols and reproducible methods to take into consideration the availability and scaled-up application of the novel element.

Sample information management

The availability of information on the samples stored in a repository and the level of details of such information is critical to the value of the conserved material. Data on sample origin, processing methods, inventory tracking, and biological characteristics at the individual and population levels are essential for gene banking. Without such information, the value of the repository risks being greatly unexploited. It is also important to foresee that the collection and storage of such information is compatible with other gene banks and user groups.

The information associated with samples can be classified in three groups:

1. Data related to the sample donor at the levels of individual (e.g., sex, age, phenotypic data) and population (e.g., GPS coordinates of collection site, environmental data, genetic characteristics);
2. Data related to the sample itself (e.g., method of collection, method of processing, sample quality);
3. Inventory (e.g., number of samples and current location in the repository); and
4. Linkages with genomic databases, production information, and future data on performance of the preserved material (e.g., fertilization percent in the case of male gametes) and the offspring produced with the samples (e.g., genetic merit).

Repository managers should develop a data management plan (DMP) identifying the data to be collected and managed, and defining rules for data collection and entry, storage and sharing, while taking into account also relevant rules and regulations on data protection (Boes, 2023). Minimum requirements for specific data fields and for the structuring of the gene bank database should be identified in cooperation with user communities. The information needed will depend on the specific objectives, applications, and species in the repository, and it should consider the potential for novel future uses of samples and information. Although it is typically understood that it is imperative to maintain a current and accurate database, the effort required is often substantially underestimated in staffing plans.

Each sample should be assigned an accession number: a distinct, unique identifier which identifies that specific sample, also called accession, stored in the gene bank. The DMP should also include a specification of metadata which is the set data fields associated with and describing a gene bank sample, as well as

information that defines the data. Examples of metadata are: (i) type of material; (ii) date of collection; iii) identification number, etc. The information collected in the database should follow the FAIR principle, according to which scientific data should be: findable, accessible, interoperable and re-usable (Boes, 2023).

The database used by the repository should be able to link with genomic databases, agricultural production information, ecological characters, and future data collected on performance of the preserved gametes (e.g., fertilization and survival success and genetic merit). In this sense, the Animal Germplasm Resource Network (A-GRIN) constitutes a larger scale example. If possible, the database should provide a public-facing interface to enable users to interact with the repository. There are many software options for sample information management, including programs specifically designed for inventory control of samples maintained in *ex situ in vitro* collections, but most are customized for individual use. Custom gene banking databases can be developed for smaller gene banks with commercially available generalist software (such as Microsoft Excel or Access), but larger gene banks need more comprehensive databases that are compatible with other gene banks and are resilient to software updates. Unfortunately, none are available commercially. In any case, backup of the database (e.g., secure cloud storage) and hard copies kept at different sites are important security considerations.

Health of broodstock

The quality of gametes is influenced by the health and condition of the parents (e.g., Yang *et al.*, 2007). Well-conditioned ripe donors should be prioritized for gamete collection, and stress should be minimized during the collection and holding of animals.

Biosecurity risks and precautionary measures

The transfer of cells and tissues from aquatic species poses concerns for unintended transfer of microbial organisms from infected broodstock or water. This is a special concern for broodstock that have become immunosuppressed as a result of stress from capture, transport, or handling for spawning (Tiersch and Jenkins 2003). All samples should be considered potentially contaminated, and precautions should always be made to avoid cross-contamination. This is especially important when transporting samples between regions. Special care should be taken when sampling wild animals when their disease-free status cannot be guaranteed. With adequate care, the level of contamination in cryopreserved material is generally not problematic. However, contamination in tissue culture of microalgae requires particular attention, as described for algae in Chapter 7.

Proper sanitation during collection is essential for limiting the spread and growth of microorganisms such as bacteria, viruses, fungi, archaea, and parasites. Materials and equipment used to freeze samples should ideally be sterile, though this may not be possible under field conditions. Nevertheless, the sampling strategy should minimize contamination and be recorded for input into sample databases.

Contamination of samples can occur during collection, processing, storage, and transport. Viruses, bacteria, archaea and fungi can survive cryogenic temperatures and are a potential source of cross-contamination in frozen samples. Pathogenic or commensal microorganisms could impair in vitro fertilization (IVF) efforts or development of embryos or larvae after thawing (Christensen and Tiersch, 1996; Jenkins and Tiersch, 1997; Sooksawat *et al.*, 2020). Microorganisms can enter liquid nitrogen via contaminated surfaces or leaks, or directly from the atmosphere when airborne water droplets freeze above an open liquid nitrogen container. While liquid nitrogen can itself be contaminated, contaminants originating from stored samples

are more likely and show larger effects on stored biomaterials (Bajerski *et al.*, 2021). Microbial load could increase with time, via incorporation of new samples or by opening of the storage unit, so the risk of sample contamination could rise with use (Bajerski *et al.*, 2020). Therefore, it is good practice to clean storage units periodically.

Additives such as egg yolk or powdered milk, used in some protocols for finfish cryopreservation as a way to protect sperm cell membranes from damage, may also carry biological contamination. While there is no good evidence that this is problematic for finfish, protocols should balance any positive effects of such additives with the potential and actual costs.

Genetic considerations

An important practical question in gene banking is often how many individuals need to be represented. There is no definitive answer that addresses all situations and purposes. For conservation breeding, information on wild and hatchery genetic diversity is needed. It has been estimated that a minimum of 150 wild-sourced broodstock finfish (50:50 sex ratio) were needed to retain rare alleles in a white seabass hatchery program (Bartley *et al.*, 1995). These rare alleles occurred at an estimated two percent frequency, and they were primarily responsible for distinction of local populations. In this case, 75 males would be a minimum target for preservation. In aquaculture, inbreeding is a more common concern. An effective population number (Ne) of 50 has been put forward as the most common recommendation (Tave, 2008), using the following formula where Ne is calculated based on the number of females and the number of males that reproduce and leave viable offspring:

$$Ne = 4 \times (\text{number females}) \times (\text{number males})/(\text{females} + \text{males})$$

This calculation refers specifically to the first generation of offspring, with deterioration in subsequent generations. Some approaches, relevant to the use of gene banks, to reduce genetic deterioration in subsequent generations, particularly in an aquaculture environment where the number of breeders used is limited (and particularly species are highly fecund), include:

1. Introduction of new broodstock from the wild (or from another gene bank);
2. Avoiding use of pooled sperm (as the effective number of males can be reduced by gametic competition) followed by a pooling scheme of the separate groups at later intervals; and
3. Use of pedigree information on the mating scheme.

Genetic analytical tools are increasingly used to help guide selective breeding and/or maintain genetic health of aquaculture broodstock. These also help qualify selection of individuals that contribute to frozen gene banks.

Community interaction level

The *community interaction level* (Figure 2) encompasses all the processes that take place at the repository, and involves research, preservation pathways, community outreach, equipment, database systems and their management, personnel and supplies. The community interaction level also identifies operational interactions, such as the exchange of materials and information that can take place among the repository

and surrounding community members. The community members may include university and non-academic research institutes, hatcheries, and grow-out farms. Fresh or frozen gametes can be contributed by farms or researchers, and preserved gametes would be returned as needed; researchers from university, government, private enterprise, or non-profit entities can contribute to methodologies; and governments can contribute policies and funding.

For example, a repository focused on aquaculture may ship cryopreserved sperm from selectively bred fish to a hatchery. The hatchery spawns the fish, raises the larvae, and distributes them to farms where they are grown out. During the harvest, the farm can collect sperm from high quality broodstock as part of their selective breeding program and send it to the repository for storage and future use (Figure 2). This is how repositories of terrestrial genetic material largely work, as well as governmental repositories that include AqGR. However, to date, the few aquaculture-focused repositories for finfish and shellfish are in-house or privately held, and farmed types are traded as fertilized ova (e.g. salmonids and *Artemia* cysts), larvae (finfish and shrimp), or juveniles (finfish). For algae, central repositories trade reconstituted genetic material in various forms, rather than the preserved samples.

Network level

The *network level* (Figure 2) focuses on the relationships among repositories, community members that interact with it and each other, and other entities. This level tracks the flow of information and materials among the network components. For example, in an international repository network focused on aquaculture, a repository would be expected to interact with other repositories, federal and local governments, international agencies, universities, hatcheries, and farms. These actors would exchange samples and data and collaborate with each other to address sustainability goals. Most of the gene banks now operating at this level are focused on terrestrial agricultural resources but some have an AqGR component. Some examples exist for international algae-specific and *Artemia* gene banks that have been established (see Chapter 6 and 7). This and the previous level would also encompass commercial interactions including sales of genetic material with intellectual property protections.

Overall, process mapping enables predictions of how the decisions made at one level of the repository can affect components at other levels. For example, a scientist may decide to use a particular type of expensive cryoprotectant (e.g., to yield a maximum value for post-thaw motility) while developing a cryopreservation protocol, but the chemical may be hard to source, or pose human safety concerns, and thus may not be suitable for large-scale, generalized application. Similarly, the implementation of the Nagoya Protocol on Access and benefit-sharing can have major implications for how a centralized, international repository functions and how the biological material is sourced and shared.

References

Bajerski, F., Bürger, A., Glasmacher, B., Keller, E.R.J., Müller, K., Mühldorfer, K., Nagel, M., Rüdel, H., Müller, T., Schenkel, J., & Overmann, J. 2020. Factors determining microbial colonization of liquid nitrogen storage tanks used for archiving biological samples. *Applied Microbiology and Biotechnology* 104(1): 131–144.

Bajerski, F., Nagel, M., & Overmann, J. 2021. Microbial occurrence in liquid nitrogen storage tanks: a challenge for cryobanking*? Applied Microbiology and Biotechnology* 105(20), 7635–7650.

Bartley, D.M., Kent, D.B., & Drawbridge, M.A. 1995. Conservation of Genetic Diversity in a White Sea Bass Hatchery Enhancement Program in Southern California. *American Fisheries Society Symposium* 15: 249–258.

Boes, J., Boettcher, P. & Honkatukia, M., eds. 2023. *Innovations in cryoconservation of animal genetic resources – Practical guide.* FAO Animal Production and Health Guidelines, No. 33. Rome, FAO. https://doi.org/10.4060/cc3078en

Christensen, J.M., & Tiersch, T.R. 1996. Refrigerated storage of channel catfish sperm. *Journal of the World Aquaculture Society* 27(3), 340–346.

Cuevas-Uribe R., & Tiersch, T.R. 2011. Estimation of fish sperm concentration by use of spectrophotometry. In: *Cryopreservation in Aquatic Species*, 2nd edition. T.R. Tiersch, and C.C. Green, editors. pp. 162–200. Baton Rouge, Louisiana, World Aquaculture Society.

Jenkins, J.A. and Tiersch, T.R. 1997. A preliminary bacteriological study of refrigerated channel catfish sperm. *Journal of the World Aquaculture Society* 28:282-288.

Liu,Y., Torres, L. & Tiersch, T.R. 2018. *Cryopreservation of sperm bundles (spermatozeugmata) from endangered livebearing goodeids*. Cryobiology 82, 49–56. https://doi.org/10.1016/j.cryobiol.2018.04.009

Liu, Y., Monroe, T.W., Belgodere, J., Choi, J.-W., Gutierrez-Wing, M.T., & Tiersch, T.R. 2021. The emerging role of open technologies for community-based improvement of throughput and quality management of sperm cryopreservation for repository development in aquatic species. *Animal Reproduction Science*. https://doi.org/10.1016/j.anireprosci.2021.106871. Epub 2021 Oct 16.**Sooksawat, T., Boonthai, T., Nimrat, S., & Vuthiphandchai, V.** 2020. Cryostorage of silver barb (Barbodes gonionotus) semen in dry shipper: Efficacy, risk of bacterial cross-contamination and effects of Aeromonas hydrophila on post-thaw sperm quality. *Cryobiology*, 96:184-196. https://doi.org/10.1016/j.cryobiol.2020.06.011. Epub 2020 Jul 18.**Tave, D.** 2008. 3. *Broodstock Management, Inbreeding, Genetic Drift and Domestication. FAO Technical Reports for Responsible Fisheries* 5 (3).

Tiersch, T.R., &J.A. Jenkins. 2003. Biosecurity considerations for cryopreserved gametes and early life stages of aquatic species. *In:* C.-S. Lee & P. J. O'Bryen, eds. *Biosecurity in Aquaculture Production Systems: Exclusion of Pathogens and Other Undesirables.* Baton Rouge, Louisiana, USA. World Aquaculture

Torres, L., & Tiersch, T.R. 2018. Addressing Reproducibility in Cryopreservation, and Considerations Necessary for Commercialization and Community Development in Support of Genetic Resources of

Aquatic Species. *Journal of the World Aquaculture Society*, 49(4): 644–663. https://doi.org/10.1111/jwas.12541

Torres, L., Hu, E., & Tiersch, T.R. 2016. Cryopreservation in fish: Current status and pathways to quality assurance and quality control in repository development. *Reproduction, Fertility and Development*, 28(8): 1105–1115. https://doi.org/10.1071/RD15388

Chapter 4. Putting the process into practice

Terrence Tiersch, Aquaculture Germplasm and Genetic Resources Center (AGGRC), Louisiana State University Agricultural Center, Baton Rouge, Louisiana, United States of America

Introduction

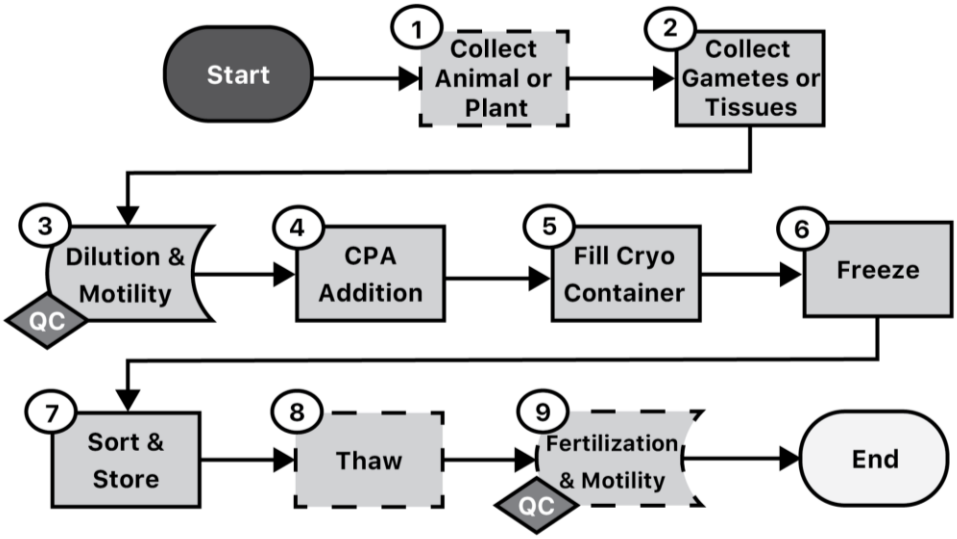
This chapter describes in more depth the in vitro preservation and management of AqGR to show how the process can be applied in the real world of gene banking, particularly for new species and newcomers to gene banking. The methods described are informed primarily by the work and experience with cryopreservation of finfish sperm but are also relevant to other organisms and biological material. More specific information for algae and shellfish is presented in the relevant succeeding chapters.

Development of preservation pathways

The bulk of aquatic species cryopreservation research has been devoted to the development of protocols. This work includes work on reproductive physiology, gamete biology, cryobiology, and genetics. At a practical level, protocols often serve as recipes that are implemented in various ways by different users. However, when protocols are placed within a broader context that accommodates community-level adoption with reproducibility arising from commonality in training, methods, equipment, and approach, they can be viewed as a pathway. If constructed properly, pathways can be independent of species and protocol. They are thus generalizable, enabling community-level interaction where multiple groups can share information and findings. If they use the same pathway, different groups can also standardize features such as terminology and identification of necessary process steps.

The pathway approach can be straightforward, incorporating the basic steps of a cryopreservation approach that could represent most species and preservation strategies. We can borrow powerful tools from industrial engineering to visualize pathways. An example is process mapping which itself can be used as a foundation for simulation modeling (Figure 3; Annex 6). An approach such as this recognizes differences but focuses on similarities. It also recognizes details but looks for generalities, and most importantly it supports cooperation rather than competition. As such, it can be used as a community-level resource. For example, as a virtual bulletin board where groups can place findings into a shared workspace where all participants can be involved in decision-making at the level of broad application. In practice, this could mean that a central germplasm repository would maintain a pathway, perhaps on a website, where researchers and other users could add new information and improvements in a common format. These advances could then be directly integrated at the repository level rather than being simply published in the context of multiple competing groups. A recent example of this approach is provided by the development of a scalable cryopreservation pathway for an endangered salamander, the Mexican axolotl (*Ambystoma mexicanum*) that is also a widely used biomedical model (Coxe *et al.*, 2024). A typical pathway-level flow of a cryopreservation process illustrated in Figure 3.

Figure 3. A pathway-level process flow diagram that outlines all the steps, in sequence, of a generalized cryopreservation process. Boxes with solid lines indicate steps in the cryopreservation pathway that take place on the same day that samples are frozen. Boxes with dashed lines indicate steps in the cryopreservation pathway that may take place in the days before or after freezing. Diamonds indicate essential nodes for quality control (QC). Arrows indicate the flow of materials through the steps. The dashed outline surrounding the diagram serves to indicate steps that can be carried out on different days than the actual freezing event. CPA = Cryoprotectant agents).



The steps of the pathway-level process illustrated in Figure 3 are described below in sequential order.

Step 1) Collection of donor animal or plant

This step includes selection of appropriate donors, and their acquisition and handling. Selection assumes an understanding of the purpose of the gene bank, which helps identify appropriate donors and their numbers. Seasonality of maturity and availability of donors can influence the timing of collection and, in some cases, may include hormonal or environmental induction of final maturation in captive animals. As indicated above, donors should be healthy, and handling should seek to minimize stress which will increase cryopreservation success, free of contamination. Collection of donors may take place together with the collection of tissues or gametes, or at a different time. Access and benefit sharing requirements to be respected in sample collection are discussed in Chapter 8.

Step 2) Collection of gametes and tissues

The collection of material to be preserved can follow protocols developed for species or species groups, often drawing on experience from captive rearing. Care needs to be taken to avoid contamination of the sample. For example, for the algae, in order to eliminate contaminants, samples have to be monoclonal and coming from an axenic culture (a culture containing only one species or farmed type of microorganism within a cultivation medium). The collection of finfish milt by stripping similarly requires drying of the abdomen, expression of feces and urine before collection of milt to avoid contamination, followed by careful expression of the clean milt into a clean vessel. Application of pressure under the pectoral fins alone may be sufficient to induce sperm flow in ripe males, helping to avoid contamination caused by a stripping method that compresses the abdominal cavity (Tiersch *et al.*, 1997). Collection of other tissues similarly requires care to avoid contamination, though protocols will vary depending on the tissues. These are

detailed for algae in Chapter 7, and corals in Annex 5. Collection of other somatic tissues is not covered in this guide, though the pathway for subsequent handling will generally apply.

The choice of a preservation strategy determines the receiving vessel for the material and the subsequent handling. For example, if the milt has to be transported to a freezing location, it can be collected in a "Whirl-pak" plastic bag or sealable vial that can be readily closed with an air (or oxygen) pocket. ensuring that the vessel is prechilled and includes an airspace (e.g., 1/3 sperm to ⅔ air). These samples can be securely transported in a cooler, and this packaging facilitates subsequent processing (see steps 6 and 7 below). If the dilution with an extender (solution of balanced salts and sugars that inhibits the activation of motility; Box 1) is planned, clean tubes with volume markings and closures (e.g., Falcon tubes) are more appropriate, but care should be taken to ensure that an airspace is maintained above the samples (i.e., do not fill tubes completely).

Finfish milt and invertebrate gametes can also be obtained by dissection. Care in this case must be taken to avoid contamination with bacteria or other material from surrounding tissues; this is especially important if dissection could open or rupture the gastrointestinal tract. Once removed, the testis or reproductive tracts can be rinsed with an extender, isotonic saline, or filtered seawater, depending on the species, to remove blood and other tissues. When removed, gonads lose their blood supply, and thus become depleted of oxygen and metabolic wastes accumulate. Therefore, it is important to keep whole gonads cool, and to dissociate them without undue delay, especially for large structures. The cleaned gonad is weighed and placed in a container with an appropriate amount of the chosen extender solution before it is pressed and homogenized. The resulting solution is filtered to remove pieces of tissue before use. Alternatively, a gonad can be lanced to allow milt to flow out, which is then aspirated with a pipette for direct use.

Gametes from several individuals may be pooled to increase volume or to increase procedural efficiency of the preservation process through batch processing. However, there is greater potential for contamination with this approach and the contributions of the sperm in a pool are not equal between the donors, so genetic considerations in a breeding program are uncertain. Decisions on pooling should be made with the overall goal of the project in mind. If possible, it is best to keep individual samples separate for freezing, with pooling done after thawing, if desired.

Step 3) Dilution with extenders

Extender solutions are designed to provide an osmotic and ionic environment that maintains the quality of milt (or other tissues) *in vitro* before it is frozen, reducing freeze damage, preventing premature activation of milt, offsetting any minor contamination, and diluting small volumes of sperm to facilitate its manipulation and improve longevity (Jenkins *et al.*, 2011). The appropriate osmotic environment can offset some contamination by urine. The inclusion of organic compounds such as sugars helps speed dehydration during freezing, reducing the risk of internal ice crystal formation. The inclusion of egg yolk or milk powder can help stabilize membranes to minimize freezing damage (although they can interfere with microscopic assessments of the milt, and be a risk of biological contamination). Overall, extenders can maintain the functional life and fertilizing capability of thawed sperm, building on practice of mammalian cryobiology. Generally, milt is mixed with the extender and held at an appropriate temperature (e.g., 4° C) determined by storage trials with the species in question. Some finfish protocols do not use extenders, incorporating these functions directly into the cryoprotection solution, for immediate freezing. The removal of this step can facilitate freezing in field conditions.

There is extensive literature on different extenders and on times and temperatures for their use. Systematic testing is still rare, although comparisons among extenders are abundant at species-specific levels. In general, when starting with a particular species it can be useful to evaluate a published protocol for that species or for other related species and adapt as needed. Hanks' balanced salt solution (HBSS) has been used with the sperm of more than 100 species, but simple solutions such as 1 percent unbuffered salt (sodium chloride) have been used for short-term (i.e., < 24 hours) chilled storage. Extenders can be prepared in large batches and stored frozen until use. Bacteria may grow in the extenders, therefore sterile protocols and refrigeration are important. Filtration is a quick and effective way to decontaminate fresh solutions. In general, the use of antibiotics should be avoided, as they may also have an impact on the viability of the target tissues. Good laboratory practices and aseptic techniques (e.g., careful preparation and handling of extenders, clean excision and dissection) should reduce risk of bacterial growth in the extender and milt and thus the subsequent need for antibiotics.

The concentration of sperm (or frozen cells) is also important for planning its use and the reproducibility of results and can be adjusted with extenders. For best efficiency, hatchery or research protocols of a given species should determine how many motile sperm cells are needed for effective fertilization of the practical number of eggs. With standardization and fertilization tests, the number of likely offspring can also be calculated. Together, this process allows for optimal management of the gene bank products. However, many protocols leave this step out to save time and money, which compromises the reproducibility of results. It is common to use more cryopreserved sperm per egg than would be used for fresh sperm, to compensate for damage in the freeze-thaw process.

Overall, the decision to dilute or not, and at what ratio, is driven in practice by the needs of the species and the chosen sampling and freezing methods. Trial experiments should be carried out for new species and working conditions to corroborate effectiveness of protocols.

3.1) Refrigerated storage and shipping

Refrigerated storage may occur after tissue collection, for example to ship to a facility that has the specialized equipment and knowledge for high-quality cryopreservation. This can be done with undiluted sperm as noted above, or after dilution with a suitable extender. For example, channel catfish sperm remained viable for up to 21 days in a refrigerator under an oxygen environment, within an extender containing antibiotic (Christensen and Tiersch, 1996). Viability for 20 hours of undiluted sperm at 2–5ºC has been reported for a variety of characid species (Marquez and Godinho, 2004), and sperm of these species has been stored in Ringer solution without antibiotic for 2–3 days at 4ºC in sealed Falcon tubes (Senhorini pers. communication, 2023). This affords adequate time to ship milt in a cooler with ice to a processing facility for freezing. For general purposes with finfish, induced motility capacity of 50–70 percent is sufficient to continue freezing, though results are inferior to freezing of fresh milt. The capability to hold samples for at least 24 hours before freezing enables shipping, avoids rushing during processing (including quality management and cataloging), and allows processing in batches rather than with single individuals. It is good practice to routinely develop and test refrigerated storage protocols for each species of interest, with and without extenders and based on published protocols for similar species and conditions. A rigorous comparison of sperm quality after freezing and thawing of milt subjected to the different freezing strategies needs to be carried out when establishing routine procedures.

Step 4) Addition of cryoprotectant solution and equilibration

During this phase, samples are mixed and equilibrated with cryoprotectant agents (CPAs), loaded into containers (e.g., straws or vials), and cooled to a final cryogenic temperature (-80 or -196°C). Cryoprotectants reduce the damage that a cell experiences during freezing (Box 1). Equilibration, generally carried out at about 4 ºC, allows time for the cryoprotectant to enter the cells in preparation for freezing. The choice of cryoprotectant, concentration, and equilibration time have been the focus of many studies on different species, determining the best balance between cryoprotection and toxicity of the cryoprotectant. For reasons still unknown, this balance can be different between tissues, species, and CPAs, so will require some experimentation with new species. The most used cryoprotectants are DMSO and methanol, although others, like the original glycerol, are also being used. Other than in vitrification, cocktails of different cryoprotectants have not generally been used. As indicated above, an external (non-penetrating) cryoprotectant such as egg yolk, milk powder, sucrose, or trehalose can also be added. The freezing solution usually contains compounds that provide buffering capacity (to minimize solute effects such as pH shifts induced by dehydration), and sometimes sugars (to modify glass-transition temperatures). These can be included in extenders. Novel components, such as fresh coconut milk, are sometimes useful in remote field situations, but have not been shown to be broadly applicable. Considerable time and resources have been expended in the past seven decades in empirical studies that identify “optimal" new cryoprotectants and freezing conditions for aquatic species. Although this is a standard empirical approach in science, it has not generally helped in establishing a comprehensive approach that transitions from research to application.

The literature contains substantial information on species-specific cryoprotectant approaches, with differences in monitoring protocols often making accurate comparisons difficult or impossible. Factors that may influence the adoption or adaptation of a protocol include the availability of the cryoprotectant or other components (e.g., DMSO may be hard to obtain, or be a controlled substance in some regions), local conditions, or differences between species. For example, a protocol for freezing of salmon milt in dry shippers was adapted for Brazilian fish species (Carolsfeld *et al.*, 2003). The DMSO-based protocol was effective for all characid species tested, although the volume of milt frozen was reduced to match hatchery practices. However, the protocol was not as effective with the more viscous milt of a silurid catfish, despite varying the details; methanol-based protocol adapted from a tilapia protocol was more effective (based on pre-and post-freezing motility tests). Both protocols were based on effective protocols of other quite different species, adjusted pragmatically to local conditions and species through logical adjustment of details and monitoring of potential for milt motility. These have been the basis of fish cryopreservation practices in Brazil (Garcia *et al.*, 2016), though there are numerous subsequent publications testing empirically various components and new protocols (Garcia *et al.*, 2016).

Step 5) Fill cryo-container

After the addition of the cryoprotectant and during the equilibration time, samples are filled into cryo-containers. Common cryo-containers include French straws and cryogenic vials, available in different sizes and manufactured to withstand liquid nitrogen temperatures. Whilst straws were originally designed for mammalian use, their use is transferrable and practical because they come with associated holders, organizers, and labels that make them easy to use. Most finfish species of interest to aquaculture broadcast semen over eggs and therefore produce large semen volumes. While livestock semen is frozen in small plastic straws (e.g.,0.25 and 0.5 ml) larger straws are often used for finfish. Protocol development that maximizes sperm concentration can allow practical usage of 0.5-ml French straws (Hu *et al.*, 2013) to take

advantage of their availability, ease of labeling, and automation and storage options. In the case of salmonids, the 11-ml SquarePack®, developed by Cryogenics, has been considered a key innovation to allow scaling-up and practical use of salmonid cryopreservation in Norway (Annex 13) (Boe *et al.*, 2021). Macro-tubes of 5 ml and 110-ml couchette bags are also available, designed for use with swine, but seem to have had little application also with AqGR (Lang *et al.*, 2003). The 0.5-ml French straw appears to be useful for most finfish, whereas 4-ml cryovials are used for invertebrates (Chapter 5).

Straws generally have a cotton plug at one end that allows aspiration of the sample but can also be sealed with polyvinyl chloride (PVC) powder at the other end once filled. Cryovials are cylindrical vials and have a gasket and screw-top lid to prevent liquid nitrogen from entering. A risk is that if liquid nitrogen does enter and evaporates upon thawing, the container may explode. Some practitioners leave the container or straw unsealed for this reason, although there may be contamination transferred through the liquid nitrogen.

Choosing the appropriate cryo-container depends on several factors, and higher levels of organization (community and network-level needs) must also be taken into consideration. Factors to be considered when choosing a container include: physical characteristics of the gametes, scale of the operation (number of samples), cost of the container, availability of the container, time required to fill each container, labeling method, sealing method, and storage space. Regardless of which container is chosen, all samples mixed with the cryoprotectant must be transferred into containers, labeled, and be ready for freezing before the equilibration time ends.

The choice of container influences freezing rates, so protocols need to be tested with the containers that are being used.

Step 6) Freezing the sample

Preparation of the various options for the freezing apparatus is part of the preliminary work that needs to be done before the cryopreservation flow starts. Often, in smaller operations, freezing is done in vapor-phase nitrogen or solid CO_2, with the rate of cooling regulated by the distance above liquid nitrogen or dry ice in a custom-built (or improvised) rack or float. Such devices can become more available and standardized with open technology and 3-D printers (Liu *et al.*, 2021).

Open-source cryopreservation equipment

Yue Liu, Aquaculture Germplasm and Genetic Resources Center (AGGRC), Louisiana State University Agricultural Center, Baton Rouge, Louisiana, United States of America
Terrence Tiersch, Aquaculture Germplasm and Genetic Resources Center (AGGRC), Louisiana State University Agricultural Center, Louisiana, United States of America

Introduction

Open technologies can be used to address problems of access to standardized equipment for optimized cryopreservation and other aspects of AqGR genetic conservation. This is primarily through the fostering of user, maker, and developer communities for open sharing of designs and protocols. Collectively, these communities can develop open hardware in a distributed (i.e., non-centralized) fashion, using consumer-level tools, supplies, softwares, and equipment, free exchange of designs and modifications of the equipment and protocols, and a shared sense of mission mediated through internet platforms. These can yield aggregate throughput comparable to facilities that specialize in expensive high-throughput capabilities. For example, cryopreservation researchers or hatcheries can then become makers to share, produce, and distribute designs, and eventually become developers to modify and improve hardware. Overall, open technologies are based on rapid and continued sharing of advances among communities based on granting of freedoms with a range of protections and licensing agreements that can include existing provisions such as trademarks and patents. These freedoms can include permission to fabricate, modify, or distribute designs created and shared by others, with specified restrictions on use (e.g., with or without commercialization) or provisions for ascribing credit. This thinking traces its roots to open-source software initiatives and has coalesced into organizations such as Creative Commons (www.creativecommons.org) which provides a system of licensing and provides structures for placing innovations into the public domain.

Examples include open scientific hardware fabricated by 3-D printing (Huene *et al.*, 2022, Tiersch *et al.*, 2023), devices operated with microcontrollers and mechanical components (Zuchowicz *et al.*, 2023), sensing and monitoring platforms (Shamkhalichenar *et al.*, 2019, 2021), and microfluidic systems (Scherr *et al.*, 2012, Belgodere *et al.*, 2022). Collaboration among multiple disciplines is the core of such programs, integrating experience in cryobiology and aquatic species reproduction with expertise in fields such as biological engineering, electrical engineering, industrial engineering, mechanical engineering, environmental engineering, computer science, veterinary medicine, outreach, sociology, and agricultural economics (Figure 4).

As more groups enter this area in the future, interdisciplinary facilities can be developed to specifically provide novel multi-level approaches to local and global problems. For example, the Aquatic Germplasm and Genetic Resources Center (AGGRC),[1] described in Annex 7, was created to enable confluence of expertise in reproductive biology, cryobiology, multiple engineering disciplines, arts, economics, and social sciences specifically to support the development of germplasm repositories to protect genetic resources of aquatic species for, biomedical, conservation, aquaculture and fisheries purposes. It is important to recognize that small initial steps can be extremely beneficial. For example, biological research groups could simply employ undergraduate engineering students to bring technologies such as computer-aided drawing (CAD) and 3-D printing to their work. This would also create an entire generation of biologically exposed engineers.

Figure 4. *Examples of different open hardware devices that can be produced by using consumer-level technologies such as CAD, 3-D printing, laser cutting, microprocessors, simple electronics, and printed circuitry. Devices such as these can be used for sample processing, cryopreservation, and quality management for aquatic species, and can be made available as printer or design computer files that can be shared and modified* (www.aggrc.com).

References

Belgodere, J., Liu, Y., Reich, E., Eades, J., Tiersch, T.R. and Monroe, W.T. 2022. Development of a single-piece sperm counting chamber (SSCC) for aquatic species. *Fishes*, 7:231. https://doi.org/10.3390/fishes7050231. PMC9838223.

Huene, A., Koch, J., Arregui, L., Liu, Y., Nicotra, M., Weis, V. and Tiersch, T.R. 2022. Cryopreservation of *Hydractinia symbiolongicarpus* sperm to support community-based repository development for preservation of genetic resources. *Animals*, 12, 2537. https://doi.org/10.3390/ani12192537 PMC9559378.

Liu, Y., Monroe, W.T., Belgodere, J., Choi, J.W., Gutierrez, M.T. and Tiersch, T.R. 2022. The emerging role of open technologies for community-based improvement of cryopreservation and quality management for repository development in aquatic species. *Animal Reproduction Science*, 246:106871. https://doi.org/10.1016/j.anireprosci.2021.106871 PMC9012811.

Scherr, T., Quitadamo, C., Tesvich, P., Park, D., Tiersch, T.R., Hayes, D., Choi, J.W., Nandakumar, K., and Monroe, W.T. 2012. A planar microfluidic mixer based on logarithmic spirals. *Journal of Micromechanics and Microengineering*, 22:055019(1-10). PMC3745025.

Shamkhalichenar, H., Choi, J. and Tiersch, T.R. 2019. Three-dimensional printing can provide customizable probes for sensing and monitoring in cryobiology applications. *Cryobiology*, 88:64-69. https://doi.org/10.1016/j.cryobiol.2019.03.010 PMC6536324.

Shamkhalichenar, H., Tiersch, T.R. and Choi, J.W. 2021. An impedimetric sensing probe based on printed circuit board technology for monitoring in cryobiology applications. *Journal of The Electrochemical Society*, 168, 067505. https://doi.org/10.1149/1945-7111/ac045a PMC9910178.

Liquid nitrogen “dry shippers” can provide acceptable cooling rates for freezing in the field (e.g., Wayman *et al.*, 1997). The freezing rate may be influenced by the position in the dewar (Childress *et al.*, 2021), the volume of the containers being frozen, the number of previous samples stored in the shipper and the charge state of the shipper. Nevertheless, this method is widely used for finfish milt in Brazil (Carolsfeld *et al.*, 2003) and has been used for field gene banking of Pacific salmon populations in Canada (Annex 14). An open hardware 3-D printed device has been developed to position samples within a dry shipper for better

control of freezing rates, and to eject them after completion of freezing (Childress *et al.*, 2021). Devices such as this can provide improved standardization for freezing in field conditions.

Simple commercially available containers (e.g., "Mr Frosty", Thermo Scientific) intended for laboratory freezing, can provide a standardizable freezing option when using cryovials to hold the samples but typically only provide slow freezing rates that may not be effective for many tissues (see Finfish and Shellfish chapters). Samples are placed in the container with an isopropanol jacket, which is placed in a -80 ºC freezer, providing a cooling rate of about 1 ºC per minute. After about 4 hours, samples are transferred to liquid nitrogen or a -80 ºC freezer for storage.

Zebrafish sperm has also been successfully cryopreserved by direct deposition in a -150ºC freezer, in cryovials or straws, and stored for more than 3 months without compromising the viability (Diogo *et al.*, 2018). This has the advantage of storage in standard vial boxes but does not provide the same longevity as storage in liquid nitrogen. Nevertheless, this may be useful for some other species with appropriate testing.

Material can be frozen as drops on dry ice (Stoss and Refstie, 1983), though this does not integrate well with modern manipulation and storage systems. However, oyster sperm can be routinely frozen in straws dipped into a slurry of methanol and dry ice (Chapter 6).

Programmable freezers provide controlled freezing rates in cryovials or straws of different conformations. These allow for better standardization, including the use of non-linear freezing profiles, which may be beneficial. Generally, they also function with nitrogen vapor, using computer-controlled exposure monitored by thermistors. Other models work with freezer plates, metal plates that are chilled by nitrogen vapour. A variety of sizes are available, including some that can be taken to the field or be used in adequately equipped trucks. These are more expensive than other freezing alternatives but provide a controlled freezing environment and reproducible results. They are particularly good for developing and optimizing freezing protocols in research environments, after which an easier field protocol can be designed. Nevertheless, larger gene banks, service providers, or hatcheries may use them for reproducibility, often after chilled transport of fresh milt to the freezing facility.

6.1) Cooling rate

The cooling rate from ambient to -80ºC is a key factor in determining freezing success, as it affects the rate of crystal formation and solute movement that cause freeze damage (Box 1). Cooling rate has been a major focus of numerous studies of sperm cryopreservation. It varies with container size, materials and geometry, how closely the containers are packed, and especially with the equipment used. Fortunately, fish sperm cells are generally forgiving in terms of cooling rate. Variance from the "ideal" cooling rate is often less important when using fresh sperm that has been handled properly and frozen within minutes of collection – i.e., the trade-offs associated with a simpler, field friendly protocol.

In general, cooling rate should be recorded within a representative sample, as well as within the chamber itself (the freezer environment). Factors affecting cooling rates include the ambient room temperature, the number of containers being frozen at one time (thermal mass), the design and material of the containers, whether freezing cycles are being repeated (successive runs can be different), whether frost has accumulated within the freezer (a problem in humid environments) and levels of liquid nitrogen in the pressure tank that drives the controlled-rate freezer. How can cooling rate be tested in a practical manner? The best accuracy is obtained with a thermocouple capable of low temperature measurement, or by monitoring with a

programmable freezer. Rough range estimates, such as comparison of 4 ºC/min ("slow") and 40 ºC/min ("fast") may be adequate to develop protocols. Based on these results, decisions can be made to focus on the specific range of cooling rates to be tested or to change other parameters.

Step 7) Sorting and storage

Storage of cryopreserved material is generally in liquid or vapor-phase nitrogen at -196ºC. While shorter term storage in -80 ºC freezers can be done for weeks or months, this is not adequate for longer-term storage, as physiological deterioration processes are not adequately halted. Storage in a household freezer is not adequate for the same reason.

Liquid nitrogen storage units come in a variety of sizes, from 35 litres research dewars to vats of thousands of litres for large gene banks. A key element is that the nitrogen level needs to be monitored and periodically replenished. This is easily overlooked in smaller applications (such as a research dewar) or if funds or personnel are limited. These risks are generally lower in larger repositories, where nitrogen vats are larger, professionally monitored, and equipped with alarms for temperature increases. It is advisable for smaller gene banks to combine efforts or make use of larger facilities that are established for livestock or specifically for AqGR. Adequate cold- and abrasion tolerant labeling of containers and holding canes is essential, as is a map of sample locations within the storage container, to avoid losing samples (see item 8 below).

Of particular importance is that there are adequate ventilation and safety protocols to support work with liquid nitrogen to avoid suffocation and burns from splashing or spillage.

7.1) Sorting, labeling, packaging, inventory and database

Improperly labeled samples can cause delays in processing, genetic contamination of pure stocks and loss of important genetic material. At the minimum, containers should be permanently labeled as to species, population or farmed type, sex, date and originating facility. With the use of French straws and SquarePack packaging, automated processing and sophisticated labeling, such as individualized printing and bar coding, are possible.

Efficient management and tracking of samples once they are received, processed, stored, and shipped are important. Maintaining an accurate inventory becomes increasingly more demanding as a gene bank grows. It is advisable to plan for this, possibly including development of, or linkage to, uniform coding systems for sample identification. For example, a powerful interactive database is the Animal Germplasm Resources Information Network (A-GRIN),[9] established by the National Animal Germplasm Program Department of Agriculture (USDA) of the of the United States of America, (Annex 8) that has archival collections, although no international standard exists.

While small gene banks may work primarily with a spreadsheet, well-designed computerized databases are better and more forward-looking. Modifiable user-friendly databases can be developed with commercially available database software (such as Microsoft Access).

Information associated with samples can be classified in four groups:

[9] https://data.nal.usda.gov/dataset/animal-germplasm-resources-information-network-grin

5. Data related to the sample donor at the levels of individual (e.g., sex, age, phenotypic data) and population (e.g., GPS coordinates of collection site, environmental data, genetic characteristics);
6. Data related to the sample itself (e.g., method of collection, method of processing, sample quality);
7. Inventory (e.g., number of samples and current location in the repository); and
8. Linkages with genomic databases, production information, and future data on performance of the preserved material (e.g., fertilization percent in the case of male gametes) and the offspring produced with the samples (e.g., genetic merit).

As indicated above, gene banking managers should establish minimum data submission requirements in relation to specific data fields and structure of the database. The information needed will depend on the specific objectives, applications, and species in the repository, and should consider the potential for future uses of samples and information. Staffing plans often underestimate the required workload for information management, especially as a collection grows.

7.2 Shipping of frozen samples

Shipping is an essential part of the gene banking process, either to transport remotely collected fresh samples to a freezing facility or to ship frozen samples. Protocols for transport are well established, although sensitive to misuse or accidents, and rely on appropriate technology, education, experience and vigilance. For example, boxes are sometimes thrown or dropped, left sitting outside in the sun or on a loading dock, or opened for inspection and improperly repackaged. Liquid nitrogen dry shippers are designed for frozen transport at the temperature of nitrogen vapor, but in inexperienced hands, may be dropped and broken, held too long, be stored horizontally or upside down, or be denied transport by some airlines. Transport of large liquid nitrogen dewars designed for laboratory use is not advisable if they contain nitrogen, due to the potential for damage to the brittle internal structure and loss of vacuum insulation. Services accustomed to transporting terrestrial genetic materials can be used for shipping frozen AqGR but it is dangerous to assume appropriate knowledge and experience.

Likewise, successful storage of fresh samples in the refrigerator for 24 hours does not necessarily mean shipping will not be a problem. Practical trials should involve using the actual packaging of choice and doing test shipping of the package overnight. The cost of shipping may also be important. Increased concentration of milt in the samples can be used to reduce costs (Hu *et al.*, 2011).

Step 8) Thawing

Thawing and subsequent use of cryopreserved genetic material should minimize delays for two reasons: 1) rapid thawing minimizes the potential for small intracellular ice crystals to recrystallize and to damage the cell; 2) premature sperm activation can occur upon thawing (i.e., without addition of water), thereby reducing usable swimming time. Samples should thus be removed from the storage dewar and transferred immediately to a water bath. Specific warming times and temperatures should account for sample volume, container type (volume, materials, and geometry all affect the rate of heating), and species (e.g., cold water or warm water fishes). A range of thawing temperatures (e.g., from 0 to 60 °C) with a thawing time of several seconds can be tested, but it is best to ensure that the final sample temperature is not damaging to the sperm. Importantly, resilience to thawing rates can vary between types of tissues and even among closely related species. This is a factor that needs to be tested for new species for optimization, taking into account experience with related species and what is known about cryobiology. For example, Cryogenetics

has found that thawing for 30 seconds at 25ºC is a critical detail for good results with 5ml Cryopacks, measured as fertilization rates of subsequent use (M. Ritter, pers. Com. 2024).

9) Fertilization and quality control

Rapid use of thawed materials is generally important to ensure best results. This consists of moving materials into appropriate growth media in the case of algae and somatic tissue, and proceeding to fertilization of ova in the case of thawed milt. Artificial "dry" fertilization of eggs in finfish is often carried out in a specific solution that activates the gametes and dilutes the cryoprotectant. This requires advance planning to have appropriate eggs and equipment prepared before thawing. Often the activating solution can double as the thawing solution. It should be noted that when using relatively non-toxic cryoprotectants such as methanol, post-thaw sperm can sometimes be refrigerated for days with subsequent satisfactory motility (e.g., Tiersch *et al.*, 1994).

Sometimes it is beneficial to treat sperm after thawing to improve performance. Usually, it is sufficient to thaw the samples, place them on eggs, and add water to activate the gametes. However, activation solutions, containing energy supplements or ATPase stimulators can help extend the functional life of the thawed milt, with some available commercially for salmon. In practical work, amendments are sometimes needed. For example, a protocol for live-bearing fishes of the genus *Xiphophorus* includes rinsing and concentration of the sperm cells (by centrifugation and resuspension), before they are transferred into the female for artificial insemination (e.g., Dong *et al.*, 2006).

After thawing, a post-thaw motility evaluation can be conducted (see section below). Post-thaw motility can be compared to motility recorded just after collection and just before cryopreservation. This QC step indicates if the cryopreservation protocol effectively preserved the sample and if sperm quality meets the standards for fertilization.

Backups and contingency plans

Planning for germplasm repositories should cover operation for periods of at least several decades. Samples and associated data face *internal* and *external* risks.

Internal risks can be classified according to the source: human mistakes (e.g., mislabeled data, internet outages, lack of liquid nitrogen supply), or equipment malfunction or infrastructure damage (e.g., fire, flooding, reductions in base funding). Contingency plans should have a comprehensive risk management approach that includes assessment and mitigation. These must be considered "living" documents which undergo annual review and updates. Common risks can be minimized by basic precautions. For example, staff need to be properly trained, and storage vessels need to be equipped with an automatic monitoring system with alarms linked to computer or phone notifications.

External risks include natural disasters, flooding, and power failures. Risk management of this type often involves trade-offs. For example, germplasm collections can be split into multiple storage locations for safety, but this is expensive and complicates record-keeping and inventory demands. The rarest risks are sometimes the most damaging, and assessments must balance the frequency of events against their severity. Preparation can involve costs. For example, at least one empty storage unit could be held available (with liquid nitrogen) for emergency sample relocation in case of equipment failure. Duplicate collections at off-site storage locations in different geographical regions could be used to diminish losses caused by devastating disasters (e.g., building collapse in an earthquake-prone area).

Data security is integral to the reliable and efficient management of a gene bank. Information loss can render biological material useless. Therefore, on-site and off-site data backup should be implemented, and policies instituted to maintain data integrity and security.

Evaluating success and quality control

Monitoring the vitality of the genetic material at stages of the preservation process that could compromise quality is important for deciding if preservation of the material should be carried through, or if it should be discarded. Low quality samples can waste banking resources and lead to false expectations of security. Quality monitoring allows for screening of samples during the preservation process, decisions on acceptable quality for the particular tissue, indicators for protocol development, and measures of success of the process. A minimum set of monitoring points, used when a protocol is well established, is shown in Figure 2, whereas a more complete set of monitoring points are listed in Table 1, applicable for protocol development, troubleshooting, or more secure knowledge of sample quality. It is important to recognize that if the genetic information carried in the frozen material is particularly valuable, low-quality samples can still be considered useful for propagation to maintain a genetic line, farmed type or genetic stock. For example, molluscs produce large numbers of gametes and larvae, so despite the typically low survival rate of cryopreserved milt, it can still produce substantial numbers of progeny.

In the case of finfish or shellfish sperm, measures include spontaneous and induced sperm motility, sperm concentration, fertilization rates, and survival of fertilized eggs to later stages of development (see below). Quality assessment methods for algae are covered in the related chapter (Chapter 7), but those of other somatic samples are not dealt with in this document. Nevertheless, the points where quality assessment is important are basically the same for all tissues.

Quality assessment in sperm cryopreservation programs

There are quality assessment assays that are generally used in the cryopreservation of finfish sperm and can also be used for shellfish milt. These are not fully standardized, which can create problems in comparing results from different studies, but they are demonstrably adequate for developing and monitoring protocols (Table 1). Nevertheless, it is important to record the methods used or to follow methods used by past practitioners in the area of interest to ensure usefulness of results.

Table 1. List of critical points, in a cryopreservation program of milt, where a quality assessment should be conducted, and related types of assessments to apply and indications.

Monitoring points	Type(s) of assessment	Note
*Immediately after collection of sample	Spontaneous and activated motility; sperm concentration	May be skipped in field collection, if protocol is well established; better to split milt of individual into several aliquots to offset risk of contamination. Concentration assessment is for estimating eventual volume of use but may happen at later steps of the pathway

After addition of extender	Spontaneous and activated motility	Part of development process; essential for quality control of established pathway
24 hours after extender addition	Spontaneous and activated motility	Part of development process; essential for quality control of established pathway
After shipping of fresh milt	Spontaneous and activated motility; smell and appearance	Should be done regularly; should also observe any unusual odors, color, or leakage
After addition of cryoprotectant solution and equilibration	Spontaneous and activated motility	Part of development process; essential for quality control of established pathway
After sample freezing	Spontaneous and activated motility	Shortly after freezing; periodic subsampling of batches during storage (annually or more)
At time of sample use	Thawed spontaneous and activated motility	Essential QC check to ensure thawed sperm is viable
After fertilization	Proportion of normal embryos post – morula stage	Measures effectiveness of process
After hatching	Proportion of normal larvae; observation of deformaties	Further measure of effectiveness and indicator of other potential problems

Types of assessment

A detailed description of the different types of quality assessment that can be implemented at the different steps of the cryopreservation pathway is provided below.

a) Sperm motility

Sperm is generally inactive while in the testis and becomes motile once activated by exposure to water (at lower salinity than blood in freshwater species, higher salinity for marine species) (Tiersch and Yang, 2012). Due to the limited energy stored in the sperm, this motility may only last for 30 seconds or less, depending on the species and activating environment. Motility is generally the main indicator used for sperm quality assessment, although there is some evidence of immotile sperm fertilizing eggs, and motile sperm being incapable of doing so. Motility is assessed with a microscope at a magnification of at least 100x, ideally with darkfield or similar lighting to enhance contrast. This magnification is generally not adequate to distinguish the sperm cell structure but allows for a rough estimation of the proportion of the moving sperm, and the type of movement. A small drop of undiluted, fresh milt is spread onto a microscope (or observation chamber) slide and examined immediately for existing motility, indicating contamination by water or urine. Subsequently, a small drop of water or activation fluid is added and mixed into the milt, and the presence of forward motility noted. Generally, an estimate of the percentage of sperm that are motile and the time of vigorous motion are recorded. A coverslip should not be used, as this slows the examination process and can influence the time of motility by suffocating the milt.

There is substantial literature on quantifying the level of induced motility, but a rough semi-quantitative scale is often adequate (none, <50 percent motile, > 50 percent motile, 100 percent vigorously motile), though estimates to the nearest 10 percent are often used. The time after activation and level of dilution will influence such measures. It is important for observers to practice, so they can distinguish milt and milt activity from other material (like bacteria) and random Brownian motion (Jenkins and Tiersch, 1997). The sperm cells are often at the limit of individual recognition in light microscopes at this magnification, so motility may be seen as wriggling masses and requires a trained eye. Quantification of motility thus easily

leans towards a subjective opinion. Documentation of methodologies and the reporting observer is thus very important (Blackburn *et al.*, 2022).

Unexpected anomalies are also important to monitor. For example, the gelling of post-thaw samples has been observed for Pacific oysters, *Magallana gigas*, (Dong *et al.*, 2007a) when calcium is included in the media. Sub-optimal conditions (e.g. cooling rate, cryoprotectant concentration, or cell concentration) can cause damage or activation of the acrosome in this species. This causes the cells to form aggregates, reducing fertilization capacity despite otherwise acceptable cell morphology. Computer-assisted sperm analysis (CASA) offers many benefits for standardized the assessment of sperm motility, but commercially available instruments are expensive (e.g., >US$ 35,000) (Yang and Tiersch, 2011). Motion analysis based on capture of digital or video images is also possible but often requires some ability to write computer code or assemble a system in a facility. Open-source motility analysis programs have been developed in recent years (Wilson-Leedy *et al.*, 2007)

Ambient environmental conditions can influence motility characteristics of sperm that may need to be considered. Fish collected at different sites or held under different conditions (such as different salinities) can vary in activation and duration of motility. There is no substitute for performing experiments that evaluate motility activation in response to osmotic or ionic gradients. Such activation studies provide essential information for delineating conditions for storage (e.g., isotonic conditions) and those necessary for gamete activation (e.g., hypotonic solutions for freshwater fishes).

b) Sperm morphology

In general, finfish sperm are relatively small cells (e.g., compared to mammals), although exceptions exist for species with large genome sizes such as salmonids. In general, sperm morphology is not readily observed with a simple light microscope but can be studied with electron microscopy with appropriate control of conditions (Dong *et al.*, 2006). This is not used as a routine measure of sperm quality but is used in research, for example to understand the effects of cryoprotectants.

c) Sperm concentration

Sperm concentration is valuable in the planning for eventual use, and controlling it is important in optimizing the application of the cryopreservation process. Assessment of concentration can be done by use of a counting chamber (e.g., hemocytometer), spectrophotometry (e.g., Tan *et al.*, 2010, Dong *et al.*, 2005a), or flow cytometry. Quantified sperm concentration is not often reported, except in more sophisticated processing laboratories, but semi-quantitative observations of “watery” or “thick” milt can be important indicators of the maturation of the donor and sperm quality or, with experience, as a rough guide for calculation of the volume needed for dilution.

d) Fertilization assays

An important assay of effectiveness is how many eggs are fertilized by the thawed milt. This can be assessed at several points during incubation but should be done at least after blastula formation, as unfertilized eggs of some species can develop at least to this stage. On the other hand, as incubation progresses, other factors may influence survival of the embryos. Proper controls with fresh milt and the same females, and reliable baseline records are essential in interpreting and reporting of fertilization success and troubleshooting any problems.

Generalization, standardization and harmonization

Developing *de novo* cryopreservation protocols for a species requires substantial funding, labor, and time. Therefore, a good strategy is to make use of the experience with other species to facilitate the development of protocols for new species. Similarly, other elements of repository pathways described above can be applied and refined based on the experience of practitioners.

Literature on the practical components of setting up an AqGR gene bank is, however, limited. A lack of standardization can present real costs at the level of repositories. An example is the case of the zebrafish, an important model for biomedical research: protection of its genetic resources is jeopardized by a systemic lack of standardization, reliability, and efficiency. This has led to over-collection of individuals and the processing and storage of multiple duplicative samples. After thawing, non-reproducible fertility leads to increased use of females and eggs to ensure production of offspring.

Researchers and established germplasm repositories do not generally use the same equipment, live in the same environment, or use the same protocols. This makes standardization more difficult. Harmonization can be used as a process for alignment of results (rather than methods) through controlled comparative studies and fostering collaboration among groups. This is particularly important for groups working in different locations.

References

ALELO PORTAL.2022 *Alelo Genetic Resources Portal*: *Curadoria Animal.* [Cited 13 December 2022].http://alelo.cenargen.embrapa.br/numeros/.

Brazilian Germplasm Bank of Sheep and Goats Semen and Embryos. 2022. *Brazilian Germplasm Bank of Sheep and Goats Semen and Embryos*. [Cited 2022]. www.researchgate.net/publication/357880205_Brazilian_Germplasm_Bank_of_Sheep_and_Goats_Semen_and_Embryos.**Bajerski, F., Bürger, A., Glasmacher, B., Keller, E.R.J., Müller, K., Mühldorfer, K., Nagel, M., Rüdel, H., Müller, T., Schenkel, J. & Overmann, J**. 2020. Factors determining microbial colonization of liquid nitrogen storage tanks used for archiving biological samples. *Applied Microbiology and Biotechnology*, 104(1): 131–144.

Bajerski, F., Nagel, M. & Overmann, J. 2021. Microbial occurrence in liquid nitrogen storage tanks: A challenge for cryobanking? *Applied Microbiology and Biotechnology*, 105(20): 7635–7650.

Bartley, D.M., Kent, D.B. & Drawbridge, M.A. 1995. Conservation of genetic diversity in a white sea bass hatchery enhancement program in Southern California. *American Fisheries Society Symposium*, 15: 249–258.

Blackburn, H., Torres, L., Liu, Y. & Tiersch, T.R. 2022. The need for a framework addressing the temporal aspects of fish sperm motility leading to community-level standardization. *Zebrafish*, 19(4): 119–130. doi: 10.1089/zeb.2022.0006. PMID: 35969383; PMCID: PMC9419943.

Bodenstein, S., Nahmens, I. & Tiersch, T.R. 2022. Simulation modeling of a high-throughput oyster cryopreservation pathway. *Journal of Shellfish Research*, 41(2): 209–221. https://doi.org/10.2983/035.041.0206

Bøe, K., Bjøru, B., Bårdsen, M.T., Wist, A.N., Wolla, S. & Sivertsen, A. 2021. Opportunities and challenges related to sperm cryopreservation in Atlantic salmon gene banks. *Conservation Science and Practice*, 552: 1–14. https://doi.org/10.1111/csp2.552

Cabrita, E., Robles, V. & Herráez, P. 2009. *Methods in Reproductive Aquaculture: Marine and Freshwater Species*. CRC Press, Boca Raton, Florida, USA, 549 pp.

Caffey, R.H. & Tiersch, T.R. 2000a. Cost analysis for integration of sperm cryopreservation into an existing fish hatchery. *Journal of the World Aquaculture Society*, 31: 51–58.

Caffey, R.H. & Tiersch, T.R. 2000b. Economics and marketing of cryopreserved fish sperm. In: *Cryopreservation in Aquatic Species* (pp. 388–408).

Carolsfeld, J., Godinho, H.P., Zaniboni Filho, E. & Harvey, B.J. 2003. Cryopreservation of sperm in Brazilian migratory fish conservation. *Journal of Fish Biology*, 63(2): 472–489.

Certified Semen Services. 2001. CSS minimum requirements for disease control of semen produced for AI. Available at: www.naabcss.org

Childress, W.M., Liu, Y. & Tiersch, T.R. 2021. Design, alpha testing, and beta testing of a 3-D printed open-hardware portable cryopreservation device for aquatic species. *Journal of Applied Aquaculture*, DOI: 10.1080/10454438.2021.1955805.

Christensen, J.M. & Tiersch, T.R. 1996. Refrigerated storage of channel catfish sperm. *Journal of the World Aquaculture Society*, 27(3): 340–346.

Correa, P. & Schmidt, C. 2014. Public research organizations and agricultural development in Brazil: How did Embrapa get it right? Technical Report.

Coxe, N., Liu, Y., Arregui, L., Upton, R., Bodenstein, S., Voss, S.R., Gutierrez-Wing, M.T. & Tiersch, T.R. 2024. Establishment of a practical sperm cryopreservation pathway for the axolotl (*Ambystoma mexicanum*): A community-level approach to germplasm repository development. *Animals*, 14: 206. https://doi.org/10.3390/ani14020206

Cryogenetics. Nd. *Reproduction Handbook for use at Hatcheries: Salmonids*. 19 pp. https://s33002.pcdn.co/wp-content/uploads/Reproduction-Handbook-Samonids.pdf

Dong, Q., Eudeline, B., Huang, C. & Tiersch, T.R. 2005a. Standardization of photometric measurement of sperm cell concentration from diploid and tetraploid Pacific oysters, *Crassostrea gigas* (Thunberg). *Aquaculture Research*, 36: 86–93.

Dong, Q., Eudeline, B., Huang, C., Allen, S.K. & Tiersch, T.R. 2005b. Commercial-scale sperm cryopreservation of diploid and tetraploid Pacific oysters, *Crassostrea gigas*. *Cryobiology*, 50: 1–16.

Dong, Q., Huang, C. & Tiersch, T.R. 2006. Post-thaw amendment of cryopreserved sperm for use in artificial insemination of a viviparous fish, the green swordtail *Xiphophorus helleri*. *Aquaculture*, 259: 403–414.

Dong, Q., Huang, C. & Tiersch, T.R. 2007a. Control of sperm concentration is necessary for standardization of sperm cryopreservation in aquatic species: Evidence from sperm agglutination in oysters. *Cryobiology*, 54: 87–98.

Dong, Q., Huang, C., Eudeline, B. & Tiersch, T.R. 2007b. Cryoprotectant optimization for sperm of diploid Pacific oysters by use of commercial dairy sperm freezing facilities. *Aquaculture*, 271: 537–545.

FAO. 2019. *The State of the World's Aquatic Genetic Resources for Food and Agriculture*. FAO Commission on Genetic Resources for Food and Agriculture assessments. Rome.

Fonseca, J. & Silva, K. 2021. Brazilian Germplasm Bank of Sheep and Goats Semen and Embryos (No. 142). Ministry of Agriculture, Livestock and Food Supply.

Garcia, R.R.F., Streit, D.P., Cabrita, E. & Godoy, L.C. 2016. Semen cryopreservation in Brazilian freshwater fish: Advances and main challenges. In: *Cryopreservation in Eukaryotes* (Francisco Marco-Jiménez & Hülya Akdemir, eds.). IntechOpen. https://doi.org/10.5772/65037

Hagedorn, M., Varga, Z., Walter, R.B. & Tiersch, T.R. 2018. Workshop report: Cryopreservation of aquatic biomedical models. *Cryobiology*, 86: 120–129. https://doi.org/10.1016/j.cryobiol.2018.10.264

Hu, E., Liao, T.W. & Tiersch, T.R. 2015. Simulation modelling of high-throughput cryopreservation of aquatic germplasm: A case study of blue catfish sperm processing. *Aquaculture Research*, 46(2): 432–445. https://doi.org/10.1111/are.12192

Hu, E., Yang, H., and Tiersch, T.R. 2011. High-throughput cryopreservation of spermatozoa of blue catfish (*Ictalurus furcatus*): establishment of an approach for commercial-scale processing. *Cryobiology* 62(1):74–82. doi:10.1016/j.cryobiol.2010.12.006.

Jenkins, J.A., and Tiersch, T.R. 1997. A preliminary bacteriological study of refrigerated channel catfish sperm. *Journal of the World Aquaculture Society* 28:282–288.

Jenkins, J.A., B.E. Eilts, A. Guitreau, C.R. Figiel, R.O. Draugelis-Dale, and T.R. Tiersch. 2011. Sperm quality assessments for endangered razorback suckers (*Xyrauchen texanus*). *Reproduction* 141:55–65.

Lahnsteiner, F. (ed). 2000. Cryopreservation of Gametes in Aquatic Species. *Aquaculture Research* (Special Issue) 31:229–336.

Lang, R.P., K.L. Riley, J.E. Chandler, and T.R. Tiersch. 2003. The use of dairy protocols for sperm cryopreservation of blue catfish (*Ictalurus furcatus*). *Journal of the World Aquaculture Society* 34:66–75.

Liu, Y., Monroe, T.W., Belgodere, J., Choi, J-W, Gutierrez-Wing, M.T., and Tiersch, T.R. 2021. The emerging role of open technologies for community-based improvement of throughput and quality management of sperm cryopreservation for repository development in aquatic species. *Animal Reproduction Science*. doi: 10.1016/j.anireprosci.2021.106871. Epub 2021 Oct 16.

National Institutes of Health. 2007. Achieving High-Throughput Repositories for Biomedical Germplasm Preservation. Final Workshop Report available at: http://esi-bethesda.com/ncrrworkshops/Biomedical/pdf/Cryopreservation%20Workshop%20-10-April%20-2007%20-%20FINAL%20REPORT%20(2).pdf.

Sooksawat, T., Boonthai, T., Nimrat, S., and Vuthiphandchai, V. 2020. Cryostorage of silver barb (*Barbodes gonionotus*) semen in dry shipper: efficacy, risk of bacterial cross-contamination, and effects of *Aeromonas hydrophila* on post-thaw sperm quality. *Cryobiology* 96:184–196.

Stoss, J., and Refstie, T. 1983. Short-term storage and cryopreservation of milt from Atlantic salmon and sea trout. *Aquaculture* 30:229–236. doi.org/10.1016/0044-8486(83)90165-5.

Tan, E., H. Yang, and T.R. Tiersch. 2010. Determination of sperm concentration for small-bodied biomedical model fishes by use of microspectrophotometry. *Zebrafish* 7:233–240.

Tave, D. 2008. Broodstock Management, Inbreeding, Genetic Drift, and Domestication. FAO Technical Reports for Responsible Fisheries 5 (3).

Tiersch, T.R., W.R. Wayman, C.R. Figiel, O.T. Gorman, J.H. Williamson, and G.J. Carmichael. 1997. Field collection, handling, and storage of sperm of the endangered razorback sucker. *North American Journal of Fisheries Management* 17:167–173.

Tiersch, T., and P. Mazik (eds). 2000. *Cryopreservation in Aquatic Species*. World Aquaculture Society, Baton Rouge, Louisiana, USA, 439 pp.

Tiersch, T.R. 2001. Cryopreservation in aquarium fishes. *Marine Biotechnology*, Special Issue: *Aquaria Fish Models of Human Disease* 3:S212-S223.

Tiersch, T.R., and J.A. Jenkins. 2003. Biosecurity considerations for cryopreserved gametes and early life stages of aquatic species. In: C.-S. Lee and P.J. O'Bryen, editors. *Biosecurity in Aquaculture Production Systems: Exclusion of Pathogens and Other Undesirables*. World Aquaculture Society, Baton Rouge, Louisiana, USA. Pp. 171–198.

Tiersch, T.R., H. Yang, J.A. Jenkins, and Q. Dong. 2007. Sperm cryopreservation in fish and shellfish. In: *Spermatology*. E.R.S. Roldan and M. Gomendio, editors. Society of Reproduction and Fertility Supplement 65, Nottingham University Press, Nottingham, England. Pp. 493–508.

Tixier-Boichard, M., Marthey, S., Marthey, N. 2018. The CRB-Anim web portal: access to biological resources for animal sciences. 69th Annual Meeting of the European Federation of Animal Science (EAAP), Aug 2018, Dubrovnik, Croatia. ffhal-02737602f.

Torres, L., Hu, E., and Tiersch, T.R. 2016. Cryopreservation in fish: Current status and pathways to quality assurance and quality control in repository development. *Reproduction, Fertility and Development* 28(8):1105–1115. https://doi.org/10.1071/RD15388.

Torres, L., and Tiersch, T.R. 2018. Addressing reproducibility in cryopreservation, and considerations necessary for commercialization and community development in support of genetic resources of aquatic species. *Journal of the World Aquaculture Society* 49(4):644–663. https://doi.org/10.1111/jwas.12541.

Wayman, W.R., Thomas, R.G., and Tiersch, T.R. 1997. Refrigerated storage and cryopreservation of black drum (*Pogonias cromis*) spermatozoa. *Theriogenology* 47:1519–1529.

Wilson-Leedy, J.G., and Ingermann, R.L. 2007. Development of a novel CASA system based on open-source software for characterization of zebrafish sperm motility parameters. *Theriogenology* 67(3):661–672.

Childress, W.M., Liu, Y., and Tiersch, T.R. 2021. Design, alpha testing, and beta testing of a 3-D printed open-hardware portable cryopreservation device for aquatic species. *Journal of Applied Aquaculture*. DOI: 10.1080/10454438.2021.1955805.

Yang, H., L. Hazlewood, R.B. Walter, and T.R. Tiersch. 2006. Effect of osmotic immobilization on refrigerated storage and cryopreservation of sperm from a viviparous fish, the green swordtail (*Xiphophorus helleri*). *Cryobiology* 52:209–218.

Yang, H., M. Norris, R. Winn, and T.R. Tiersch. 2010. Evaluation of cryoprotectant and cooling rate for sperm cryopreservation in the euryhaline fish medaka (*Oryzias latipes*). *Cryobiology* 61:211–219.

Yang, H.Y., and Tiersch, T.R. 2011. Application of computer-assisted sperm analysis (CASA) to aquatic species. In: Tiersch T.R., and Green C.C., (eds), *Cryopreservation in Aquatic Species*, 2nd edition. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 240–254.

Chapter 5. *Ex situ in vitro* conservation of finfish

Elsa Cabrita, Center for Marine Science (CCMAR), University of Algarve, Faro, Portugal
Francisca Felix, Center for Marine Science (CCMAR), University of Algarve, Faro, Portugal
José Beirão, Nord University, Bodo, Norway

Introduction

Finfish are the most diverse category of vertebrates, with over 35 000 species occupying virtually all aquatic environments of the world. They are also an essential source of food for people and support livelihoods for billions. Farmed finfish dominate the world production of farmed aquatic animal species and some of the major aquaculture species are represented by finfish species that most contribute to global food security (e.g. carps, catfishes, cichlids) (FAO, 2024). They also include some of the species more commonly conserved in *in vitro* gene banks, such as the Common carp (*Cyprinus carpio*) and the rainbow trout (*Oncorhynchus mykiss*) (FAO, 2019).

Aquaculture of finfish ranges from capturing wild fish and holding them for a period of time before harvest to completing the life cycle entirely in captivity. Gene banking is usually carried out for those species where captive rearing is possible, though gene banking of endangered species without this proven ability also occurs.

In 1953, Blaxter published the first gamete cryoconservation protocol for aquatic species, particularly for a finfish species (Blaxter, 1953). In the subsequent 70 years, protocols using controlled freezing techniques have been developed to conserve several types of cells and tissues of hundreds of finfish species (Gallego and Asturiano, 2019). However, only a limited number of fish groups, mostly freshwater, have received significant attention. Finfish are extremely diverse and this diversity is also seen in the gamete (especially male gametes) morphology and biology (Mattei 1991; Beirão *et al.*, 2015; Emel'yanova and Pavlov, 2020). Specific protocols have been established for aquacultured species amongst salmonids, cyprinids, sturgeons and some siluriformes. Successful cases of sperm cryopreservation in marine finfish are more recent and less advanced than in freshwater finfish. One of the main reasons is that for most of marine finfish species, fertilization in aquaculture facilities occurs naturally and does not require artificial fertilization, which implies that management of gametes is less critical (Gallego and Asturiano, 2019; Martínez-Páramo *et al.*, 2017). However, with the greater development of marine farming and domestication of marine species, cryoconservation can be crucial to improving the reproductive management in captivity.

Gene banks for finfish have been developed with different aims, benefiting finfish farming through improved reproductive management and genetic selection. Protocols have been developed for species new to aquaculture and conservation, species with problematic captive reproduction, and for established commercial or model species. Effective cryopreservation methods are quite diverse, as are the fish, suggesting that protocols may need to be adapted to each species, cells or tissue types of interest (Asturiano *et al.*, 2017, Cabrita *et al.*, 2009, 2014, 2022), although protocols for species groups may be similar (see

Annex 1). This is maybe not surprising, considering the huge diversity of finfish, but generalization and standardized resolution of key limiting factors is important (Cabrita *et al.*, 2022).

As reviewed by Martínez-Páramo *et al.* (2017), over the past decades, several national gene banks have been developed in different countries over the world as germplasm repositories, for freshwater and marine finfish, with goals of conservation of the genetic diversity from wildlife, support for selective breeding and conservation of farmed types in aquaculture. Other gene banks, public, semi-private and private, also exist, including in research institutes and aquaculture companies that have their own gene banks where their brood lines of interest are kept.

The use of cryopreservation in finfish aquaculture supports improved broodstock management at hatcheries (for example, extending the reproductive season), preserving important farmed types including genetically improved strains derived from selective breeding, or resolving problems of asynchronous gamete production of males and females, or low sperm production (Asturiano *et al.*, 2017). However, the use of centralized finfish germplasm gene banks in aquaculture is relatively small compared with that seen in terrestrial livestock farming. Nonetheless, there are companies that make use of this technology for their broodstock management, sometimes in collaboration with research institutes or companies specialized in providing cryoconservation services. For example, the Norwegian company Cryogenetics offers cryopreservation services for several farmed finfish species in Europe, North America and South America, Australia and New Zealand. Much of the salmon industry now makes regular use of sperm cryopreservation in their production units and selective breeding programs, either through in-house expertise or with the help of service providers or research institutes (Martínez-Páramo *et al.*, 2017). However, the application is still limited to a few species: mostly Atlantic salmon (*Salmo salar*), Pacific salmon (*Oncorhynchus spp*), halibut (*Hippoglossus* spp.), cods and trouts. To overcome this limitation, cryobiologists need to reduce ambiguities in applications, results, and reporting, as well as maintaining more collaborations with the industry to promote knowledge transfer.

Another potential application of cryopreservation for finfish species, is the maintenance of wild-type genetic material to form the broodstock for a selective breeding program. This germplasm can be used in the future to recover the original wild genotypes if needed (Asturiano *et al.*, 2017).

Finally, finfish germplasm gene banking has conservation-related applications, including: the storage of genetic resources of threatened and potentially threatened species; and as a tool in restocking and conservation programs (Martínez-Páramo *et al.*, 2017). As in commercial aquaculture, sperm cryopreservation can be used in conservation breeding to resolve asynchrony in male and female gamete production, difficulty in obtaining males at the same time as females, or when genetic analysis of an individual is needed before use (Martínez-Páramo *et al.*, 2017).

Finfish reproduction

Finfish are characterized by an extraordinary diversity of reproductive behaviours and fertilization modes compared to other vertebrate groups. Fertilization can occur either outside (i.e. external fertilization) or within (i.e. internal fertilization) the female reproductive tract (Fitzpatrick, 2020). Most species have external fertilization, wherein eggs and sperm are released into the environment, generally associated with mating behavior that maximizes the contact between eggs and sperm. In many species, the motility of sperm cells is activated by contact with the surrounding water and lasts only 30seconds to several minutes. Motility is an indicator of viability and fertilization capability of the sperm, so proximity of the two gametes is

essential in natural environments and rapid processing is critical to effective artificial reproduction in fish culture (Beirão *et al.*, 2019).

Once fertilized, the oocyte membrane expands and forms a protective shell, preventing further sperm penetration and protecting the developing embryos. Larvae hatch out of the eggs after a species-specific and temperature dependent incubation period of a day to months, generally with an attached yolk sac to provide nutrition for several days to a week. Some species have internal fertilization and live birth, and some are protective of the egg masses and larvae.

Types of finfish cells and tissues that can be conserved *in vitro*

Many studies have been performed during the last decades to design cryopreservation protocols for *ex situ in vitro* conservation of finfish for different applications. Research on germplasm cryobanking includes diverse cell and tissue types, including sperm, spermatogonia, oogonia, primordial germ cells (PGCs), somatic cells, and embryos (Labbé *et al.*, 2013). Each type of cell or tissue presents different challenges. The possibility of preservation and gene banking of fish genomes, both paternal and maternal, would contribute greatly to the advance of conservation and selective breeding. However, freezing of fish oocytes and embryos has so far not been very successful, so the attention is shifting to cryopreservation of PGC and germinal stem cells (GSC).

Sperm

Sperm cryopreservation has been the main focus of *in vitro* conservation of finfish with commercial applications. Sperm is easy to collect in most fish species. Spermatozoa are small, with a relatively simple structure and have high chilling resistance. Although it is difficult to indicate the exact number of finfish species whose sperm has been successfully cryopreserved, Tiersch *et al.* (2011) estimated between 50 and 200 for marine and freshwater species, respectively at that time. The key elements in cryoconservation protocols are similar amongst species (see Chapter 3), allowing for development of effective processes for many species. Standardization of approaches is nevertheless elusive.

Laboratory protocols for the cryoconservation of sperm cells of some commercially important finfish species are provided in Annex 1, that can be used as reference for developing protocols of similar species.

Oocytes and ovarian

The focus on sperm preservation has been criticized as preserving only part of a species' genetic information. Cryopreservation of fish oocytes would allow the preservation of female germplasm. However, this has been difficult due to the large cell volume relative to the surface area, high lipid content, low permeability of the oocytes' cellular membrane to cryoprotectants and high chilling sensitivity (Diwan *et al.*, 2020; Martínez-Páramo *et al.*, 2017). Different studies have been carried out in zebrafish *(Danio rerio*) and marine finfish species, evaluating oocyte cryopreservation at different stages of development (Martínez-Páramo *et al.*, 2017). These indicate that early-stage ovarian follicles are less sensitive to chilling injury than late-stage ovarian follicles (Tsai *et al.*, 2009) and that vitrification is a better alternative for fish ovarian tissue banking (Lujić *et al.*, 2017, Marques *et al.*, 2015). Nevertheless, cryopreservation of finfish oocytes, ovarian follicles, and embryos is still far from practical application.

Primordial germ cells

PGCs are the first germline cells to develop during early embryonic development, migrating eventually to the genital ridge and colonizing the gonads. These cells subsequently proliferate and differentiate into spermatogonial stem cells (SSCs) or oogonial stem cells (OSCs). Cryoconservation of PGCs from early embryonic development, during migration and from the genital ridges has been attempted (Franěk and Pšenička, 2020; Riesco *et al.*, 2012). Cryoconservation of cell suspensions containing isolated microencapsulated PGCs gave the best results (Riesco *et al.*, 2012). Genital ridges of rainbow trout (Kobayashi *et al.*, 2007) and zebrafish (Riesco *et al.*, 2012) were also cryopreserved with high PGC recovery and viability. In the case of rainbow trout, cryopreserved PGCs have been transplanted into abdominal cavities of allogenic trout larvae, where they migrated into the recipient's genital ridges and differentiated to give rise to donor-derived gametes and offspring.

Even though cryopreservation of PGCs appears feasible, several factors hamper the application of this technology in a practical manner. PGCs are present only in larvae up to a certain age, and each larva contains only a limited number of PGCs. The identification and visualization of these cells is also challenging, relying on the use of specific fluorescent markers or the collection in transgenic lines. The collection of PGCs from a large number of marine larvae is laborious and time-consuming, particularly for species with small larvae (Cabrita *et al.*, 2022).

Isolated germinal stem cells

Germinal stem cells (GSCs) are an alternative for cryoconservation. These are diploid cells that develop into gametes, containing both paternal and maternal genomes in a single cell (Yoshizaki and Lee, 2018; Yoshizaki and Yazawa, 2019). Šćekić *et al.* (2020) describe cryopreservation of ovarian or testis tissue fragments that contain these GSCs. After thawing, these are dissociated and GSCs isolated, maintained in culture for enrichment, and transplanted into the body cavity of recipient larvae. Once in the larvae, they migrate towards the recipient's genital ridge and colonize it, start to proliferate and differentiate, and ultimately give rise to donor-derived gametes in the host animal.

As in the case of PGCs, more work is needed for the optimization of the biotechnological tools. Spermatogonia and PGCs have been cryopreserved in several fish species (Lee *et al.*, 2016; Marinović *et al.*, 2019; Robles *et al.*, 2017; Yoshizaki *et al.*, 2011; Cabrita *et al.*, 2023). However, a bottleneck of the application of these biological materials is the development of tools such as transplantation at a larger scale, required to establish the germline (Franek *et al.*, 2019a, b) and the production of donor-derived progeny. So far, there are no applications at the commercial level.

Embryos

Embryo cryopreservation would also ensure the preservation of paternal and maternal genomes in the same individual. It could also significantly improve hatchery production, reducing costs and facilitating out-season reproduction. Challenges facing fish embryo cryopreservation have been summarized in Martinez-Paramo *et al.*, (2017), including low surface-to-volume ratio, large size of yolk globules, low membrane permeability and high chilling sensitivity. Recent reports on zebrafish embryo cryopreservation have shown

some promising results (Khosla *et al.*, 2020), but this is still far from large scale application, requiring further development.

Somatic cells

Differentiated somatic cells, including adult cells and those collected from the embryos after epiboly have been studied for genome preservation. Somatic cells are diploid, so their advantage, similarly to embryo cryopreservation, is that they carry both maternal and paternal genomes. In addition, they can be collected from finfish of any age or sex. However, so far, the use of this biological material for reconstitutive breeding of finfish is restricted to research applications in model species and not so much for production or conservation (Martinez-Paramo *et al.*, 2017; Chenais *et al.*, 2014). Several bottlenecks are still present. Cells from the skin and fins have been the best candidates due to their regenerative capacity (Chenais *et al.*, 2014). While these cells do well in cryopreservation, their use in the restoration of an organism remains problematic, as with spermatogonia or PGCs. Techniques are needed in species and tissue-specific cell culture, nuclear transfer into a host oocyte with microinjection, and cell reprogramming to avoid clone failure. Although some advances have been made in different species, we are still far from the success obtained with spermatogonia or PGCs.

Factors affecting finfish cryoconservation and practical challenges

It is often debated why, despite years of research, sperm cryopreservation of finfish has not achieved the same scale as that of terrestrial livestock. Opinions expressed by different authors include that

- Sperm is rarely a limitation in finfish breeding, resulting in low investments in cryobiology.
- Selective breeding programs only exist for a few finfish species; and
- Protocols are not standardized adequately to allow for broad adoption. Finfish are extremely diverse, which makes standardization difficult, and protocol development approaches are also diverse, making standardization even more complicated.

  Lack of consensus among researchers on the standards for the application of sperm cryopreservation to marine finfish, for example, is reflected in the variety of terminology, methods and reporting (Asturiano *et al.*, 2017; Torres *et al.*, 2017). As a result, different laboratories often use different protocols (different extenders, cryoprotectants, freezing and thawing rates, etc.) for the same species, based on historical research trajectories rather than biological characteristics. This creates challenges in comparing results of published methods and thus in the scaling up of the technologies (Asturiano *et al.*, 2017). Asturiano *et al.* (2017) and Torres *et al.* (2017) have recently published comprehensive analyses towards the standardization of fish sperm cryopreservation practices. Whereas each species may require their own adaptation of standard methods to allow effective sperm cryopreservation, the general framework is similar for most species. These steps include collection of the sperm, preparation of the sperm sample, cryoprotectant addition, cryopreservation package, freezing, storage, thawing, fertilization, and quality assurance. These are largely outlined in other sections of the Practical Guide, with a view to building greater standardization in the field, facilitating an associated increased application.

Future directions and challenges

To fulfill the potential of its application, cryoconservation faces several challenges which are discussed below.

Improving quality of genetic material subject to cryopreservation

Despite the progress made in sperm cryopreservation of over 200 finfish species, the sperm quality of most of these species declines significantly during freeze-thaw process, consequently affecting the fertilization and hatching rate (Xin et al., 2020). More research is needed to identify the key parameters determining the sensitivity to the cryopreservation process, especially in the freeze-thaw steps. For example, proteome analysis or the identification of specific mRNAs transported into finfish spermatozoa during gametogenesis and spermiation may be key elements to understand changes in gamete quality and identify heterogeneity quality in some samples (Cabrita *et al.*, 2022). These aspects will be important also to identify molecular players responsible for gamete quality. At the same time, cryoresistance also needs to be improved, at least in some species. The addition of novel compounds such as seminal plasma proteins, antioxidants and antifreeze proteins to protect sperm cells from cell damages, is a new research trend which could offer opportunity for improvement but requires further investigation (Cabrita *et al.*, 2014, 2022; Sandoval-Vargas *et al.*, 2020, Xin et al.).

Recent research also suggests that the cryopreservation process may cause direct genetic damage (Cabrita *et al.*, 2022). Further analysis of specific genes that can be altered by cryopreservation is needed, for example, changes in the methylation profile (Chatterjee *et al.*, 2017; Depincé *et al.*, 2019; Riesco and Robles, 2014) should be investigated. Epigenetic risks associated with DMSO (Depincé *et al.*, 2020; Almeida *et al.*, unpublished data) must be better investigated to evaluate the risk of altering progeny from cryopreserved material. Of note, less toxic cryoprotectants are favoured in newer protocols (Ritter, pers com., 2024).

Standardization of approaches

The application of cryopreservation methods as part of a standard methodology used by the finfish farming industry is needed and can contribute to increase production through appropriate broodstock management. Standardized evaluation of effectiveness and reporting can help, but collaborations with the industry to promote knowledge and technology transfer are essential to facilitate large-scale protocol application. Of note, cryogenic companies have developed and use standardized protocols in Norway, North and South America, New Zealand, and Australia, adapted to a number of farmed species in large scale production as well as unfarmed wild species including 8 salmonid species, halibut, Atlantic cod, sablefish, cobia, lumpsucker, blue catfish, and zebrafish (www.cryogenetics.com/species/).

Continued development of alternatives for gamete cryopreservation

Due to the impossibility of cryopreserving fish oocytes and embryos, new alternatives for germplasm preservation have been shown to address the need to preserve both fish paternal and maternal genome. Research has focused on the use of primordial germ cells, oogonia and spermatogonia where cryopreservation of these materials has shown promising results at laboratory scale. The Brazilian CEPTA/ICMBIO gene bank has focused on this issue (Annex 9). However, as mentioned before, associated techniques such as cell culture for proliferation has so far limited the use of these materials. Technology to overcome unsuccessful embryo cryopreservation will also require more investigation, both for freezing and thawing (Khosla *et al.*, 2020).

References

Asturiano, J.F., Cabrita, E., and Horváth, Á. 2017. Progress, challenges, and perspectives on fish gamete cryopreservation: a mini-review. *Gen. Comp. Endocrinol.* 245, 69–76.

Babiak, I. 2009. Cryopreservation of Atlantic halibut sperm (*Hippoglossus hippoglossus*). In: *Methods in Reproductive Aquaculture: Marine and Freshwater Species*, Cabrita E, Robles V, Herráez MP (Eds), Taylor and Francis Group, CRC Press, Boca Raton, pp. 433–436.

Beirão, J., Boulais, M., Gallego, V., O'Brien, J.K., Peixoto, S., Robeck, T.R., and Cabrita, E. 2019. Sperm handling in aquatic animals for artificial reproduction. *Theriogenology* 133, 161–178.

Beirão, J., Lewis, J., and Purchase, C.F. 2015. Spermatozoa ultrastructure of two osmerid fishes in the context of their family (Teleostei: Osmeriformes: Osmeridae). *Journal of Applied Ichthyology* 31, 28–33.

Bernáth, G., Żarski, D., Kása, E., Staszny, Á., Várkonyi, L., Kollár, T., Hegyi, Á., Bokor, Z., Urbányi, B., and Horváth, Á. 2016. Improvement of common carp (*Cyprinus carpio*) sperm cryopreservation using a programmable freezer. *General and Comparative Endocrinology* 237, 78–88.

Billard, R., Cosson, J., Noveiri, S.B., and Pourkazemi, M. 2004. Cryopreservation and short-term storage of sturgeon sperm: a review. *Aquaculture* 236(1–4), 1–9.

Blaxter, J.H.S. 1953. Sperm storage and cross-fertilization of spring and autumn spawning herring. *Nature* 26, 1189–1190.

Bokor, Z., Bernáth, G., Várkonyi, L., Molnár, J., Láng, Z.L., Tarnai-Király, Z., Solymosi, E., and Urbányi, B. 2019. The applicability of large-scale sperm cryopreservation in wels catfish (*Silurus glanis*) optimized for hatchery practice. *Aquaculture* 506, 337–340.

Bokor, Z., Urbányi, B., Horváth, L., and Horváth, Á. 2010. Commercial-scale cryopreservation of wels catfish (*Silurus glanis*) semen. *Aquaculture Research* 41(10), 1549–1551.

Boryshpolets, S., Kholodnyy, V., Cosson, J., and Dzyuba, B. 2020. Fish sperm quality evaluation after cryopreservation. In: Betsy, J., Kumar, S. (Eds.), *Cryopreservation of Fish Gametes*, Springer, Singapore, pp. 117–133.

Cabrita, E., Engrola, S., Conceição, L.E.C., Pousão-Ferreira, P., and Dinis, M.T. 2009. Successful cryopreservation of sperm from sex-reversed dusky grouper, *Epinephelus marginatus*. *Aquaculture* 287, 152–157.

Cabrita, E., Horváth, A., Marinović, Z., and Asturiano, J.F. 2022. Technologies and strategies for ex situ conservation of aquatic organisms: the role of cryopreservation in long-term management. In: Monzón, I., Fernandes, J. (Eds.), *Cellular and Molecular Approaches in Fish Biology*, Springer, Berlin, pp. 1–48.

Cabrita, E., Ma, S., Diogo, P., Martínez-Páramo, S., Sarasquete, C., and Dinis, M.T. 2011. The influence of certain amino acids and vitamins on post-thaw fish sperm motility, viability, and DNA fragmentation. *Anim. Reprod. Sci.* 125 (1–4), 189–195.

Cabrita, E., Martínez-Páramo, S., Gavaia, P.J., Riesco, M.F., Valcarce, D.G., Sarasquete, C., Herráez, M.P., and Robles, V. 2014. Factors enhancing fish sperm quality and emerging tools for sperm analysis. *Aquaculture* 432, 389–401.

Cabrita, E., Pacchiarini, T., Fatsini, F., Sarasquete, C., Herráez, M.P. 2023. Post-thaw quality assessment of testicular fragments as a source of spermatogonial cells for surrogate production in the flatfish *Solea senegalensis*. Fish Physiology and Biochemistry. https://doi.org/10.1007/s10695-023-01232-2

Cabrita, E., Pacchiarini, T., Fatsini, F., Sarasquete, C., and Herráez, M.P. 2023. Post-thaw quality assessment of testicular fragments as a source of spermatogonial cells for surrogate production in the flatfish *Solea senegalensis*. *Fish Physiology and Biochemistry*. https://doi.org/10.1007/s10695-023-01232-2.

Cabrita, E., Robles, V., Cuñado, S., Wallace, J.C., Sarasquete, C., and Herráez, M.P. 2005. Evaluation of gilthead sea bream, *Sparus aurata*, sperm quality after cryopreservation in 5 ml macrotubes. *Cryobiology* 50(3), 273–284.

Cabrita, E., Sarasquete, C., Martínez-Páramo, S., Robles, V., Beirão, J., Pérez-Cerezales, S., Herráez, M.P. 2010. Cryopreservation of fish sperm: applications and perspectives. *Journal of Applied Ichthyology* 26, 623–635.

Chatterjee, A., Saha, D., Niemann, H., Gryshkov, O., Glasmacher, B., and Hofmann, N. 2017. Effects of cryopreservation on the epigenetic profile of cells. *Cryobiology* 74, 1–7.

Chenais, N., Depince, A., Le Bail, P.-Y., and Labbé, C. 2014. Fin cell cryopreservation and fish reconstruction by nuclear transfer stand as promising technologies for the preservation of fish genetic resources. *Aquacult. Int.* 22, 63–76.

Ciereszko, A., Dietrich, G. J., Nynca, J., Dobosz, S., and Zalewski, T. 2014. Cryopreservation of rainbow trout semen using a glucose-methanol extender. *Aquaculture* 420, 275–281.

Cosson, J. 2019. Fish sperm physiology: structure, factors regulating motility, and motility evaluation. IntechOpen, London.

Depincé, A., Gabory, A., Dziewulska, K., Le Bail, P. Y., Jammes, H., and Labbé, C. 2019. DNA methylation stability in fish spermatozoa upon external constraint: Impact of fish hormonal stimulation and sperm cryopreservation. *Molecular Reproduction and Development* 87(1), 124–134.

Diogo, P., Martins, G., Quinzico, I., Nogueira, R., Gavaia, P.J., and Cabrita, E. 2018. Electric ultrafreezer (-150°C) as an alternative for zebrafish sperm cryopreservation and storage. *Fish Physiol. Biochem.* 1–13.

Emel'yanova, N.G., and Pavlov, D.A. 2020. Spermatozoa morphology of Goatfishes (*Mullidae*): *Upeneus sulphureus*. *Journal of Ichthyology* 60, 71–78.

FAO. 2019. The State of the World's Aquatic Genetic Resources for Food and Agriculture. FAO Commission on Genetic Resources for Food and Agriculture Assessments. Rome.

FAO. 2024. The State of World Fisheries and Aquaculture 2024. Blue Transformation in Action. Rome. https://openknowledge.fao.org/items/ef79a6ba-d8df-41b9-9e87-2b6edd811511

Félix, F., Oliveira, C.C.V., and Cabrita, E. 2021. Antioxidants in fish sperm and the potential role of melatonin. *Antioxidants* 10, 36.

Fitzpatrick, J.L. 2020. Sperm competition and fertilization mode in fishes. *Phil. Trans. R. Soc. B* 375: 20200074. http://dx.doi.org/10.1098/rstb.2020.0074.

Fopp-Bayat, D., Nitkiewicz, A., Sarosiek, B., and Kowalski, R.K. 2023. The successful production of "sterbel" hybrids using beluga (*Huso huso*) cryopreserved sperm. *Animal Reproduction Science* 249, 107197.

Franěk, R., Marinović, Z., Lujić, J., Urbányi, B., Fučíková, M., Kašpar, V., Pšenička, M., and Horváth, Á. 2019a. Cryopreservation and transplantation of common carp spermatogonia. *PLoS One* 14(4), e0205481.

Franěk, R., and Pšenička, M. 2020. Cryopreservation of germ stem cells in fish. In: Betsy, J., Kumar, S. (Eds.), *Cryopreservation of Fish Gametes*. Springer, Singapore.

Franěk, R., Tichopád, T., Steinbach, C., Xie, X., Lujić, J., Marinović, Z., Horváth, Á., Kašpar, V., Pšenička, M. 2019. Preservation of female genetic resources of common carp through oogonial stem cell manipulation. *Cryobiology* 87, 78–85.

Gallego, V., Asturiano, J.F. 2019. Fish sperm motility assessment as a tool for aquaculture research: A historical approach. *Rev. Aquac.* 11, 697–724.

Horváth, Á., Urbányi, B. 2000. The effect of cryoprotectants on the motility and fertilizing capacity of cryopreserved African catfish *Clarias gariepinus* (Burchell 1822) sperm. *Aquaculture Research* 31(3), 317–324.

Hu, E., Yang, H., Tiersch, T.R. 2011. High-throughput cryopreservation of spermatozoa of blue catfish (*Ictalurus furcatus*): Establishment of an approach for commercial-scale processing. *Cryobiology* 62(1), 74–82.

Igna, V., Telea, A., Florea, T., Popp, R., Grozea, A. 2022. Evaluation of Russian sturgeon (*Acipenser gueldenstaedtii*) semen quality and semen cryopreservation. *Animals* 12(16), 2153.

Jawahar, K.T.P., Betsy, J. 2020. Cryopreservation of fish gametes: An overview. In: Betsy, J., Kumar, S. (Eds.), *Cryopreservation of Fish Gametes*, Springer, Singapore, pp. 151–175.

Judycka, S., Nynca, J., Ciereszko, A. 2019. Opportunities and challenges related to the implementation of sperm cryopreservation into breeding of salmonid fishes. *Theriogenology* 132, 12–21.

Judycka, S., Nynca, J., Liszewska, E., Dobosz, S., Grudniewska, J., Ciereszko, A. 2018. Optimal sperm concentration in straws and final glucose concentration in extender are crucial for improving the cryopreservation protocol of salmonid spermatozoa. *Aquaculture* 486, 90–97.

Judycka, S., Szczepkowski, M., Ciereszko, A., Dietrich, G.J. 2015. New extender for cryopreservation of Siberian sturgeon (*Acipenser baerii*) semen. *Cryobiology* 70(2), 184–189.

Judycka, S., Zarski, D., Dietrich, M.A., Palinska-Zarska, K., Karol, H., Ciereszko, A. 2019. Standardized cryopreservation protocol of European perch (*Perca fluviatilis*) semen allows to obtain high fertilization rates with the use of frozen/thawed semen. *Aquaculture* 498, 208–2016.

Khosla, K., Kangas, J., Liu, Y., Zhan, L., Daly, J., Hagedorn, M., Bischof, J. 2020. Cryopreservation and laser nanowarming of zebrafish embryos followed by hatching and spawning. *Adv. Biosyst.* 4(11): e2000138.

Kobayashi, T., Takeuchi, Y., Takeuchi, T., Yoshizaki, G. 2007. Generation of viable fish from cryopreserved primordial germ cells. *Mol. Reprod. Dev.* 74, 207–213.

Kovács, É., Müller, T., Márián, T., Krasznai, Z., Urbányi, B., Horváth, Á. 2010. Quality of cryopreserved African catfish sperm following post-thaw storage. *J. Appl. Ichthyol.* 26(5), 737–741.

Kowalski, R.K., Cejko, B.I. 2019. Sperm quality in fish: Determinants and affecting factors. *Theriogenology* 135, 94–108.

Labbé, C., Robles, V., Herráez, M.P. 2013. Cryopreservation of gametes for aquaculture and alternative cell sources for genome preservation. In: Allan, G., Burnell, G. (Eds.), *Advances in Aquaculture Hatchery Technology*, Woodhead Publishing, pp. 76–116. ISSN: 0213–3911.

Lee, S., Yoshizaki, G. 2016. Successful cryopreservation of spermatogonia in critically endangered Manchurian trout (*Brachymystax lenok*). *Cryobiology* 72, 165–168.

Lujić, J., Marinović, Z., Sušnik Bajec, S., Djurdjevič, I., Kása, E., Urbányi, B., Horváth, Á. 2017. First successful vitrification of salmonid ovarian tissue. *Cryobiology* 76, 154–157.

Marcantonini, G., Bartolini, D., Zatini, L., Costa, S., Passerini, M., Rende, M., Luca, G., Basta, G., Murdolo, G., Calafiore, R., Galli, F. 2022. Natural cryoprotective and cytoprotective agents in cryopreservation: A focus on melatonin. *Molecules* 27.

Marinović, Z., Li, Q., Lujić, J., Iwasaki, Y., Csenki, Z., Urbányi, B., Yoshizaki, G., Horváth, Á. 2019. Preservation of zebrafish genetic resources through testis cryopreservation and spermatogonia transplantation. *Sci. Rep.* 9, 13861.

Marques, L.S., Mikich, A.B., Leandro, G., Laura, A.S., Daniel, M., Zhang, T., Streit Jr, D.P. 2015. Viability of zebrafish (*Danio rerio*) ovarian follicles after vitrification in a metal container. *Cryobiology* 71, 1–7.

Martínez-Páramo, S., Horváth, Á., Labbé, C., Zhang, T., Robles, V., Herráez, M.P., Suquet, M., Adams, S., Viveiros, A., Tiersch, T.R., Cabrita, E. 2017. Cryobanking of aquatic species. *Aquaculture* 472, 156–177.

Mattei, X. 1991. Spermatozoon ultrastructure and its systematic implications in fishes. *Can. J. Zool.* 69(12), 3038–3055.

Nascimento, J.P., Horokhovatskyi, Y., Kholodnyy, V., Rodina, M., Dzyuba, V., Stechkina, T., Boryshpolets, S., Dzyuba, B. 2021. Optimization of sterlet sperm concentration for cryopreservation. *Aquaculture* 540, 736682.

Nynca, J., Judycka, S., Liszewska, E., Dobosz, S., Ciereszko, A. 2017. Standardization of spermatozoa concentration for cryopreservation of rainbow trout semen using a glucose-methanol extender. *Aquaculture* 477, 23–27.

Paredes, E. 2016. Biobanking of a marine invertebrate model organism: The sea urchin. *J. Mar. Sci. Eng.* 4(1), 7.

Riesco, M.F., Martínez-Pastor, F., Chereguini, O., Robles, V. 2012. Evaluation of zebrafish (*Danio rerio*) PGCs viability and DNA damage using different cryopreservation protocols. *Theriogenology* 77, 122–130.

Riesco, M.F., Robles, V. 2014. Cryopreservation causes genetic and epigenetic changes in zebrafish genital ridges. *PLoS ONE* 9(1).

Robles, V., Riesco, M.F., Pšenicka, M., Saito, T., Valcarce, D.G., Cabrita, E., Herráez, M.P. 2017. Biology of teleost primordial germ cells (PGCs) and spermatogonia: Biotechnological applications. *Aquaculture* 472, 4–20.

Routray, P. 2020. Cryopreservation and short duration storage of germ cells and male gametes of freshwater fish. In: *Cryopreservation of Fish Gametes*, Betsy, J., Kumar, S. (Eds.), Springer, pp. 177–186.

Routray, P., Choudhary, A.K., Dash, S.N., Verma, D.K., Dash, C., Swain, P., Jena, J.K., Gupta, S.D., Sarangi, N. 2006. Cryopreservation of dead fish spermatozoa several hours after death of Indian major carp, *Labeo rohita*, and its successful utilization in fish production. *Aquaculture* 261(4), 1204–1211.

Sandoval-Vargas, L., Mauricio, S.J., González, J.R., Figueroa, E.V., Cabrita, E., Valdebenito, I.I. 2020. Oxidative stress and use of antioxidants in fish semen cryopreservation. *Rev. Aquac.* 13, 365–387.

Šćekić, I., Marinović, Z., Lujić, J., Müller, T., Kitanović, N., Urbányi, B., Horváth, Á. 2020. A novel strategy for conservation of European eel (*Anguilla anguilla*) genetic resources: Cryopreservation of ovarian stem cells. *Cryobiology* 95, 151–156.

Suquet, M., Chereguini, O., Fauvel, C. 2009. Cryopreservation of sperm from turbot (*Psetta maxima*). In: *Methods in Reproductive Aquaculture: Marine and Freshwater Species*, Cabrita, E., Robles, V., Herráez, M.P. (Eds.), Taylor and Francis Group, CRC Press, Boca Raton, pp. 463–468.

Tiersch, T.R., Green, C.C. (Eds.) 2011. *Cryopreservation in Aquatic Species*, second edition. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Torres, L., Liu, Y., Guitreau, A., Yang, H., Tiersch, T.R. 2017. Challenges in development of sperm repositories for biomedical fishes: Quality control in small-bodied species. *Zebrafish* 14, 552–560.

Tsai, S., Rawson, D.M., Zhang, T. 2009. Studies on chilling sensitivity of early-stage zebrafish (*Danio rerio*) ovarian follicles. *Cryobiology* 58, 279–286.

Tsai, S., Rawson, D.M., Zhang, T. 2010. Development of in vitro culture method for early-stage zebrafish (*Danio rerio*) ovarian follicles for use in cryopreservation studies. *Theriogenology* 74, 290–303.

Viveiros, A.T.M. 2011. Current status of sperm cryopreservation in siluriform catfishes. In: *Cryopreservation in Aquatic Species*, 2nd edition, Tiersch, T.R., Green, C.C. (Eds.), World Aquaculture Society, Baton Rouge, Louisiana, pp. 387–397.

Xin, M., Niksirat, H., Shaliutina-Kolešová, A., Siddique, M.A.M., Sterba, J., Boryshpolets, S., Linhart, O. 2019. Molecular and subcellular cryoinjury of fish spermatozoa and approaches to improve cryopreservation. *Rev. Aquac.* 12, 909–924.

Yoshizaki, G., Fujinuma, K., Iwasaki, Y., Okutsu, T., Shikina, S., Yazawa, R., Takeuchi, Y. 2011. Spermatogonial transplantation in fish: A novel method for the preservation of genetic resources. *Comp. Biochem. Phys. D* 6, 55–61.

Yoshizaki, G., Lee, S. 2018. Production of live fish derived from frozen germ cells via germ cell transplantation. *Stem Cell Res.* 29, 103–110.

Yoshizaki, G., Yazawa, R. 2019. Application of surrogate broodstock technology in aquaculture. *Fish. Sci.* 85, 429–437.

Chapter 6. *Ex situ in vitro* conservation of shellfish

Serean L. Adams, Cawthron Institute, Nelson, New Zealand
Julien Vignier, Cawthron Institute, Nelson, New Zealand
Huiping Yang, Fisheries and Aquatic Science Program, School of Forest, Fisheries, and Geomatics Sciences, University of Florida, Gainesville, United States of America

1. Introduction

Shellfish are a diverse group of species that in the context of the Practical Guide refer to molluscs, crustaceans, and echinoderms. To date, only a small number of shellfish have been the subject of *ex-situ* gene banking, and only a few species have been banked using *in vitro* cryopreservation, despite extensive research efforts to develop protocols for a number of different species. Among shellfish, the most studied species for germplasm cryopreservation are molluscan bivalves (oysters, clams, mussels, and scallops), abalones, shrimps, and sea urchins, which are all commercial aquaculture species. For shellfish, *in vitro ex-situ* gene banking has several potential applications:

1) ***Preservation of genetics from breeding individuals or specific lines or farmed types***. Selective breeding for aquaculture is often a key impetus for developing methods for cryopreserving shellfish gametes, embryos, and larvae. Cryobanks provide breeders with a backup of germplasm if breeding lines are lost in the field (e.g. severe weather, theft, and lost tags or marks) or breeding objectives change. For example, a sperm repository of the Eastern oyster (*Crassostrea virginica*), saved all the male breeders for three generations from a breeding program (Yang *et al.*, 2021). For the Pacific cupped oyster (*Magallana gigas*, previously named *Crassostrea gigas*), breeding objectives shifted from fast growth and other commercial traits to disease resistance following outbreaks of the syndrome associated with the Ostreid herpesvirus-1 (OsHV-1) (Divilov *et al.*, 2019; FAO, 2023; Symonds, 2018). A cryopreserved sperm bank of oyster families has enabled researchers to create susceptible family lines and to study interactions between the virus and its host (Delisle *et al.*, 2022).

2) ***Creation of self-fertilized inbred lines***. Inbred lines are valuable resources for breeding programs, but they are difficult to produce in animals and require repeated crossing of brothers and sisters or backcrossing. Some molluscan bivalves, including oysters, can change sex during their lifespan (Broquard *et al.*, 2020; Heller, 1993). Sperm cryopreservation can be used to create self-fertilized lines by using the cryopreserved sperm to fertilize oocytes produced by the same individual after it has changed sex (Yang *et al.*, 2015).

3) ***Preservation of sperm from tetraploids***. Tetraploid oysters are widely used in oyster aquaculture to produce triploid oysters which are commercially farmed because of their fast growth and sterility (Guo *et al.*, 2009). Tetraploid broodstock is expensive to produce and because of cytogenic instability, they can only be used in commercial triploid production for about two years once mature (J. Vignier, personal communication). Cryopreservation of sperm from tetraploids can provide cost savings and security for maintaining tetraploid stock and breeding lines (Dong *et al.*, 2006).

4) ***Preservation of natural biodiversity***. These apply particularly to shellfish species that are threatened by climate change and environmental degradation.

The number of shellfish cryopreservation banks established globally, their primary purpose, and their accessibility are not known. Some banks may be in the private domain, accessible only to the companies whose breeding programs they support. Others may be public banks established for national breeding

initiatives, conservation, or research purposes. Some existing shellfish cryopreservation banks (Table 2) are all for molluscan bivalves. For many species, there are living gene banks consisting of farms dedicated to the holding of broodstock for breeding programs of cultured shellfish (Camara *et al.*, 2017; Dégremont *et al.*, 2015). These are typically more commonplace than cryopreservation banks. Cryobanks can be complementary to living gene banks and enable a greater number of genetic strains (such as rare strains) that are currently not actively being used in a breeding program to be held more cost-effectively.

Table 2. Molluscan cryobanks and their locations.

Species	Country	Bank Name/Hosting Institution	Reference
Pacific oyster	United States of America	United States Department of Agriculture – National Animal Germplasm Program	https://agrin.ars.usda.gov/database_collaboration_page_dev
Pacific oyster	France	French National Cryobank	www.crb-anim.fr/crb-anim_eng/
Pacific oyster	New Zealand	The private bank held at Cawthron Institute	J. Vignier, Cawthron Institute, New Zealand, pers. comm.
Eastern oyster	United States of America	Gulf of Mexico Oyster Genetics and Breeding Research Consortium and United States Department of Agriculture – National Animal Germplasm Program	(Yang *et al.*, 2021) https://agrin.ars.usda.gov/database_collaboration_page_dev
Green-lipped mussel	New Zealand	The private bank held at Cawthron Institute	J. Vignier, Cawthron Institute, New Zealand pers. comm.
Jinjiang oyster	Vietnam	Research Institute for Aquaculture No 1	Pham Hong Nhat, Research Institute for Aquaculture No 1 (RIA1), Vietnam, pers. comm.
Various freshwater mussel species	United States of America	Warm Springs Fish Technology Center	www.fws.gov/project/cryopreservation-lab
Various species	UK	Cryoarks	www.cryoarks.org/activities/

2. Shellfish reproduction

Shellfish have different strategies for reproduction (Figure 5). Most shellfish have separate sexes, though some are hermaphrodites either simultaneously or sequentially, and some reproduce parthenogenetically.

For molluscan bivalves, sea urchins, and abalone, most species are broadcast spawners whilst others, such as oysters from the Genus *Ostrea* and many freshwater mussels, are brooders (Foighil, 1989; Rusk *et al.*, 2017; Soler *et al.*, 2018). Environmental changes in factors such as temperature, salinity, and chemical or physical stimuli can trigger spawning (Helm *et al.*, 2004). In natural populations, spawning duration varies between species and locations. Some species (e.g., Pacific oysters) release their entire gonadal contents over a short period whereas other species (e.g., Green-lipped mussels) spawn over longer periods of several weeks, releasing their mature eggs and sperm gradually in occasional small bursts. Broadcast spawners release millions of oocytes and billions of spermatozoa into the water column where they mix, fertilize, and develop into free-swimming larvae (Figure 5). The free-swimming larvae feed on phytoplankton for several days or weeks in the water column, depending on water temperature, until they metamorphose and settle as juveniles with the same lifestyle as their parents. Newly settled oysters, mussels, and scallops attach themselves to a hard surface. Oysters remain permanently fixed, whereas mussels and scallops may move if conditions are unfavorable. Clams adopt a burrowing lifestyle, while abalone and sea urchins remain mobile after settlement to graze on biofilm made of algal diatoms, bacterial films, and drift macroalgae. In brooding species, eggs are fertilized within the gills or mantle cavity by sperm released from males in balls known as spermatozeugmata (Foighil, 1989; Waller and Lasee, 1997). The adult females generally hold on to the developing larvae (or brood) and release them into the water column until the larvae are competent for settlement. In some freshwater mussel species, the larvae go through a parasitic stage during which they are hosted on fish gills (Vikhrev *et al.*, 2019), but the details of this lifecycle stage are unknown for many species (Zieritz *et al.*, 2018).

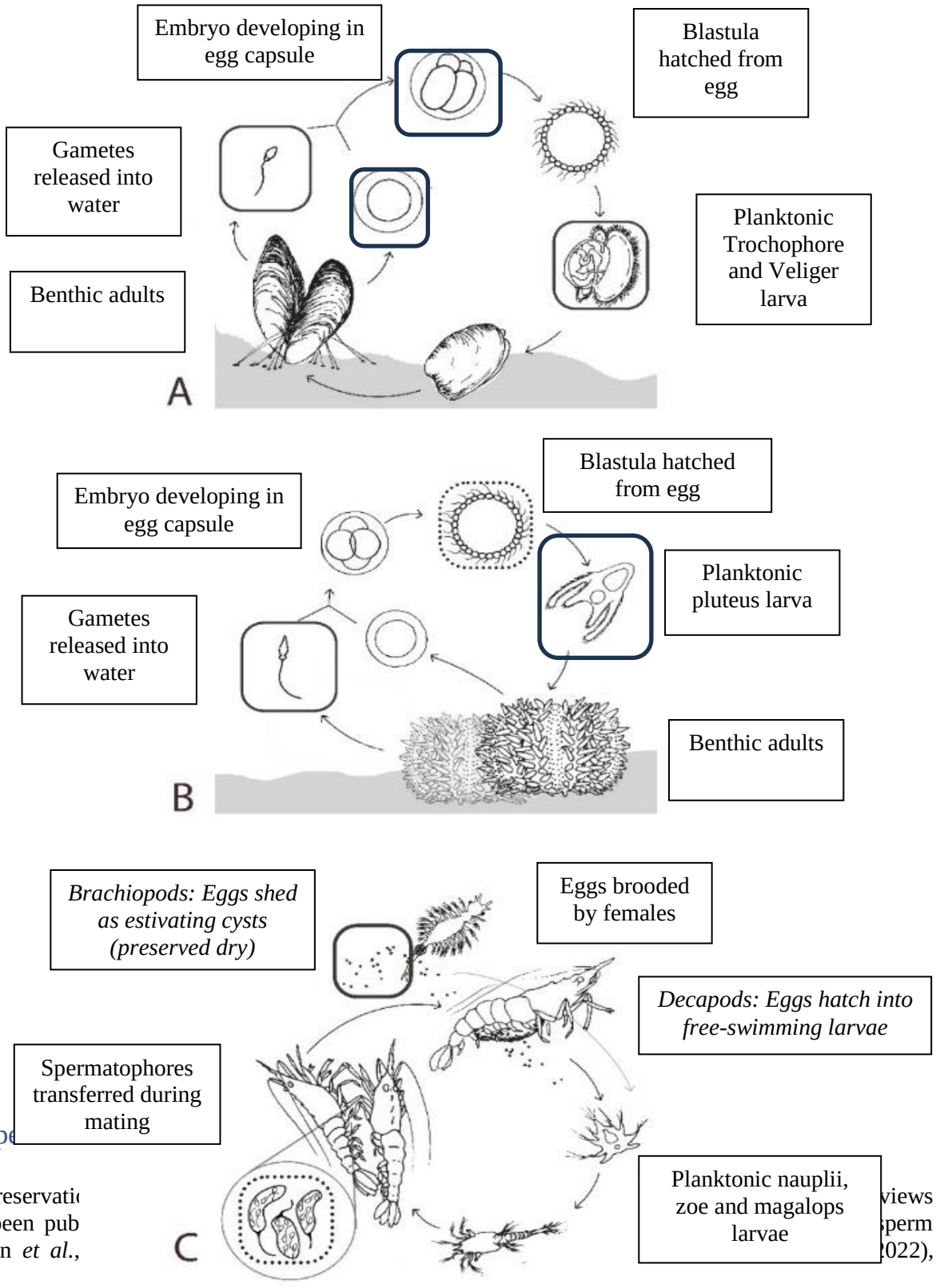
Molluscan bivalves release oocytes at the prophase of meiosis I. Upon fertilization, the fertilized eggs release two polar bodies and go through rapid cell division to become morula, blastula, swimming ciliated trochophores, and D-stage larvae (24–36 hrs). Depending on species, food availability, and water temperature, the D-larvae grow into the umbo larvae and become pediveliger larvae when a foot and an eye are developed. Within 1–3 days, pediveligers go through metamorphosis and turn into juveniles (Galtsoff, 1964). To date, germplasm cryopreservation has been studied in sperm, oocytes, and all larval stages.

Many crustaceans, including shrimp, prawns, crabs, and lobsters, have mating behaviour that involves males transferring sperm to females in capsules or packages called spermatophores (Subramoniam, 1993). The spermatophores are used in internal or external fertilization to produce embryos that are brooded by females amongst their abdominal swimmerets (in decapods) or at the tail (in branchiopods). These embryos hatch as free-swimming nauplius larvae that grow through several larval stages before metamorphosing into benthic juveniles (Figure 5). Under some adverse conditions, branchiopod crustacean species, such as brine shrimp (Annex 4), form dormant embryos in cysts that can persist for years before hatching out into nauplii larvae once conditions improve (Lavens and Sorgeloos, 1987). To date, cryopreservation has been reported in spermatophores, embryos, and larvae.

Most echinoderms are broadcast spawners. For sea urchins, the eggs are released after meiotic maturation and upon fertilization, embryo development initiates and goes through the blastula, gastrula, and prism stages to become two-arm pluteus larvae. Pluteus larvae take over 4–6 weeks (developing from two-arm, four-arm, six-arm, and eight-arm pluteus) to develop into competent larvae for metamorphosis (occurring within a few hours) into juveniles (Harris and Eddy, 2015). Cryopreservation has been reported in sperm, eggs, embryos, and larvae at different stages.

Figure 5. Generalized life histories of shellfish showing developmental stages that have either established or advanced research stage cryopreservation protocols. Not all larval stages are represented. ***A****. Molluscan bivalves – sperm,*

oocytes, embryos, and veliger larvae can be cryopreserved; ***C****. Crustaceans, including shrimps where spermatophores are cryopreserved and Artemia brine shrimps where diapaused embryos are stored dried as cysts. (Illustration by Z. Cilliers and A. Young).* ***B****. Echinoderms (sea urchin) – sperm, blastula, and larvae can be cryopreserved.*



3. Type

Cryopreservati views
have been pub sperm
(Hassan *et al.*, 022),

crustaceans in general (Guo and Weng, 2020), crustacean sperm (Aquino *et al.*, 2022; Morales-Ueno and Paniagua-Chávez, 2020), sea urchin sperm, eggs, and larvae (Campos *et al.*, 2021; Paredes *et al.*, 2019), fish and shellfish in general (Diwan *et al.*, 2020), and aquatic species (Martinez-Paramo *et al.*, 2017). Limited research has been conducted on cryopreservation of shellfish primary germ cells and gonadal tissues (Potts *et al.*, 2020). Despite these extensive research efforts on a broad range of germplasm, *ex-situ in vitro* germplasm cryopreservation is not routine for most shellfish species, and gene banking continues to be mainly *ex-situ in vivo*.

3.1 Mollusc germplasm cryopreservation

Cryopreservation research in molluscs has primarily focused on sperm (Liu *et al.*, 2015; Yang, 2017). Dimethyl sulfoxide (DMSO) with or without sugar additives has predominantly been used as an effective cryoprotectant in different cooling systems with cooling rates. However, propylene glycol (PG) and ethylene glycol (EG) have also been used successfully in sperm cryopreservation. Post-thaw fertility varies considerably ranging from 0 to 97 percent (Liu *et al.*, 2015).

Oocyte cryopreservation in molluscs is more challenging with no or limited success (Naidenko, 1997; Smith *et al.*, 2001) except in one study on Pacific oyster (*Crassostrea gigas*) where post-thaw fertility ranged from <1–74 percent when either 10 percent EG or 15 percent DMSO were used as cryoprotectants (Tervit *et al.*, 2005). Some oocytes were reportedly able to further develop to spat.

Embryo and larval cryopreservation have been attempted for several molluscan species with significantly varied post-thaw survival (Diwan *et al.*, 2020; Martinez-Paramo *et al.*, 2017; Yang and Huo, 2022). Most studies focus on oysters due to their global commercial aquaculture importance. Overall, the most suitable larval stage for cryopreservation varies between species. For example, the trochophore stage in Pacific oysters was reported to be the most amenable for cryopreservation of this species (Gwo, 1995) but in surf clams (*Spisula sachalinensis*), the D-stage/umbo veliger stage was reported to be more suitable (Choi *et al.*, 2008). Cryoprotectants for larval cryopreservation include PG, EG DMSO, and glycerol at 5–15 percent with sugar additives. Cooling rates are primarily slow, ranging from 0.2 to 5°C/min. Post-thaw larval survival varies greatly ranging from 0 to 100 percent (Yang and Huo, 2022), but some studies reported normalized survival to the controls (Paredes *et al.*, 2013). Furthermore, many studies report active motility upon thawing as survival rather than subsequent larval development post-thawing. Often, post-thaw larvae after immediate thawing were reported as slow-swimming (Horvath *et al.*, 2012; Usuki *et al.*, 2002). Despite these caveats, survival to the juvenile stage or beyond has been reported in a few publications (Liu *et al.*, 2020; Paniagua-Chavez *et al.*, 1998; Suquet *et al.*, 2014), indicating the promising future of this technology.

3.2 Crustacean germplasm cryopreservation

For crustaceans, cryopreservation of spermatophores, embryos, and larvae has been studied, but no practical cryopreservation protocols have been commercially implemented (Aquino *et al.*, 2022; Diwan *et al.*, 2020; Guo and Weng, 2020; Morales-Ueno and Paniagua-Chávez, 2020).

Effective cryoprotectants for spermatophore cryopreservation, include glycerol, DMSO, methanol, and EG at concentrations ranging from 5–15 percent (Nimrat *et al.*, 2022; Selvakumar *et al.*, 2018; Uberti *et al.*, 2014). Cells were generally exposed to cryoprotectants for 15–60 min at room temperature and then frozen at cooling rates of 0.5–2.5°C/min to an intermediate sub-zero temperature (-32 or -80°C) before plunging in liquid nitrogen for storage. Sperm of crustaceans are immobile and the assessment of post-thaw survival has mostly been conducted using viability stains of eosin-nigrosine dye (Björndahl *et al.*, 2003). To date, post-thaw fertility has been reported in three species: *Penaeus merguiensis* (Nimrat *et al.*, 2020), *Macrobrachium rosenbergii* (Chow *et al.*, 1985) and *Penaeus monodon* (Nimrat *et al.*, 2008)While for most

farmed *Penaeus vannamei*, no post-thaw fertility was reported, while the immediate post-thaw survival was 30–80 percent (Uberti *et al.*, 2014).

For crustacean embryo and larval cryopreservation, most studies focused on cryoprotectant toxicity only, and no or limited post-thaw survival was reported (Diwan and Kandasami, 1997). There is one report investigating cryopreservation of crustacean germ cells (Rakbanjong *et al.*, 2021).

3.3 Sea urchin germplasm cryopreservation

For sea urchins, cryopreservation has been reported in sperm, eggs/embryos, and larvae at a range of developmental stages (Campos *et al.*, 2021; Paredes *et al.*, 2019) In general, sperm and blastula stage embryos have been successfully cryopreserved for several species (Paredes *et al.*, 2022). In contrast, success with eggs has been limited (Campos *et al.*, 2021). Despite successful cryopreservation procedures being reported, gene banks have not been established for echinoderms.

4. General methodologies for shellfish cryopreservation

4.1 Gamete collection

For molluscan bivalves, gamete collection can be conducted through stripping the dissected gonads (strip-spawn, oysters) or natural or induced spawning (scallops, clams, and mussels). Generally, bivalve spawning is induced by cycling animals through a series of thermal shocks or by applying a chemical treatment. To collect sperm of appropriate density for cryopreservation, animals are often removed from the water upon initiation of spawning and left to continue to spawn "dry" (watery sperm is often not suitable for downstream cryopreservation as density impacts longevity and post-thaw fertility). For crustacean shrimps, spermatophores must be collected by gently squeezing the spermatophore sac beneath the first pleopods. Echinoderms can also be induced to spawn using thermal cycling or chemical treatment. For sea urchins, the injection of potassium chloride into the body cavity is often used to stimulate spawning. For sperm collection, the urchins can be removed from the water once the spawning begins so that the sperm can be collected as concentrated as possible for cryopreservation.

Fertilization can often be achieved by mixing sperm and oocytes (or eggs) at a suitable ratio. When the proportion of males producing sperm is low, an alternative is to fertilize as many eggs as possible with the available sperm. Hatching fertilized eggs for embryo or larval collection usually follows the aquaculture practice at proper density and water quality. Collection of oocytes/eggs or swimming larvae can be conducted by passing the suspension through a screen and resuspending in seawater at the required density for cryopreservation.

Quantification and standardization of germplasm density is an important step to ensure effective and efficient cryopreservation and is often overlooked by many researchers or users. A hemocytometer or similar cell-counting chambers or cell-counting instruments (spectrophotometer or flow cytometer) can be used to determine cell concentrations. Additionally, the quality of freshly collected germplasm needs to be examined.

4.2 Addition and equilibration of cells with one or more cryoprotective agents

Mixing cryoprotectant solutions with germplasm suspension is often carried out slowly to avoid osmotic damage to cells and at lowered temperatures (such as on ice) to reduce any toxic effects of the cryoprotectant chemicals. A range of cryoprotectants may be used: DMSO, ethylene glycol, and propylene glycol are commonly used with shellfish species at concentrations varying from about 5 – 15 percent (v/v) for most cell types. Besides the cryoprotectant, additional additives, such as sugars (trehalose or sucrose), other molecules (e.g., polyvinylpyrrolidone), metal chelator (EDTA), antioxidants (tempol or sodium pyruvate), or lipids (Drobnis *et al.*, 1993) have been studied incorporation with cryoprotectants to increase the post-thaw viability. The solvent for dissolving cryoprotectants and additives is made up of either seawater, distilled or deionized water, or saline solutions (often calcium-free) and the ratio of dilution of cells to cryoprotectant solution may also vary (typically from 1:1 to 1:9).

Equilibration time (from mixing with cryoprotectants to initiation of the cooling process) can be an important consideration for post-thaw survival. The time required for loading straws or cryovials with cell suspension needs to be factored in and should be standardized for cryobanking.

4.3 Cooling of germplasm samples

Following an equilibration period, cells are loaded into cryopreservation straws or vials and cooled to a frozen state before being plunged into liquid nitrogen for long-term storage. The cooling rates are sometimes dependent on the equipment available. Racks at determined distances over liquid nitrogen, Mr Frosty® cooling chambers, methanol/dry ice slurries, and programmable freezers are used. For example, a rack suspended 5 cm over liquid nitrogen in a Styrofoam cooler can provide a cooling rate of 11ºC/min (Paredes *et al.*, 2022), and different cooling rates can be achieved by adjusting different factors using a Styrofoam box (Huo *et al.*, 2022). "Mr Frosty" (Nalgene) is a freezing chamber with an isopropanol sleeve that is placed in a -80°C freezer, providing a nominal freezing rate of 1ºC/min in cryovials. Freezing in "dry shippers" charged with nitrogen vapor has been reported for oysters, providing freezing rates of 1–64°C/min, depending on the position in the chamber (Childress *et al.*, 2023). Open-source 3-D printed hardware can be used to access standardized equipment for sample cooling (Childress *et al.*, 2023; Hu *et al.*, 2017). Comparison of different chilling mechanisms for the Pacific oyster sperm cooling showed similar post-thaw viability (Adams *et al.*, 2004). The type and dimensions of the storage container (straws or cryovials) also play a role; a 0.25- or 0.5-ml plastic straw, with a high surface area to volume, will have a much faster cooling rate in a given cooling system than a cryovial.

Different germplasm types require different cooling rates. Generally, shellfish sperm seem tolerant of a relatively wide range of cooling rates and usually need faster cooling rates than eggs, embryos, or larvae. Eggs and larvae are cooled to an ice-seeding temperature (around -10 to -12°C) and then at rates ranging from 0.3 to 2.5°C per min to eutectic temperatures, typically around -35°C before samples are plunged into liquid nitrogen for storage.

4.4 Storage

Cryopreserved germplasm is usually stored in liquid nitrogen or vapor of liquid nitrogen in different-sized dewars or -150°C deep freezers. An inventory or database of cryopreservation samples is often managed for easy retrieval of samples. Importantly, emergency backup for the sample safety and sample inventory of cryopreserved samples should be planned as a priority.

4.5 Thawing

Thawing is generally carried out in a water bath or air at temperatures ranging from ambient to 75°C. Practitioners must remove the straws or cryovials promptly (for example immediately upon ice melting in the sample) to make sure that the cells are not exposed to a lethally high temperature.

4.6 Post-thaw sample use

After thawing, sperm, oocytes, and eggs need to be immediately used in fertilization while others such as embryos and larvae, can be ongrown. Some published protocols for various species and the expected outcome in terms of fertility and survival are provided in Annex 2.

Viability evaluation can be performed by thawing a single straw or vial before further samples are thawed. The fertilization and thawing procedures can be adjusted based on the results of the evaluation. For example, it may be necessary to remove the cryoprotectants completely if the cryoprotectants are deleterious to certain germplasm, or gradual dilution of the cryoprotectants (or a combination of the two) may be required to avoid osmotic shock.

To offset the lower quality of frozen gametes, it can be useful to: use relatively more sperm; increase the time of sperm exposure to the eggs; and/or increase the concentration of the sperm/egg mixture (Adams *et al.*, 2004; Yang *et al.*, 2012). Overall, these strategies are generally sufficient for use in selective breeding, where several thousand of larvae from each cross are needed to maintain a particular line. An adaptive management approach is to thaw a straw from a given individual or family and record the fertilization rate as indicated by survival at the stage of the first one or two cleavages. If the fertilization rate is too low to provide enough larvae, more straws can be thawed to fertilize more eggs. Luckily, shellfish eggs can be fertilized up to a few hours after release or sometimes even longer if kept cool, e.g., at 4°C.

5. Future directions and challenges

Cryopreserving sperm alone enables the male genes of a particular family or population to be banked and then to be recovered later. The cryopreservation of both male and female gametes from a given species allows complete control of parental crosses and back-crossing in breeding and restoration programs, however, oocyte/egg cryopreservation is more challenging than sperm cryopreservation and there is only one study that reports successful oocyte cryopreservation oocytes/eggs in shellfish (Adams *et al.*, 2009; Tervit *et al.*, 2005). Alternatively, whole-genome conservation may be obtained through the cryopreservation of embryos or larvae.

Current progress on larval cryopreservation of several bivalve species indicates that this may be a more promising pathway (Yang and Huo, 2022). It may be particularly important in the context of conservation efforts, where sperm banking alone will not suffice for the preservation of a species. However, for breeding program, embryo and larval cryopreservation is less useful than sperm cryopreservation because the embryos and larvae cannot be crossed immediately and must first be reared to maturity.

In the future, researchers should work closely with breeders and conservation managers to identify cryopreservation methods and approaches that meet the objectives of breeding and conservation programs. For example, whilst a high survival rate is always desirable, a method that gives moderate survival may be sufficient to achieve the objective of a breeder or a conservation manager who aims to recover a sufficient number of individuals to introduce or re-introduce specific genotypes into a breeding or conservation program.

BOX 2

The *Artemia* gene bank of the Asian Regional Artemia Reference Center

Sui Liying, Director, Asian Regional Artemia Reference Center, College of Marine and Environmental Sciences, Tianjin University of Science and Technology, Tianjin, China

The Asian Regional *Artemia* Reference Center (ARARC) at the College of Marine and Environmental Sciences, Tianjin University of Science and Technology in China is an Artemia gene bank established in cooperation with the Food and Agriculture Organization of the United Nations (FAO) in 2016. ARARC promotes and coordinates interdisciplinary and interregional *Artemia* research, provides a platform for information exchange on *Artemia*, and provides technical consultation and services to produce *Artemia* cysts and their use.

ARARC contains collections from 300 sites representing 50 *Artemia* populations, from different parts of the world. The collections include seven recognized bisexual *Artemia* species and many parthenogenetic populations (not assigned to species). The samples were collected from inland salt lakes and artisanal solar saltworks, including China, Central Asian countries, Russia, the United States of America, and Vietnam. Some of these samples (between 10 and 20) were contributed by the Artemia Reference Centre at the Gent University (Belgium). The earliest samples were obtained in 1990. The samples are vacuum-packed and kept in a freezer at -20 ºC.

ARARC maintains a database of its collection, including characteristics of the cysts; hatching performance and nutritional value (fatty acid profile, crude protein, and lipid content) of the resulting nauplii; and genetic identity based on mitochondrial DNA sequencing (cytochrome oxidase subunit 1 (COI) for species identification, and single-nucleotide polymorphism (SNP) fingerprinting for strain identification. The cyst materials and the information in the database are used for scientific studies such as on genetic diversity, germplasm evaluation, and genetic improvement (including the hybridization between natural and introduced *Artemia)* (Deji et al., 2021; Han et al., 2019; Han et al., 2021; Han et al., 2022). It also provides a repository of *Artemia* genetic resources that are currently threatened or may become so in the future.

The ARARC gene bank is vital for *Artemia* resource conservation and sustainable exploitation, for the efficient application of *Artemia* in aquaculture, and for scientific research. This last remains the primary focus of the gene bank and the material is mainly used by Chinese researchers. In the future, more demands from international organizations and companies are expected. However, ARARC is facing financial difficulties in supporting new collections, developing techniques for prolonged viability of the stored cysts, and for research using gene bank materials.

References

Deji, G.S., Zhang, C., Sui, L.Y. & Han, X.K. 2021. The complete mitochondrial genome of *Artemia salina* Leach, 1819 (Crustacea: Anostraca). *Mitochondrial DNA Part B-Resources*, 6(11): 3255-3256. https://doi.org/10.1080/23802359.2021.1992315

Han, X.K., Tashi, L., Sui, L.Y., Wang, G.S., Deji, G.S. & Zhang, C. 2022. The complete mitochondrial genome of *Artemia persimilis* Piccinelli and Prosdocimi, 1968 (Crustacea: Anostraca). *Mitochondrial DNA Part B-Resources*, 7(3): 464-465. https://doi.org/10.1080/23802359.2022.2036258

Han, X.K., Ren, Y.Z., Ouyang, X.M., Zhang, B. & Sui, L.Y. 2021. Construction of a high-density genetic linkage map and QTL mapping for sex and growth traits in *Artemia franciscana*. *Aquaculture*, 540. https://doi.org/10.1016/j.aquaculture.2021.736692

Han, X.K., Xu, R.Y., Zheng, Y.Y., Gao, M.R. & Sui, L.Y. 2019. Development of Est-Ssr markers and genetic diversity analysis among three *Artemia* species from different geographic populations. *Crustaceana*, 92(7): 841-851. https://doi.org/10.1163/15685403-00003916

References

Adams, S.L., Tervit, H.R., McGowan, L.T., Smith, J.F., Roberts, R.D., Salinas-Flores, L., Gale, S.L., Webb, S.C., Mullen, S.F., Critser, J.K. 2009. Towards cryopreservation of Greenshell™ mussel (*Perna canaliculus*) oocytes. *Cryobiology* 58(1): 69–74. https://doi.org/10.1016/j.cryobiol.2008.10.130

Adams, S.L., Smith, J.F., Roberts, R.D., Janke, A.R., Kaspar, H.F., Tervit, H.R., Pugh, P.A., Webb, S.C., King, N.G. 2004. Cryopreservation of sperm of the Pacific oyster (*Crassostrea gigas*): Development of a practical method for commercial spat production. *Aquaculture* 242(1–4): 271–282. https://doi.org/10.1016/j.aquaculture.2004.08.034

Aquino, J.I.L., Elliott, L., Zeng, C.S., Paris, D.B.B.P. 2022. Recent developments in male fertility evaluation, sperm cryopreservation and artificial fertilisation, and their potential application to decapod crustacean aquaculture. *Reviews in Aquaculture* 14(2): 848–889. https://doi.org/10.1111/raq.12627

Björndahl, L., Söderlund, I., Kvist, U. 2003. Evaluation of the one-step eosin-nigrosin staining technique for human sperm vitality assessment. *Human Reproduction* 18(4): 813–816. https://doi.org/10.1093/humrep/deg199

Broquard, C., Martinez, A.-S., Maurouard, E., Lamy, J.-B., Dégremont, L. 2020. Sex determination in the oyster *Crassostrea gigas* – A large longitudinal study of population sex ratios and individual sex changes. *Aquaculture* 515: 734555. https://doi.org/10.1016/j.aquaculture.2019.734555

Camara, M.D., Yen, S., Kaspar, H.F., Kesarcodi-Watson, A., King, N., Jeffs, A.G., Tremblay, L.A. 2017. Assessment of heat shock and laboratory virus challenges to selectively breed for ostreid herpesvirus 1 (OsHV-1) resistance in the Pacific oyster, *Crassostrea gigas*. *Aquaculture* 469: 50–58. https://doi.org/10.1016/j.aquaculture.2016.11.031

Campos, S., Troncoso, J., Paredes, E. 2021. Major challenges in cryopreservation of sea urchin eggs. *Cryobiology* 98: 1–4. https://doi.org/10.1016/j.cryobiol.2020.11.008

Childress, W.M., Liu, Y., Tiersch, T.R. 2023. Design, alpha testing, and beta testing of a 3-D printed open-hardware portable cryopreservation device for aquatic species. *Journal of Applied Aquaculture* 35(1): 213–236. https://doi.org/10.1080/10454438.2021.1955805

Choi, Y.H., Lee, J.Y., Chang, Y.J. 2008. The influence of developmental stages and protective additives on cryopreservation of surf clam (*Spisula sachalinensis*) larvae. *Journal of Environmental Biology* 29(4): 461–463. https://jeb.co.in/journal_issues/200807_jul08_spl/paper_08.pdf

Chow, S., Taki, Y., Ogasawara, Y. 1985. Cryopreservation of spermatophore of the freshwater shrimp, *Macrobrachium rosenbergii*. *Biological Bulletin* 168(3): 471–475. https://doi.org/10.2307/1541526

Dégremont, L., Nourry, M., Maurouard, E. 2015. Mass selection for survival and resistance to OsHV-1 infection in *Crassostrea gigas* spat in field conditions: Response to selection after four generations. *Aquaculture* 446: 111–121. https://doi.org/10.1016/j.aquaculture.2015.04.029

Delisle, L., Laroche, O., Hilton, Z., Burguin, J.F., Rolton, A., Berry, J., Pochon, X., Boudry, P., Vignier, J. 2022. Understanding the dynamics of POMS infection and the role of microbiota composition in the survival of Pacific oysters *Crassostrea gigas*. *Microbiology Spectrum* 10(6): 1–20. https://doi.org/10.1128/spectrum.01959-22

Divilov, K., Schoolfield, B., Morga, B., Dégremont, L., Burge, C.A., Cortez, D.M., Friedman, C.S., Fleener, G.B., Dumbauld, B.R., Langdon, C. 2019. First evaluation of resistance to both a California OsHV-1 variant and a French OsHV-1 microvariant in Pacific oysters. *BMC Genetics* 20(1): 96. https://doi.org/10.1186/s12863-019-0791-3

Diwan, A.D., Harke, S.N., Gopalkrishna, Panche, A.N. 2020. Cryobanking of fish and shellfish eggs, embryos and larvae: An overview. *Frontiers in Marine Science* 7: 00251. https://doi.org/10.3389/fmars.2020.00251

Diwan, A.D., Kandasami, K. 1997. Freezing of viable embryos and larvae of marine shrimp, *Penaeus semisulcatus* de Haan. *Aquaculture Research* 28(12): 947–950. https://doi.org/10.1046/j.1365-2109.1997.00926.x

Dong, Q., Huang, C., Eudeline, B., Allen, S.K., Tiersch, T.R. 2006. Systematic factor optimization for sperm cryopreservation of tetraploid Pacific oysters, *Crassostrea gigas*. *Theriogenology* 66(2): 387–403. https://doi.org/10.1016/j.theriogenology.2005.12.004

Drobnis, E.Z., Crowe, L.M., Berger, T., Anchordoguy, T.J., Overstreet, J.W., Crowe, J.H. 1993. Cold shock damage is due to lipid phase transitions in cell membranes – A demonstration using sperm as a model. *Journal of Experimental Zoology* 265(4): 432–437. https://doi.org/10.1002/jez.1402650413

FAO 2023. Proactive approach proved key to survival for the Australian Pacific oyster industry – Genetics aquaculture. A case study. https://doi.org/10.4060/cc4389en

Foighil, D.O. 1989. Role of spermatozeugmata in the spawning ecology of the brooding oyster *Ostrea edulis*. *Gamete Research* 24(2): 219–228. https://doi.org/10.1002/mrd.1120240209

Galtsoff, P.S. 1964. The American oyster, *Crassostrea virginica* Gmelin, Fishery Bulletin Volume 64. Washington, D.C., United States Government Printing Office.

Guo, J.H., Weng, C.F. 2020. Current status and prospects of cryopreservation in aquatic crustaceans and other invertebrates. *Journal of Crustacean Biology* 40(4): 343–350. https://doi.org/10.1093/jcbiol/ruaa034

Guo, X., Wang, Y., Xu, Z., Yang, H. 2009. Chromosome set manipulation in shellfish. In: G. Burnell and G. Allen, eds. *New Technologies in Aquaculture: Improving Production Efficiency, Quality and Environmental Management*, pp. 165–194. Abington, Cambridge, Woodhead Publishing Limited.

Gwo, J.C. 1995. Cryopreservation of oyster (*Crassostrea gigas*) embryos. *Theriogenology* 43(7): 1163–1174. https://doi.org/10.1016/0093-691X(95)00088-P

Harris, L.G., Eddy, S.D. 2015. Sea urchin ecology and biology. In: *Echinoderm Aquaculture*, pp. 1–24. Maitland, FL, John Wiley and Sons, Inc.

Hassan, M.M., Qin, J., Li, X. 2015. Sperm cryopreservation in oysters: A review of its current status and potentials for future application in aquaculture. *Aquaculture* 438: 24–32. https://doi.org/10.1016/j.aquaculture.2014.12.037

Heller, J. 1993. Hermaphroditism in molluscs. *Biological Journal of the Linnean Society* 48(1): 19–42. https://doi.org/10.1111/j.1095-8312.1993.tb00874.x

Helm, M.M., Bourne, N., Lovatelli, A. 2004. *Hatchery Culture of Bivalves. A Practical Manual*. FAO Fisheries Technical Paper No. 471. Rome, FAO.

Horvath, A., Bubalo, A., Cucevic, A., Bartulovic, V., Kotrik, L., Urbanyi, B., Glamuzina, B. 2012. Cryopreservation of sperm and larvae of the European flat oyster (*Ostrea edulis*). *Journal of Applied Ichthyology* 28(6): 948–951. https://doi.org/10.1111/jai.12066

Hu, E., Childress, W., Tiersch, T.R. 2017. 3-D printing provides a novel approach for standardization and reproducibility of freezing devices. *Cryobiology* 76: 34–40. https://doi.org/10.1016/j.cryobiol.2017.03.010

Huo, Y., Yee, J.C., Yang, H. 2022. Development of a cost-effective and portable aeration freezing system for germplasm cryopreservation for eastern oysters and other aquatic species. *Aquaculture Research* 53(2): 453–466. https://doi.org/10.1111/are.15587

Lavens, P., Sorgeloos, P. 1987. *Artemia Research and its Applications*. In: Ecology, Culturing, Use in Aquaculture, p. 556. Wetteren, Belgium, Universa Press.

Liu, Y., Gluis, M., Miller-Ezzy, P., Qin, J., Han, J., Zhan, X., Li, X. 2020. Development of a programmable freezing technique for larval cryopreservation in *Mytilus galloprovincialis*. *Aquaculture* 516: 734554. https://doi.org/10.1016/j.aquaculture.2019.734554

Liu, Y., Li, X., Robinson, N., Qin, J. 2015. Sperm cryopreservation in marine mollusks: A review. *Aquaculture International* 23(6): 1505–1524. https://doi.org/10.1007/s10499-015-9900-0

Martinez-Paramo, S., Horvath, Á., Labbé, C., Zhang, T.T., Robles, V., Herráez, P., Suquet, M., Adams, S., Viveiros, A., Tiersch, T.R., Cabrita, E. 2017. Cryobanking of aquatic species. *Aquaculture* 472: 156–177. https://doi.org/10.1016/j.aquaculture.2016.05.042

Morales-Ueno, K., Paniagua-Chávez, C.G. 2020. Sperm cryopreservation in crustaceans. In: J. Betsy and S. Kumar, eds. *Cryopreservation of Fish Gametes*, pp. 211–238. Singapore, Springer Singapore.

Naidenko, T. 1997. Cryopreservation of *Crassostrea gigas* oocytes, embryos and larvae using antioxidant echinochrome A and antifreeze protein AFP1. Cryoletters, 18(6): 375–382.

Nimrat, S., Sonwat, S., Matharatanukul, B., Boonthai, T. and Vuthiphandchai, V. 2022. Chilled and cryopreserved spermatophores of banana shrimp (*Fenneropenaeus merguiensis*): Effects of antibiotics on sperm quality and bacterial abundance. Aquaculture, 560: 738551. https://doi.org/10.1016/j.aquaculture.2022.738551

Nimrat, S., Noppakun, S., Sripuak, K., Boonthai, T. and Vuthiphandchai, V. 2020. Cryopreservation of banana shrimp (*Fenneropenaeus merguiensis*) spermatophores with supplementation of medicinal plant extracts: Development of a programmable controlled-rate method and a practical method. Aquaculture, 515: 734537. https://doi.org/10.1016/j.aquaculture.2019.734537

Nimrat, S., Bart, A.N., Keatsaksit, A. and Vuthiphandchai, V. 2008. Microbial flora of spermatophores from black tiger shrimp (*Penaeus monodon*) declines over long-term cryostorage. Aquaculture, 274(2): 247–253. https://doi.org/10.1016/j.aquaculture.2007.11.018

Paniagua-Chavez, C.G., Buchanan, J.T., Supan, J.E. and Tiersch, T.R. 1998. Settlement and growth of eastern oysters produced from cryopreserved larvae. Cryo-Letters, 19(5): 283–292.

Paredes, E., Campos, S., Lago, A., Bueno, T., Constensoux, J. and Costas, D. 2022. Handling, reproducing and cryopreserving five European sea urchins (Echinodermata, Klein, 1778) for biodiversity conservation purposes. Animals, 12(22): 3161. https://doi.org/10.3390/ani12223161

Paredes, E., Adams, S.L. and Vignier, J. 2019. Cryopreservation of sea urchin sperm and early life stages. Echinoderms, Pt A, 150: 47–69. https://doi.org/10.1016/bs.mcb.2018.11.008

Paredes, E., Bellas, J. and Adams, S.L. 2013. Comparative cryopreservation study of trochophore larvae from two species of bivalves: Pacific oyster (*Crassostrea gigas*) and blue mussel (*Mytilus galloproviricialis*). Cryobiology, 67(3): 274–279. https://doi.org/10.1016/j.cryobiol.2013.08.007

Potts, R.W.A., Gutierrez, A.P., Cortés-Araya, Y., Houston, R.D. and Bean, T.P. 2020. Developments in marine invertebrate primary culture reveal novel cell morphologies in the model bivalve. Peerj, 8: e9180. https://doi.org/10.7717/peerj.9180

Rakbanjong, N., Okutsu, T., Chotigeat, W., Songnui, A. and Wonglapsuwan, M. 2021. Cryopreservation of germ cells of banana shrimp (*Fenneropenaeus merguiensis*) and black tiger shrimp (*Penaeus monodon*). Marine Biotechnology, 23(4): 590–601. https://doi.org/10.1007/s10126-021-10048-1

Rusk, A.B., Alfaro, A.C., Young, T., Watts, E. and Adams, S.L. 2017. Investigation of early mussel development using histology, SEM imaging, immunochemistry and confocal microscopy. Marine Biology Research, 13(3): 314–329. https://doi.org/10.1080/17451000.2016.1257812

Selvakumar, N., Dhanasekar, K., Carmel, D.B. and Munuswamy, N. 2018. Retrieval and cryopreservation of sperm in spermatophores from cadaveric Indian white shrimp, *Fenneropenaeus indicus* (H. Milne Edwards, 1837). Animal Reproduction Science, 192: 185–192. https://doi.org/10.1016/j.anireprosci.2018.03.008

Smith, J.F., Pugh, P.A., Tervit, H.R., Roberts, R.D., Janke, A.R., Kaspar, H.F. and Adams, S.L. 2001. Cryopreservation of shellfish sperm, eggs and embryos. Proceedings of the New Zealand Society of Animal Production, 61: 31–34.

Soler, J., Wantzen, K.M., Jugé, P. and Araujo, R. 2018. Brooding and glochidia release in *Margaritifera auricularia* (Spengler, 1793) (Unionoida: Margaritiferidae). Journal of Molluscan Studies, 84: 182–189. https://doi.org/10.1093/mollus/eyy008

Subramoniam, T. 1993. Spermatophores and sperm transfer in marine crustaceans. Advances in Marine Biology, Vol 29, 29: 129–214. https://doi.org/10.1016/S0065-2881(08)60131-5

Suquet, M., Labbe, C., Puyo, S., Mingant, C., Quittet, B., Boulais, M., Queau, I., Ratiskol, D., Diss, B. and Haffray, P. 2014. Survival, growth and reproduction of cryopreserved larvae from a marine invertebrate, the Pacific oyster *Crassostrea gigas*. PLOS One, 9(4): e93486.

https://doi.org/10.1371/journal.pone.0093486**Symonds, J.E**. 2018. New Zealand aquaculture selective breeding: From theory to industry application for three flagship species. In: Proceedings of the World Congress on Genetics Applied to Livestock Production, p. 1035.**Tervit, H.R., Adams, S.L., Roberts, R.D., McGowan, L.T., Pugh, P.A., Smith, J.F., Janke, A.R.** 2005. Successful cryopreservation of Pacific oyster (*Crassostrea gigas*) oocytes. *Cryobiology* 51(2): 142–151. https://doi.org/10.1016/j.cryobiol.2005.06.001

Uberti, M.F., Vieira, F.D., Salencia, H.R., Vieira, G.D., Vinatea, L.A. 2014. Assessment of viability of sperm cells of *Litopenaeus vannamei* on cryopreservation. *Brazilian Archives of Biology and Technology* 57(3): 374–380. https://doi.org/10.1590/S1516-89132014005000013

Usuki, H., Hamaguchi, M., Ishioka, H. 2002. Effects of developmental stage, seawater concentration and rearing temperature on cryopreservation of Pacific oyster *Crassostrea gigas* larvae. *Fisheries Science* 68(4): 757–762. https://doi.org/10.1046/j.1444-2906.2002.00490.x

Vikhrev, I.V., Makhrov, A.A., Artamonova, V.S., Ermolenko, A.V., Gofarov, M.Y., Kabakov, M.B., Kondakov, A.V., Chukhchin, D.G., Lyubas, A.A., Bolotov, I.N. 2019. Fish hosts, glochidia features and life cycle of the endemic freshwater pearl mussel *Margaritifera dahurica* from the Amur basin. *Scientific Reports* 9: 8300. https://doi.org/10.1038/s41598-019-44752-9

Vogeler, S., Bean, T.P., Lyons, B.P., Galloway, T.S. 2016. Dynamics of nuclear receptor gene expression during Pacific oyster development. *BMC Developmental Biology* 16: 33. https://doi.org/10.1186/s12861-016-0129-6

Waller, D.L., Lasee, B.A. 1997. External morphology of spermatozoa and spermatozeugmata of the freshwater mussel *Truncilla truncata* (Mollusca: Bivalvia: Unionidae). *American Midland Naturalist* 138(1): 220–223. https://doi.org/10.2307/2426669

Yang, H., Huo, Y.Z. 2022. Review of molluscan larval cryopreservation and application to germplasm cryobanking and commercial seed production. *Aquaculture* 547: 737491. https://doi.org/10.1016/j.aquaculture.2021.737491

Yang, H., Huo, Y., Yee, J.C., Rikard, S., Walton, W.C., Saillant, E. 2021. Sperm repository for a breeding program of the eastern oyster *Crassostrea virginica*: Sample collection, processing, cryopreservation, and data management plan. *Animals* 11(10): 2836. https://doi.org/10.3390/ani11102836

Yang, H. 2017. Application of germplasm preservation in breeding programs for molluscan shellfish aquaculture and restoration. *Bulletin of Japan Fisheries Research and Education Agency* 45: 15–20. www.fra.affrc.go.jp/bulletin/bull/bull45/45-0502.pdf

Yang, H., Wang, Y., Guo, X., Tiersch, T.R. 2015. Production of inbred larvae through self-fertilization using oocytes and cryopreserved sperm from the same individuals after sex reversal in eastern oyster *Crassostrea virginica*. *Aquaculture Research* 46(9): 2153–2165. https://doi.org/10.1111/are.12371

Yang, H., Hu, E., Cuevas-Uribe, R., Supan, J., Guo, X., Tiersch, T.R. 2012. High-throughput sperm cryopreservation of eastern oyster *Crassostrea virginica*. *Aquaculture* 344: 223–230. https://doi.org/10.1016/j.aquaculture.2012.03.018

Zieritz, A., Bogan, A.E., Froufe, E., Klishko, O., Kondo, T., Kovitvadhi, U., Kovitvadhi, S., Lee, J.H., Lopes-Lima, M., Pfeiffer, J.M., Sousa, R., Van Do, T., Vikhrev, I., Zanatta, D.T. 2018. Diversity, biogeography and conservation of freshwater mussels (Bivalvia: Unionida) in East and Southeast Asia. *Hydrobiologia* 810(1): 29–44. https://doi.org/10.1007/s10750-017-3104-8

Chapter 7. *Ex situ in vitro* conservation of algae

Cecilia Rad-Menéndez, Culture Collection of Algae and Protozoa (CCAP), The Scottish Association for Marine Science, Oban, United Kingdom
Michael Ross, Culture Collection of Algae and Protozoa (CCAP), The Scottish Association for Marine Science, Oban, United Kingdom
Michael W. Lomas, National Center for Marine Algae and Microbiota, Bigelow Laboratory for Ocean Sciences, Bigelow, United State of America

Introduction

Algae are a polyphyletic group of organisms that contain single cell microalgae and multicellular macroalgae. Microalgae are a diverse group of eukaryotic unicellular organisms, mostly photosynthetic, that cover a wide range of phylogenetic positions and can be found in marine, freshwater and terrestrial environments. They range in size from a few micrometres to a few hundred micrometres; some species, although unicellular, can exist as filaments, chains, or colonies. Macroalgae are eukaryotic multicellular photosynthetic organisms that can be found in marine and freshwater environments. They vary in size from a few millimetres to around 60 metres (Schiel and Foster, 2015). Both microalgae and macroalgae have a broad spatial and biological diversity and complex life cycles that feature sexual and asexual stages. Furthermore, this diversity is physically expressed across a multitude of morphologies, and a range of cell types (e.g., flagellated gametes and spores), tissues and biochemicals synthesized.

Algae improve the lives of many people, often without their awareness. For example, macroalgae that support natural fisheries and *in situ* aquaculture are worth many billions of dollars, and are a source of protein for millions worldwide (FAO, 2012, 2022). Algae cultivated on land are increasingly used as additives in livestock feed formulations to provide a sustainable non-competing source of proteins, essential fatty acids and oils and natural colorants, and are also used as biostimulants for terrestrial agricultural crops (Colla and Rouphael, 2020). Furthermore, algae are increasingly investigated as a potential solution to many societal needs, such as sequestering carbon dioxide through blue-carbon storage (Krause-Jensen *et al.*, 2018), amending livestock feed to reduce enteric methane production (Roque *et al.*, 2019), remediating domestic waste streams (Cole *et al.*, 2016; Li *et al.*, 2019) and providing natural products for industrial applications (Chu, 2012; Raposa *et al.*, 2013). Algae are also being used as cell factories for the sustainable production of plastic products (Dang *et al.*, 2022). All of these uses are predicated on access to well-curated and robust algal germplasm collections, as such, *ex situ in vitro* conservation is a critical tool for the management and sustainability of collections (National Academies of Sciences, 2020).

Algal culture collections are central to the long-term maintenance and preservation of well-characterized cultures and reference strains with well curated associated metadata (Day *et al*., 1999). The importance of these organisms for industry, aquaculture, and biodiversity conservation creates a need for their *ex situ* preservation and highlights the importance of algal culture. Several algal culture collections have in-house cryopreservation facilities where the cryopreserved holdings are kept, usually in cryostats filled with liquid nitrogen, or in ultra-cold freezers. Cryopreservation helps reduce labor, operational costs, and consumables

requisite for algal cultures maintained by serial transfer. Furthermore, cryopreservation also reduces other risks associated with serial transfer, including contamination, genetic drift and culture loss.

A list of worldwide, publicly accessible, algal culture collections that curate, maintain, and distribute some of the wide natural diversity of live and preserved marine and freshwater algae strains, both microalgae and macroalgae, are listed in Table 3**.** This table includes links to their websites, where information on cryopreservation and cryopreserved strains can be obtained. Algal culture collections are cross-cutting and facilitate ecological, evolutionary, and biotechnological science, including: i) basic scientific research on taxonomy, evolution and ecological relationships (Brawley *et al.*, 2017; Caron *et al.*, 2016; Sexton and Lomas, 2018); ii) translation of basic scientific research for industrial applications and commercialization, including genetic enhancement for improved biomass or product synthesis; and iii) conservation of biological diversity (especially macroalgae) threatened by climate change and other anthropic stressors (Wade *et al.*, 2020).

Table 3. Worldwide algae culture collections that are publicly accessible. Approximate number of strains represent total holdings, both micro- and macroalgae, with the number of strains relevant to aquaculture in parentheses.

Acronym	Collection Name	Country	Approximate No. of strains	Website/other
ANACC	Australian National Algae Culture Collection	Australia	1000	www.csiro.au/en/Research/Collections/ANACC
BACA	Azorean Bank of Algae and Cyanobacteria	Azores (Portugal)	780	http://cibio.uac.pt/en/baca
BCCM/DCG	Belgian Coordinated Collections of Microorganisms: Microalgae	Belgium	434	https://bccm.belspo.be/catalogues/catalogue-search?collection=DCG
BCCM/ULC	Belgian Co-ordinated Collections of Microorganisms: Cyanobacteria	Belgium	221	https://bccm.belspo.be/catalogues/catalogue-search?collection=ULC
BMAK	Banco de Microorganismos Aidar and Kutner (IOUSP)	Brazil	230	http://bmak.io.usp.br/bmak/algae/
BCMD	Freshwater Microalgae Collection Cultures	Brazil	35	
CCMA-UFSCar	The Culture Collection of Freshwater Microalgae from the Federal University of Sao Carlos	Brazil	790	https://zenodo.org/record/6619002
CCCM	Canadian Center for the Culture of Microorganisms	Canada	300	http://cccm.botany.ubc.ca/
CPCC	Canadian Phycological Culture Center for Algae, Cyanobacteria and Lemna at the University of Waterloo	Canada	400	https://uwaterloo.ca/canadian-phycological-culture-centre/
FACHB	Freshwater Algae Culture Collection at the Institute of Hydrobiology	China	2000	http://algae.ihb.ac.cn/Default.aspx

CCTCC	China Center for Type Culture Collection	China	214	http://cctcc.whu.edu.cn/
CAUP	Culture Collection of Algae of Charles University of Prague	Czech Republic	250	https://botany.natur.cuni.cz/algo/caup.html
CCALA	Culture Collection of Autotrophic Organisms	Czech Republic	844	https://ccala.butbn.cas.cz/index.php
HAMBI/UHCC	Biological Resource Centre HAMBI – Cyanobacteria	Finland	919	www.helsinki.fi/en/infrastructures/biodiversity-collections/infrastructures/microbial-domain-biological-resource-centre-hambi
RCC	Roscoff Culture Collection	France	5676	http://roscoff-culture-collection.org/
PCC	Pasteur Culture Collection of Cyanobacteria	France	475	https://webext.pasteur.fr/cyanobacteria/
Algobank Caen	Collection de Cultures de Microalgues – Universite de Caen Normandie	France	259	www.unicaen.fr/algobank//accueil/
SAG	Sammlung fur Algenkulturen	Germany	2315	www.uni-goettingen.de/en/184982.html
CCAC	Central Collection of Algal Cultures; previously Cologne Algal Culture Collection	Germany	2060	www.uni-due.de/biology/ccac/
CCCryo	Culture Collection of Cryophilic Algae	Germany	530	http://cccryo.fraunhofer.de/web/infos/welcome/
MZCH-SVCK	Microalgae and Zygnematophyceae Collection Hamburg Sammlung von Conjugaten-Kulturen	Germany	591	www.mzch-svck.uni-hamburg.de/
DSMZ	Leibniz Institute DSMZ – Germany Collection of Microorganisms and Cell Cultures	Germany	118	www.dsmz.de/dsmz
TAU-MAC	Aristotle University of Thessaloniki Microalgae and Cyanobacteria Culture Collection, CyanoLab	Greece	100	https://cyanolab.bio.auth.gr/?page_id=53
MACC	Mosonmagyarovar Algal Culture Collection	Hungary	970	https://plantbio.sze.hu/en_GB/mosonmagyarovar-algal-culture-collection
VBCCA	Visva-Bharati Culture Collection of Algae	India	50	
BDU	National Facility for Marine Cyanobacteria	India	407	http://nfmc.bdu.ac.in/germplasm.html
GCC	Global Collection of Cyanobacteria	India	150	
NCIM	National Collection of Industrial Microorganisms	India	28	www.ncl-india.org/files/ncim/Default.aspx
NAIMCC	National Agriculturally Important Microbial Culture Collection	India	356	https://nbaim.icar.gov.in/naimcc/

CCATM	Culture Collection of Algae at Tarbiat Modares University	Iran	22	
INAC	The Iranian National Algae Culture Collection	Iran	98	www.inacc.ir/
IBRC	Iranian Biological Resource Center	Iran	175	www.acm-mrc.asia/documents/m/ibrc/IBRC%20Product%20Catalog.pdf
NIES	NIES Microbial Culture Collection	Japan	3087	https://mcc.nies.go.jp/
KU-MACC	Kobe University Macroalgal Culture Collection	Japan	1114	https://ku-macc.nbrp.jp/
NBRC	NITE Biological Resource Center	Japan	380	www.nite.go.jp/en/nbrc/cultures/nbrc/index.html
NMCCC	North Macedonian Culture Collection of Cyanobacteria	Macedonia	12	
MACC	Malaysia Algae Culture Collection	Malaysia	52	
UMACC	University of Malaya Algae Culture Collection	Malaysia	280	
CDBB	Coleccion Nacional de Cepas Microbianas y Cultivos Celulares	Mexico		http://cdbb.cinvestav.mx/cdbb/index.html
CM-CNRG	Coleccion de Microorganismos del Centro Nacional de Recursos Geneticos	Mexico	30	http://cmcnrg.inifap.gob.mx/acerca.html
MCC-MN	Microalgae Culture Collection	Mongolia	100	
CICCM	Cawthron Institute Culture Collection of Microalgae	New Zealand	600	http://cultures.cawthron.org.nz/
NORCCA	Norwegian Culture Collection of Algae at NIVA, incorporating cultures from the former SCCAP	Norway	1712	https://niva-cca.no/
CCBA	Culture Collection of Baltic Algae	Poland	200	https://ccba.ug.edu.pl/pages/en/home.php
SZCZ	Szczecin Diatom Culture Collection	Poland	1800	http://geocentrum.usz.edu.pl/en/szczecin-diatom-culture-collection-szcz/
ACOI	Coimbra Collection of Algae	Portugal	3000	http://acoi.ci.uc.pt/
KMMCC	Korea Marine Microalgae Culture Center	Republic of Korea	2219	www.e-algae.org/upload/pdf/algae-2015-30-S-S1.pdf
IPPAS Culture Collection	Institute of Plant Physiology, Russian Academy of Sciences	Russia	300	http://en.cellreg.org/Collection-IPPAS.php
ACCS	Algae Culture Collection of Siberia	Russia		

BCAC	Bashkortostan Collection of Algae and Cyanobacteria	Russia	1220	
CALU	Collection of Algae St. Petersburg (Leningrad) State University	Russia	1000	https://researchpark.spbu.ru/en/collection-ccem-eng/1930-ccem-kollekciya-calu-eng
IBSS	Collection of Living Cultures of Plankton Microalgae	Russia	60	
MSUALGDMA	Collection of Microalgae, Moscow State University	Russia	67	
PGC	Peterhof Genetic Collection of Algae	Russia	700	
SYKOA	Strain Collection of Microalgae and Cyanobacteria from Northern and Arctic Regions in the Institute of Biology of Komi Scientific Center	Russia	400	https://ib.komisc.ru/sykoa/eng/home/
BMCC	Basque Microalgae Culture Collection	Spain	600	www.ehu.eus/en/web/bmcc/collection
BEA	Banco Espanol de Algas	Spain	895	https://marinebiotechnology.org/es/
CCVIEO		Spain	400	https://vgohab.com/coleccion-de-cultivos/
CCOS	The Culture Collection of Switzerland	Switzerland		www.ccos.ch/
BCRC	Bioresource Collection and Resource Center	Taiwan	62	https://catalog.bcrc.firdi.org.tw/
TISTR Culture Collection	Thailand Institute of Scientific and Technological Research	Thailand	430	www.tistr.or.th/tistr_culture/index.php
ASYA	Antarctic Microalgae Research	Turkey	60	
EGE-MACC	EGE – Microalgae Culture Collection	Turkey	43	
MAKU-MACC	Burdur Mehmet Akif Ersoy University Microalgae Culture Collection	Turkey	85	
Soley	Microalgae Culture Collection	Turkey		
CCAP	Culture Collection of Algae and Protozoa	UK	3116	www.ccap.ac.uk/
ACKU	Culture Collection of Algae at Kyiv University	Ukraine	1023	
CAMU	Collection of Algae at Bohdan Khmelnytskyi Melitopol State Pedagogical University	Ukraine	100	
CWU-MACC	Herbarium of Kharkiv University (CWU) – Microalgae Cultures Collection	Ukraine	22	

UTEX	Culture Collection of Algae at the University of Texas at Austin	USA	3000	https://utex.org/
NCMA	National Center for Marine Algae and Microbiota; previously CCMP	USA	4000 (1300)	https://ncma.bigelow.org/
ATCC	American Type Culture Collection	USA	2000	www.lgcstandards-atcc.org/
ARC	Algal Resources Collection	USA	412	www.algalresourcescollection.com/
Antarctic Protist Culture Collection		USA	70	www.whoi.edu/science/B/protists/classification.html
***Chlamydomonas* Resource Center**		USA	3630	www.chlamycollection.org/

Algae preservation methodologies

Several different approaches are currently used for long-term maintenance and preservation of algal tissues, with different methods specific to micro- and macroalgae. Appropriate methods tend to be group specific. There is no universal preservation methodology that is unique to algae strains relevant to aquaculture.

Agar slants and plates or liquid media

Traditionally, algal cultures have been maintained by serial subculture under specific environmental conditions (Pringsheim, 1946). Depending upon the species, microalgae can be cultivated asexually in both liquid and solid (agar) media and are generally maintained by transferring a small volume of inoculum into a sterilized growth medium. In the case of macroalgae, haploid gametophytes (e.g., kelps) and asexually propagated strains (e.g., *Ulva* spp. and *Gracilaria* spp.) can be maintained in liquid media, in some cases for weeks to months, under the required conditions and then divided by fragmentation when transferred to fresh sterilized medium. The frequency of the transfer, the composition of the medium and environmental growth variables are determined by the specific growth and requirements of each strain. However, continuous maintenance of actively growing algal strains over long periods of time requires specialized skills due to the wide diversity of the group and is time-consuming and costly when large numbers of cultures are involved (Day, J.G., 2007; Lorenz *et al.*, 2005). Furthermore, algal strains that grow *in vitro* are not immune to problems. For instance, serial transfer methods are prone to culture loss due to biological failure, whether it be due to lack of robust growth under stable conditions, contamination, or human error.

Lyophilisation

This method is preferred for bacterial and fungal germplasms, but it has not been proven to be appropriate for long-term algal cell culture maintenance due to very low initial viability (<0.001 percent; McGrath *et al.*, 1978) and further loss over time in storage (Day *et al.*, 1987). Some success has been recorded with a small group of green microalgae (Chlorococcales) using protective chemicals and complex equipment (Malik, 1993). This approach has only been used for a small number of organisms and is unlikely to be satisfactory for the longer-term preservation of more fragile organisms due to its intrusiveness, with loss of motility observed immediately post-drying and no subsequent recovery (Day *et al.*, 1997).

Cryopreservation

To avoid the limitations of continuous culturing, cryopreservation has been extensively used in algal culture collections to facilitate the long-term storage of microalgae and macroalgae by reducing time, resources, cost and several risks associated with the manipulation of cultures, serial cultivation of living tissues and genetic drift (Day *et al*., 2005; Day *et al*., 2007). Several cryopreservation protocols have been published for a variety of microalgae (Day and Brand, 2005; Day and Deville, 1995; Day and Harding, 2008; Day *et al.*, 1997; Day *et al*., 2007; Fernandes *et al*., 2019; Lorenz *et al*., 2005; Paredes *et al.*, 2021 and Taylor and Fletcher, 1998) and macroalgae (Choi *et al*., 2013; Lee and Nam 2016; Taylor and Fletcher 1999a and b; Visch *et al*., 2019; Wang *et al*., 2005, 2011; Yang *et al*., 2021; Zhang *et al*., 2008). However, all the methods have variations on the same basic steps: 1) addition of an appropriate cryoprotective agent; 2) cooling from room temperature to about -50°C followed by immersion in liquid nitrogen; 3) thawing and recovery and 4) viability assessment. Most of the procedures result from empirical studies with large variability in results and reporting (Taylor and Fletcher, 1998).

At present, in contrast to well-established cryopreservation protocols for agricultural species, there are no high-throughput, standardized methods with industrial quality controls for algal cryopreservation. Development of cryopreservation protocols and evaluation of their success have been hampered by lack of standardization of the wide range of variables including cell physiological condition (e.g. growth stage, cell size, biochemical composition), freezing rates and temperatures, cryoprotectant type, concentration, and exposure times. Currently, fewer than half of the global microalgal and macroalgal strains held in culture collections have been cryopreserved.

Cryoprotectant type and concentration

The success of a cryopreservation protocol usually depends on the use of: healthy, either in late log-phase or early stationary phase of growth cultures, and colligative chemical cryoprotectants that penetrate the cell and prevent concentration of solutes in its interior (Mazur, 1970) while decreasing the freezing-point of intracellular water (Franks, 1985) thus avoiding cellular damage. However, colligative cryoprotectants can be toxic (Fleck, 1998), so experimentation is needed to determine the type and concentration of cryoprotectant for each cell type and taxon, that is effective but non-toxic. Still the most commonly used cryoprotectants for microalgae are DMSO, methanol and glycerol (Meryman, 1966, Fleck *et al.*, 1996, Taylor and Fletcher, 1998, Visch *et al.*, 2019). These were originally chosen because of their fast penetration of the cell membrane and their relative ease of removal after thawing. A standard method commonly employed in culture collections for a wide range of microalgae involves use of the cryoprotectant to a final concentration of 5-10 percent (*v/v*) and incubating for 15 minutes at the same temperature used to grow the culture (Day and Brand, 2005).

A recent review by Yang *et al.* (2021) provides a comprehensive overview of protocols for the cryopreservation of macroalgae using different life stages, such as haploid gametophytes, diploid sporophytes, gametes and spores. While DMSO alone at 5–10 percent has been used to cryopreserve macroalgae, it is much more common to combine DMSO with different sugars for successful cryopreservation.

The use of these cryoprotectants is a classic and ubiquitously employed approach for algal tissue. While optimization of procedures is now occurring on a species-specific basis (Kapoore *et al.*, 2019, Visch, *et al.*, 2019, Yee and Yang, 2023), this tailored approach will likely limit the adoption of a broad, standardized, method to be employed by diverse algal collections and at a larger scale.

Cryopreservation and recovery[10]

Once mixed with the cryoprotective agent, the equilibrated mixture of the culture and cryoprotectant is added to cryovials and inserted into a cooling device. A routinely used cooling program for microalgae would be 1°C min^{-1} to -40 to -50°C; holding there for 10–30 minutes, followed by plunging into liquid nitrogen for long-term cryogenic storage (Day and Brand, 2005; Day *et al*., 2007; Paredes *et al*., 2021). While the cooling rate and hold temperature is the same for macroalgae, holding time can vary significantly depending on the species (Visch *et al*., 2019, Yang *et al*., 2021).

To achieve reproducible rates of cooling, different cooling devices can be used. These fall into two categories:

1. *Passive freezing*
   Passive freezing units, such as "Mr Frosty" (NalgeneTM Cryo 1°C Freezing container Cat. No 5100–001), are relatively inexpensive and provide reproducible cooling rates. They work by placing cryovials into a chilled reservoir of isopropyl alcohol which is then inserted into a -80°C freezer. The chamber cools at approximately -1°C min^{-1} over a temperature range of 0°C to -50°C after which the frozen vials are quickly plunged into liquid nitrogen (Day and Brand, 2005).

2. *Controlled cooling-rate freezers*
   Certain algal taxa require a more carefully controlled cooling rate or a complex cooling pattern that cannot be achieved using passive freezing chambers. Whilst there are similarities between both methods, the controlled cooler allows for finer temperature control. This can be achieved by direct injection of liquid nitrogen to a cooling chamber, rather than employing an isopropyl bath. Several commercial controlled-cooling-rate freezers (e.g., Planer, Biotronics, CryoMed, Thermo Electron Corporation) can be programmed to produce a wide range of customized cooling protocols. As with passive freezing devices, the frozen cryovials must be transported rapidly to permanent storage once the cooling program has ended (Day and Brand, 2005).

Thawing and recovery

Samples of both micro and macroalgae are usually thawed by immersion in a preheated water-bath and removed when visible ice crystals have melted. There have been a few studies focusing on the effect of thawing temperature and, in some species, a rapid thaw correlates with higher viability (Cañavate and Lubián, 1997). In general, 40°C has been successful across a wide range of algal taxa. However, the National Center for Marine Algae and Microbiota (NCMA)[11] found greater viability when thawing at 30°C rather than at 40°C.

Following thawing, it is recommended to immediately pelletize the cell culture in a centrifuge, aspirate the cryoprotective agent and transfer to appropriate fresh sterilized culture medium, which will allow cryoprotectants to leave the cell (Day and Brand, 2005) and allow the cell to begin growth. The thawed samples are initially stored in low light to reduce the effect of light-induced stresses/metabolic uncoupling before returning to standard growth conditions.

[10] Owing to the enormous variety in algal biodiversity, life cycles, and cultivation variables, the main steps and parameters reported in this section should be considered as of general success, and could be considered as a good starting point, rather than as optimized methodologies. Annex 3 reports specific protocols for the main aquaculture micro- and macroalgae species.

[11] https://ncma.bigelow.org/

Viability assessment

Post-thaw viability assessment is most often based upon survival; however, viability methods may vary. For example, for microalgae and macroalgal gametophytes, a suspension of cells is cryopreserved, and the viability assessed post-thaw using vital stains or by measuring increases in cell number (Day and DeVille, 1995). For each strain cryopreserved, success is often defined as when the culture grows back to the average cell density it was at when initial cryopreservation was conducted. The time between thaw and reaching the cell density threshold is called the "grow-back time" and can be considerable. The use of fluorescent membrane integrity stains may provide a more rapid assessment of potential success on the core assumption that cells with non-compromised membranes will ultimately grow back. For asexually propagated macroalgae (e.g., green algae, and some red algae), sections of whole tissue are often cryopreserved and vital stains are most commonly used to assess success (Yang *et al.*, 2021). While significant research has been performed on cryopreservation methods, comparatively few efforts have been conducted to determine the effects of cryopreservation on the phenome and genome of the population of cells that grows back (Day and Fleck, 2015; Kapoore *et al.*, 2019).

Future directions and challenges

Diversity of algae of interest

Working with algal cultures must consider the large diversity of algae. Decisions are required on priorities for maintaining relevant collections, developing related preservation protocols and integrating diverse genotype and phenotype datasets to maximize value from the collections. The great biodiversity of algae and the rapid growth of companies working with them requires responsive repositories that can service changing trends. Arguably, applied algal biotechnology began in the 1950s with coupled wastewater treatment and bioenergy production using high-rate algal ponds (Aitken *et al.*, 2012; Craggs *et al.*, 2011; Kim *et al.*, 2011). Since then, the research focus has changed with time and has included renewable biofuels, ecosystem engineering, food/feed and biochemical synthesis (Wood, 2021; National Academies of Sciences, 2022; Pereira *et al.*, 2013). A cryopreservation facility may have a maximum capacity, requiring decisions on what to preserve and store. However, it is good to remember that preserved strains may turn out to have value for applications other than the initial focus, so replication is valuable.

Capacity

While the infrastructure, resources, and workforce of existing algal collections have grown over time, their size and capabilities have not kept up with scientific advances that increase the need for such repositories. Individual researchers focusing upon safeguarding biodiversity and population genetics can accrue hundreds or thousands of samples. Research involving the creation of new "strains" from a wild-type alga biomolecular approaches (e.g., CRISPR/Cas9 technology, Zhang *et al.*, 2019) also adds to the demands for space and capacity. In recent years, these developments in research and commercialization have resulted in an upsurge in new strain holdings requiring cryopreservation that exceeds the total global accumulated holdings over the past 50 years. In addition, focused federal programs (e.g., ARPA-E Mariner Program) have led to the collection of thousands of new macroalgae isolates that need to be curated. Unambiguous regulations and guidelines for adding or removing strains from germplasm collections need to be established. Development of algal cryopreservation protocols will be an essential part of this process.

Information infrastructure

To maintain the rapid pace of algal innovation, scientists need easier access to standardized data on algal phenotypic traits, genetic information, genome-to-phenome mapping, growth methods and preservation

protocols. One needs only look to the *Chlamydomonas* Resource Center[12] to see the value of having a catalogue that not only defines algae by physiological traits, but also holds many physiological variants of the wild type strain. Currently, algal collections use different platforms for compiling their catalogues of strains and associated metadata. In addition, the characteristics listed tend to be more relevant to academic researchers rather than to application scientists.

Several national and international initiatives that function as clearing houses for diverse aggregations of data may serve as a starting point for development of consistent and informative platforms specific to cryopreserved algal and other microbial collections. They include the U.S. Culture Collection Network, UK Biological Resource Centre Network, European Culture Collections' organization, World Federation for Culture Collections, Scientific Collections International, Biodiversity Collections Network, and Global Biodiversity Information Facility.

Strain ownership and the Convention on Biological Diversity: the experiences of the National Center for Marine Algae and Microbiota and the Culture Collection of Algae and Protozoa

Clear statements on algal strain ownership are important in the light of the Convention on Biological Diversity (CBD) and its requirement of access and benefit-sharing (see Chapter 8) given the increasing use of strains for commercialization. For example, all strains in the public collection of the National Center for Marine Algae and Microbiota (NCMA) are either the property of NCMA with full right and title (all NCMA microalgae strains and a fraction of the macroalgae strains) or have been licensed from an entity and NCMA has been given full right to distribute and negotiate commercial licenses. At the current time, all algal strains are isolated from waters within the national jurisdictions of the United Sates of America, from waters beyond national jurisdiction, or were isolated internationally but the acquisition pre-dates the entry into force of the CBD in 1993. For these reasons, the NCMA has not had to engage in a benefit-sharing agreement under the CBD.

The Culture Collection of Algae and Protozoa (CCAP) is supported by the Natural Environment Research Council (NERC) of the United Kingdom, as a National Capability service. CCAP's strains have been isolated from all over the world. Many were isolated prior to the date of the CBD entry into force, but for those isolated after that date, and before the date that the Nagoya Protocol came into force (2014), CCAP include the following clause in its *"Terms and Conditions*" which is sent to each user: *"If the recipient desires to use the material (or modifications) for defined commercial purpose(s), it is incumbent upon the recipient, in advance of such use and providing that the country of origin is a signatory to the CBD, to negotiate in good faith with the depositor or appropriate authority in the country of origin, the terms of any benefit sharing in compliance with the CBD."* For strains that fall within the scope of the Nagoya Protocol, we follow the UK access and benefit sharing (ABS) regulations.[1]

Reference

[1] www.gov.uk/guidance/abs

[12] www.chlamycollection.org/

The availability of sufficient quantities of healthy cultures of cryopreserved microalgae and macroalgal gametophytes ready to inoculate large cultures could revolutionize the availability, genetic security, and maintenance costs of algal cultures for commercial enterprises. To become a reliable, cost-effective tool for gene banking, the overall process needs to be improved, and the approach needs to be integrated into an efficient large-scale platform linked with genetic and biological databases and metadata, long-term storage capabilities, inventory management, quality control, sample distribution pathways, biosecurity assurance, utilization and disposal practices, and a sound cryobiological foundation (Tiersch and Green, 2011).

References

Aitken, D. & Antizar-Ladislao, B. 2012. Achieving a green solution: limitations and focus points for sustainable algal fuels. *Energies*, 5(5): 1613–1647.

Cañavate, J.P. & Lubian, L.M. 1997. Effect of culture age on cryopreservation of marine microalgae. *Eur. J. Phycol.*, 32: 87–90.

Choi, Y.H., Nam, T.J. & Kuwano, K. 2013. Cryopreservation of gametophytic thalli of *Porphyra yezoensis* (Rhodophyceae) by one-step fast cooling. *J. Appl. Phycol.*, 25: 531–535.

Chu, W. 2012. Biotechnological applications of microalgae. *ISME Journal*, 6: S24–S37.

Cole, A., Neveux, N., Whelan, A., Morton, J., Vis, M., De Nys, R. & Paul, N. 2016. Adding value to the treatment of municipal wastewater through the intensive production of freshwater macroalgae. *Algal Research*, 20: 100–109.

Colla, G. & Rouphael, Y. 2020. Microalgae: New source of plant biostimulants. *Agronomy*, 10(9): 1240.

Craggs, R., Heubeck, S., Lundquist, T. & Benemann, J. 2011. Algal biofuels from wastewater treatment high rate algal ponds. *Water Sci. Technol.*, 63(4): 660–665.

Dang, B.-T., Bui, X.-T., Tran, D.P.H., Hao Ngo, H., Nghiem, L.D., Hoang, T.-K.-D., Nguyen, P.-T., Nguyen, H.H., Vo, T.-K.-Q., Lin, C., Yi Andrew Lin, K. & Varjani, S. 2022. Current application of algae derivatives for bioplastic production: A review. *Bioresource Technology*, 347: 126698.

Day, J.G. 2007. Cryopreservation of microalgae and Cyanobacteria. In: Day, J.G. & Stacey, G. (Eds.), *Methods Mol Biol*. Humana Press, Totowa, New Jersey, pp. 141–151.

Day, J.G., Benson, E.E. & Fleck, R.A. 1999. In vitro culture and conservation of microalgae: Applications for aquaculture, biotechnology and environmental research. *In Vitro Cellular and Developmental Biology-Plant*, 35: 127–136.

Day, J.G. & Brand, J.J. 2005. Cryopreservation methods for maintaining microalgal cultures. In: Andersen, R.A. (Ed.), *Algal Culturing Techniques*. Elsevier Academic Press, pp. 165–188.

Day, J.G. & Deville, M.M. 1995. Cryopreservation of algae. In: Day, J.G. & McLellan, M.R. (Eds.), *Cryopreservation and Freeze Drying Protocols*. Human Press, Totowa, NJ, pp. 81–89.

Day, J.G. & Fleck, R.A. 2015. Cryo-injury in algae and the implications this has to the conservation of micro-algae. *Microalgae Biotechnology*, 1: 1–11.

Day, J.G. & Harding, K. 2008. Cryopreservation of algae. In: Reed, B.M. (Ed.), *Plant Cryopreservation: A Practical Guide*. Springer New York, New York, NY, pp. 95–116.

Day, J.G., Lorenz, M., Wilding, T., Friedl, T., Harding, K., Pröschold, T., Brennan, D., Müller, J., Santos, L.A., Santos, M., Osório, H.C., Amaral, R., Lukešová, A., Hrouzek, P., Lukeš, M., Elster, J., Lukavský, J., Probert, I., Ryan, M.J., & Benson, E. 2007. The use of physical and virtual infrastructures for the validation of algal cryopreservation methods in international culture collections. *Cryoletters*, 28: 359–376.

Day, J.G., Priestley, I.M. & Codd, G.A. 1987. Storage, recovery and photosynthetic activities of immobilised algae. In: Webb, C. & Mavituna, F. (Eds.), *Plant and Animal Cells: Process Possibilities*. Chichester, West Sussex, pp. 257–261.

Day, J.G., Tytor, S., Egardt, J., Applegren, M., Rad-Menéndez, C., Chepurnova, O., ... & Godhe, A. 2017. Challenges for the maintenance and cryopreservation of multiple isolates of model microorganisms: An example using the marine diatom *Skeletonema marinoi*. *Biopreservation and Biobanking*, 15(3): 191–202.

Day, J.G., Watanabe, M.M., Morris, G.J., Fleck, R.A. & McLellan, M.R. 1997. Long-term viability of preserved eukaryotic algae. *Journal of Applied Phycology*, 9: 121–127.

FAO 2012. *The State of World Fisheries and Aquaculture*. In: FAO (Ed.), Rome.

FAO 2022. Global Aquaculture Production. Available at: www.fao.org/fishery/en/collection/aquaculture.

Fernandes, M.S., Calsing, L.C.G., Nascimento, R.C., Santana, H., Morais, P.B., de Capdeville, G. & Brasil, B.S.A.F. 2019. Customized cryopreservation protocols for chlorophytes based on cell morphology. *Algal Research*, 38: 101402.

Fleck, R.A., Day, J.G. & Benson, E.E. 1996. An investigation of viability loss and cell damage during the application of cryopreservation to the unicellular microalgae: *Euglena gracilis* and *Haematococcus pluvialis*. *Cryobiology*, 33: 645–646.

Fleck, R.A. 1998. The assessment of cell damage and recovery in cryopreserved freshwater protists. PhD Thesis, Univ. Abertay Dundee, 393 pp.

Franks, F. 1985. Complex aqueous systems at subzero temperatures. In: Simatos, D. & Multon, J.L. (Eds.), *Properties of Water in Foods*. Martinus Nijhoff, Dordrecht, pp. 497–509.

Kapoore, R.V., Huete-Ortega, M., Day, J.G., Okurowska, K., Slocombe, S.P., Stanley, M.S. & Vaidyanathan, S. 2019. Effects of cryopreservation on viability and functional stability of an industrially relevant alga. *Scientific Reports*, 9: https://doi.org/10.1038/s41598-41019-38588-41596.

Kim, D.-G., Park, J., Lee, D. & Kang, H. 2011. Removal of nitrogen and phosphorus from effluent of a secondary wastewater treatment plant using a pond–marsh wetland system. *Water Air Soil Poll.*, 214(1–4): 37–47.

Kono, S., Kuwano, K., Ninomiya, M., Onishi, J. & Saga, N. 1997. Cryopreservation of *Enteromorpha intestinalis* (Ulvales, Chlorophyta) in liquid nitrogen. *Phycologia*, 36: 76–78.

Krause-Jensen, D., Lavery, P., Serrano, O., Marba, N., Masque, P. & Duarte, C.M. 2018. Sequestration of macroalgal carbon: the elephant in the Blue Carbon room. *Biology Letters*, 14: 20180236.

Kuwano, K., Aruga, Y. & Saga, N. 1993. Cryopreservation of the conchocelis of the marine alga *Porphyra yezoensis* Ueda (Rhodophyta) in liquid nitrogen. *Plant Sci.*, 94: 215–225.

Lalrinsanga, P.L., Deshmukhe, G., Chakraborty, S.K., Dwivedi, A., Barman, N. & Kumar, N. 2009. Preliminary studies on cryopreservation of vegetative thalli of some economically important seaweeds of India. *J. Appl. Aquacult.*, 21: 250–262.

Lee, Y.N. & Nam, K.W. 2016. Cryopreservation of gametophytic thalli of *Ulva prolifera* (Ulvales, Chlorophyta) from Korea. *J. Appl. Phycol.*, 28: 1207–1213.

Li, S., Ji, L., Shi, Q., Wu, H. & Fan, J. 2019. Advances in the production of bioactive substances from marine unicellular microalgae *Porphyridium* spp. *Bioresource Technology*, 292: 122048.

Lorenz, M., Friedl, T. & Day, J.G. 2005. Perpetual maintenance of actively metabolizing microalgal cultures. In: Andersen, R.A. (Ed.), *Algal Culturing Techniques*. Academic Press, New York, pp. 145–156.

Malik, K.A. 1993. Preservation of unicellular green algae by liquid drying. *J. Microbiol. Methods*, 18: 41–46.

Mazur, P. 1970. Cryobiology: The freezing of biological systems. *Science*, 168: 939–949.

McGrath, M.S., Daggett, P.-M. & Dilworth, S. 1978. Freeze-drying of algae: *Chlorophyta* and *Chrysophyta*. *Journal of Phycology*, 14: 521–525.

Meryman, H.T. 1966. Review of biological freezing. In: Meryman, H.T. (Ed.), *Cryobiology*. Academic Press, New York and London, pp. 1–114.

Morris, G.J. 1978. Cryopreservation of 250 strains of *Chlorococcales* by the method of two-step cooling. *British Phycological Journal*, 13: 15–24.

Müller, J., Friedl, T., Hepperle, D., Lorenz, M. & Day, J.G. 2005. Distinction between multiple isolates of *Chlorella vulgaris* (Chlorophyta, Trebouxiophyceae) and testing for conspecificity using amplified fragment length polymorphism and its rDNA sequences. *Journal of Phycology*, 41(6): 1236–1247.

National Academies of Sciences, E. & Medicine 2020. *Biological Collections: Ensuring Critical Research and Education for the 21st Century*. Washington, DC, p. doi.org/10.17226/25592.

National Academies of Sciences, E. & Medicine 2022. *A Research Strategy for Ocean-based Carbon Dioxide Removal and Sequestration*. The National Academies Press, Washington, D.C.

Paredes, E., Ward, A., Probert, I., Gouhier, L. & Campbell, C.N. 2021. Cryopreservation of algae. In: Wolkers, W.F. & Oldenhof, H. (Eds.), *Cryopreservation and Freeze-Drying Protocols*. Springer US, New York, NY, pp. 607–621.

Pereira, R., Yarish, C. & Critchley, A.T. 2013. Seaweed aquaculture for human foods in land-based and IMTA systems. In: Christou, P., Savin, R., Costa-Pierce, B.A., Misztal, I. & Whitelaw, C.B.A. (Eds.), *Sustainable Food Production*. Springer, New York, pp. doi.org/10.1007/978-1001-4614-5797-1008_1189.

Pringsheim, E.G. 1946. *Pure Cultures of Algae*. Cambridge U.P., Cambridge, 119 pp.

Raposa, M., Morais, R. & Morais, A. 2013. Bioactivity and applications of sulphated polysaccharides from marine microalgae. *Marine Drugs*, 11: 233–252.

Roque, B.M., Brooke, C.G., Ladau, J., Polley, T., Marsh, L.J., Najafi, N., Pandey, P., Singh, L., Kinley, R., Salwen, J.K., Eloe-Radrosh, E., Kebreab, E. & Hess, M. 2019. Effect of the macroalgae *Asparagopsis taxiformis* on methane production and rumen microbiome assemblage. *Animal Microbiome*, 1. https://doi.org/10.1186/s42523-019-40004-42524.

Schiel, D.R. & Foster, M.S. 2015. The Biology and Ecology of Giant Kelp Forests. University of California Press. Available at: www.jstor.org/stable/10.1525/j.ctt14btfvw, Oakland, CA, pp. 416.

Taylor, R. & Fletcher, R.L. 1998. Cryopreservation of eukaryotic algae – a review of methodologies. *Journal of Applied Phycology*, 10(5): 481–501.

Taylor, R. & Fletcher, R.L. 1999a. A simple method for the freeze-preservation of zoospores of the green macroalga *Enteromorpha intestinalis*. *J. Appl. Phycol.*, 11: 257–262.

Taylor, R. & Fletcher, R.L. 1999b. Cryopreservation of eukaryotic algae – a review of methodologies. *J. Appl. Phycol.*, 10: 481–501.

Tiersch, T.R. & Green, C.C. (Eds.) 2011. *Cryopreservation in Aquatic Species: A Comprehensive Overview of Current Practices, Programmatic Development and Future Directions for Cryopreservation of Gametes, Embryos and Larvae of Aquatic Species*, 2nd edition. World Aquaculture Society, *Advances in World Aquaculture*, Baton Rouge, Louisiana, 1003 pp.

Visch, W., Rad-Menendez, C., Nylund, G.M., Pavia, H., Ryan, M.J. & Day, J.G. 2019. Underpinning the development of seaweed biotechnology: Cryopreservation of brown algae (*Saccharina latissima*) gametophytes. *Biopreserv. Biobank.*, 17: 378–386.

Wade, R., Augyte, S., Harden, M., Nuzhdin, S., Yarish, C. & Alberto, F. 2020. Macroalgal germplasm banking for conservation, food security, and industry. *PLoS Biology*, 18: e3000641.

Wang, Q., Liu, Y., Zhang, E., Li, D. & Zhang, C. 2005. Cryopreservation of clonal gametophytes of *Undaria pinnatifida* by encapsulation-dehydration. *Acta Oceanol. Sin.*, 27: 154–159.

Wang, B., Zhang, E., Gu, Y., Ning, S., Wang, Q. & Zhou, J. 2011. Cryopreservation of brown algae gametophytes of *Undaria pinnatifida* by encapsulation–vitrification. *Aquaculture*, 317: 89–93.

Wood, D.A. 2021. Microalgae to biodiesel – Review of recent progress. *Bioresource Technology Reports*, 14: doi.org/10.1016/j.biteb.2021.100665.

Yang, H., Huo, Y., Yee, J.C. & Yarish, C. 2021. Germplasm cryopreservation of macroalgae for aquaculture breeding and natural resource conservation: A review. *Aquaculture*, 544: doi.org/10.1016/j.aquaculture.2021.737037.

Yee, J.C. & Yang, H. 2023. Cryopreservation of *Tetraselmis striata* through systematic evaluation of multiple parameters in the cooling process. *Aquaculture*, 566: 739172.

Zhang, Q., Cong, Y., Qu, S., Luo, S., Yang, G. 2008. Cryopreservation of gametophytes of *Laminaria japonica* (Phaeophyta) using encapsulation-dehydration with two-step cooling method. *J. Ocean Univ. China.*, 7: 65–71.

Zhang, Y.-T., Jiang, J.-Y., Shi, T.-Q., Sun, X.-M., Zhao, Q.-Y., Huang, H. & Ren, L.-J. 2019. Application of the CRISPR/Cas system for genome editing in microalgae. *Applied Microbiology and Biotechnology*, 103(8): 3239–3248.

Zhou, W., Li, Y. & Dai, J. 2007. Study on cryopreservation of *Porphyra yezoensis* conchocelis. *J. Ocean Univ. China.*, 6: 299–302.

Chapter 8. Access and benefit-sharing in gene banking of aquatic genetic resources

Fran Humphries, Queensland University of Technology, Brisbane, Australia

Introduction

Access and benefit-sharing (ABS) can have a significant impact on how a gene bank can legally obtain, use and distribute genetic resources and associated information, with serious consequences in case of non-compliance. There is an urgent need to integrate ABS procedures and traceability requirements into the decision trees proposed in this guide for the strategic diversification of genetic resource collections and preservation protocols. Gene banks may play multiple roles as collectors, providers, intermediaries and users of genetic materials – all of which require consideration of ABS. The aim of this section is to highlight key practical considerations for creating ABS protocols in gene banking, which may be embedded into the collection and preservation protocols. ABS laws and policies apply to use and preservation of *in situ* and *ex situ* aquatic genetic resources (AqGR), but the considerations in this section focus on important issues relating to *ex situ in vitro* uses and preservation, which is the scope of this Practical Guide. For more extensive information on ABS applied to AqGR, it is recommended to consult Humphries et al. (2024).

ABS is a legal and policy tool used by many countries to:

(a) regulate access and use of genetic resources and associated Traditional Knowledge (TK), and, under some laws, digital sequence information (DSI). DSI is broadly understood to refer to nucleotide sequence data as well as being a "placeholder term" for other biological data; and
(b) share the benefits from their use with the provider in a fair and equitable way.

In the case of AqGR, ABS issues have been considered since the 1990s and were first reviewed by Greer and Harvey (2004). Despite this, only a small fraction of ABS literature relates to AqGR and associated TK, indicating that the far-reaching consequences of ABS on aquaculture is yet to be fully analyzed (Humphries *et al.*, 2018). The FAO's *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* (2019) includes considerations for developing, adapting or implementing ABS measures for genetic resources for food and agriculture, including AqGR, taking into account their distinctive features. Distinctive features of AqGR include the importance of international exchange for the functioning of the aquaculture sector and the genetic intermingling over boundaries and over different time periods (FAO, 2019).

The main international agreements providing frameworks for AqGR ABS measures, include the *Convention on Biological Diversity* (CBD) (1992), the *Nagoya Protocol on Access to Genetic Resources and the Fair and Equitable Sharing of Benefits arising from their Utilization to the Convention on Biological Diversity* (Nagoya Protocol) (2014) and the *Agreement under the United Nations Convention on the Law of the Sea on the Conservation and Sustainable Use of Marine Biological Diversity of Areas Beyond National Jurisdiction* (BBNJ) (2023). At the 15th Conference of the Parties to the CBD, countries agreed to a multilateral mechanism for benefit-sharing from the use of DSI, that includes DSI on AqGR, but the definition, scope and procedures of that mechanism are yet to be determined (COP, 2022 – Decision 15/9).

Despite having frameworks at the international level, national ABS laws and policies vary significantly. Some countries do not regulate access to, and benefit-sharing from, genetic resources under ABS laws. Others may only regulate benefit-sharing in certain areas (e.g. in national parks in the United States of America). For those countries that have specific ABS laws or policies, scope of ownership and control by ABS actors often reflect national circumstances with wide variation in geographic coverage, regulated activities and subject matter (Humphries *et al.*, 2021a). The result is a very complex matrix of ABS rules worldwide and stakeholder confusion around compliance with these rules.

Access and benefit-sharing procedures and temporal scope

ABS procedures vary significantly between countries and sometimes also within countries in which each state or province has its own approach (Humphries *et al.*, 2021b). Generally, procedures are divided into:

1. administrative procedures for the access component such as permits or registration, which document the prior informed consent (or permission) of the provider for collecting or taking AqGRs or associated DSI or TK; and
2. contractual procedures for benefit sharing, where the user agrees to share with the provider monetary and/or non-monetary benefits arising from the use of the AqGR, information or knowledge for research, development and/or commercialization.

Some national ABS laws have, or are developing, procedures for obtaining the free, prior and informed consent of indigenous people and local communities (IPLCs) to access their genetic resources and associated TK (e.g., Greer and Harvey, 2004; ICC, 2023; Oguamanam, 2018). While governments sometimes issue codes of conducts or Practical Guide for identifying the communities entitled to give permission on mutually agreed terms, communities may have their own biocultural protocols that need to be followed when carrying out the government's ABS procedures (Girard *et al.*, 2022).

Usually, procedures for the access and/or use of the AqGR will only be triggered after the relevant law has entered into force for that country, although some countries' laws are retroactive. This means that new uses of AqGR collections that were obtained years ago may be subject to ABS procedures. Countries may achieve retroactivity by referring to the time of utilization rather than date of collection. This means that genetic resources collected decades or even centuries earlier would be covered by ABS regulations once they are put into use. The gene bank or resource users should always check with the relevant government for specific advice about whether it has ABS laws or policies and if so, whether these may apply to previously accessed AqGR. The national position on ABS may change at any time, so gene banks need to stay informed about any impacts on resources in their collections by these changes.

Where a country does not have ABS laws or policy, a gene bank may decide to follow the spirit and intent of ABS for moral, reputational or economic reasons and create its own policy about how to manage these situations. At the very least, they should seek confirmation in writing from the national focal point of the country that ABS measures do not apply to their specific accessions, keeping in mind the countries' position on ABS may change. There may be other laws that have implications for collecting (permits or licenses), using or sharing the benefits from the resources or knowledge (e.g., TK intellectual property protection laws).

Considerations for ABS procedures

National ABS laws that apply to AqGR are usually designed for bilateral (transactional) systems, meaning that authorizations to access genetic material and benefit sharing agreements must be negotiated with one (or more) provider(s) for each AqGR. While a gene bank may often be more of an intermediary than collector or end user, they still have a responsibility to ensure that their accessions are properly collected and used. This means that gene banks need to determine the origin of every accession in their collection to design protocols about how to manage them, including:

(a) collection of new accessions for the gene bank, requiring negotiation with the provider, who may be a government, indigenous people, local community and/or other entity that is considered to be a 'provider' under the law (see below);
(b) new uses by the gene bank or other users of existing accessions in collections (which may require ABS negotiations between the relevant gene bank and government and/or communities depending on the provider country laws); and
(c) users accessing genetic material stored in a gene bank. These users would need to firstly comply with access requirements of the country where the gene bank is located and secondly negotiate a benefit sharing contract with either the gene bank and/or the provider country government or community, depending on the requirements of the relevant ABS laws that apply to that genetic material.

Both the administrative (access) and contractual (benefit-sharing) procedures can take months or years to negotiate and conclude (Young and Tvedt 2019), therefore applications need to be initiated early for existing and newly acquired AqGR in a collection.

In contrast to AqGR, the crop sector has developed a much more cost-effective system to regulate countries' access to genetic resources which is represented by the Multilateral System (MLS) of the *International Treaty on Plant Genetic Resources for Food and Agriculture* (ITPGRFA). Starting from the recognition that countries are strongly interdependent on plant material from many sources and the existing genetic mixing of plant stocks, the ITPGRFA developed a MLS for those plant genetic resources (PGR) that most contribute to food security and for which countries are more interdependent. The MLS has simplified and standardized procedures, including a standard material transfer agreement, that govern transfer of genetic material for research, breeding and training and has the advantage of reducing transaction costs associated with exchanges based on bilateral negotiations (FAO, 2010). While there is no international MLS to support a similar approach for AqGR, gene banks may consider designing protocols and model contract terms for standard material transfer agreements for collection, use and distribution of AqGR in their gene banks. This may include broadening the scope of existing international agreements to include aquaculture material in multilateral systems.

While AqGR procedures are more complex and varied than PGR procedures under the ITPGRFA, there are a few examples of national ABS laws accommodating the distinctive features of AqGR, such as those outlined in Box 2.

For AqGR there is also a range of regional ABS frameworks but almost no literature analyzing whether regional approaches are effective in achieving conservation, sustainable use and fairness/equity objectives in the case of AqGR and TK associated with those AqGR. Examples of regional approaches to ABS were developed by the African Union and the European Union. In the EU for example, the European ABS Regulation was implemented throughout the EU to enforce the Nagoya Protocol. Its primary goal was to create a unified compliance framework among EU Member States, aiming to ensure adherence to the ABS rules of a provider country when its resources are utilized within the EU. However, the decisions regarding

the regulation of ABS for genetic resources and TK within their own territories are left to the discretion of individual EU Member States, leading to diverse approaches across the region (Humphries et al., 2024).

Geographic scope and country of origin

Determining the country of origin of each genetic resource within a collection is essential to determining which ABS laws apply. The ABS concept was originally developed to overcome global inequities where technology-rich countries (often called the global North) were benefiting from biodiverse-rich countries (global South) that were unable to exploit their own genetic resources (primarily for agriculture and pharmaceuticals). Contractual benefit sharing was a means of compensating the providers (via non-monetary and/or monetary benefits) for the cost of conservation of their biological resources *in situ* (Lawson 2016) while preventing the misappropriation of genetic resources and TK (Robinson and Raven 2017). Since all countries have contract law, contracts were a way of ensuring compliance by resource users outside the provider country (Young and Tvedt, 2017). In practice, ABS has become a tool for the redistribution of wealth and resources across borders, with few examples of the expected conservation outcomes directly linked to ABS (Laird *et al.*, 2020). The generalization of North (user) and South (provider) inequities overlooks the more complex global movement of genetic resources for use in aquaculture, which is not predominantly North/South but relies on gene flows in all directions (Sonesson *et al.*, 2023), and the potential for ABS to provide benefits for AqGR conservation. Indeed, recent global analyses using global DSI datasets and scientific publications have disproven the provider/user dichotomy but the policy rhetoric remains (Scholz *et al.*, 2021, 2022).

The location of a genetic resource within a country also determines whether ABS procedures apply to a particular transaction. Most countries that regulate access apply their laws to *in situ* biological resources but whether the laws apply to resources held in public or private *ex situ* facilities varies in each country (Humphries *et al.*, 2021b). For example, some countries' laws (e.g. Australia other than the Northern Territory) only apply to genetic resources in public lands/waters and public *ex situ* collections, meaning that private facilities are not considered providers entitled to ABS under law. However, depending on the origin of the original resource held in the facility, another country's laws may apply to the use of that resource. It is important to understand that benefit-sharing obligations move with the genetic resource. The ABS role of the *ex situ* facility can be as provider, user or intermediary of the resource. In each role, there may be different requirements and responsibilities for each AqGR (see below).

<u>How to determine which countries' ABS laws apply to an individual AqGR:</u>

- Document the provenance (country or region of origin) of individual AqGR in a collection. This is essential for compliance with ABS laws and policy.
- Record the date of collection, date of accession (deposit into a collection), and dates of any utilization of the AqGR.
- Consider whether there is more than one provider country. In cases where there are multiple countries of origin for an access or where a modified resource contains different genetic material from a number of countries, each provider country may require prior informed consent and benefit sharing.
- Consider whether other countries require consent. The country or countries of origin may be different from the provider country but may also require prior informed consent and benefit sharing. Gene banks and proposed users of AqGR should contact the National Focal Point and the Competent National Authority of the country of origin and/or provider country to obtain detailed information on ABS laws or policies that may apply in their specific circumstance (https://absch.cbd.int/en/).

- Consider the location of the genetic resource. There is wide variation in how countries' ABS laws manage genetic resources held in private and public *ex situ f*acilities. If an AqGR originally came from an ocean area beyond national jurisdiction, the framework of the new High Seas treaty may apply, but infrastructures and procedures for benefit-sharing from this framework have yet to be determined (UNGA 2023).

Scope of subject matter and activity

While all ABS laws have genetic resources as the subject, many also include TK associated with genetic resources and some include DSI on genetic resources. The CBD defines genetic resources as 'genetic material of actual or potential value' where 'genetic material 'means any material of plant, animal, microbial or other origin containing the functional units of heredity (CBD article 2). Subject matter is often interpreted broadly (e.g. gametes, tissues, embryos, sporophytic material and potentially non-gametic tissues) and usually also applies to derivatives such as chemical compounds (Humphries *et al.*, 2021b). Recent breakthroughs in creating reproductive cells from somatic cells through *in vitro* gametogenesis make it clear that the definition of "functional units of heredity" is no longer clear-cut (Saitou and Hayashi 2021). Biodiverse-rich countries are increasingly including DSI as subject matter, including where it is used independently of the physical materials (Bagley *et al.*, 2020), although the new CBD multilateral benefit-sharing mechanism may alter this pattern (COP 2022). Many national laws include TK associated with genetic resources as subject matter, although definitions of TK vary widely between national ABS laws and policies (Humphries *et al.*, 2021b).

AqGR are generally considered to be subject matter of ABS only if they are being used in research, development and/or commercialization (i.e. utilized) for their genetic material potential and not as a commodity (e.g. for human or animal consumption). However, some countries include a broader range of activities under their ABS laws, so users of AqGR should check whether the relevant country's ABS law includes uses that do not involve human intervention on genetic material, for example, simple grow out in a hatchery. ABS does not generally distinguish between live and preserved AqGR, nor the mode of preservation, e.g. cryopreservation, desiccation and lyophilization.

The focus of regulation is access, utilization and/or distribution of genetic resources and the activities included differ by country. Broadly speaking (and this varies under each law):

1. 'Access' may include collection from the wild, public or private collections or other facilities;
2. 'Utilization' may include commercial uses (including intellectual property and commercial breeding); and/or non-commercial uses such as research, conservation and re-stocking. In some countries the laws extend to taxonomic activities, while in other countries, non-commercial uses are not regulated by ABS laws;
3. 'Distribution' may be temporary (sending samples for bioassays or temporary loan) or permanent (export to another country) and can require benefit-sharing provisions in the material or data transfer agreement (Humphries *et al.*, 2021b).

For all the above activities, there may also be additional (non-ABS) permit requirements, such as research, export, CITES, biosafety, or sanitary permits.

<u>Determining whether AqGR falls within scope:</u>

- Every AqGR accession potentially falls within scope of ABS subject matter, but some laws have exclusions (where certain subject matter and activities are not regulated), some countries do not regulate access, or some may have more simplified arrangements (Box 3).

- The activity in relation to AqGR will determine which laws or requirements are relevant in a specific circumstance for genetic resources and TK or DSI.
- While preservation protocols and pathways may be based on single species, species groups or more generalized pathways, ABS protocols need to include decision making at the individual accession level on a case-by-case basis.

ABS Actors – Providers, users and intermediaries

Once a gene bank or user of AqGR has determined which ABS laws apply and whether the activity or associated information and knowledge fall within scope, it must determine the correct provider for the purposes of prior informed consent and benefit sharing. In many cases this will be the government (at the national or local level) but under some laws, it may include Indigenous peoples, local communities, entities and individuals who control the resources, including *ex situ* facilities. In some cases, there may be more than one provider who the user may need to negotiate with. While most laws relate to providers of genetic resources on public lands, waters or facilities, some apply to privately owned areas and facilities. Determining the correct provider in cases of public/private partnerships and collaborations can be complex. Possession, ownership and control over AqGR and the information it contains are distinct legal concepts and can trigger other areas of law including contract law and intellectual property law, which may need to be taken into account when determining permission for access, use or distribution of AqGRs.

Gene banks are in a unique position because they may be a provider, user, and/or an intermediary. This means they may have different obligations depending on the circumstances of the original collection and subsequent use of AqGR or associated information. For example, different rules may apply to an AqGR that was donated to a gene bank in trust (e.g. for distribution to farmers for grow out only) as opposed to AqGR that has been developed under a research program. ABS laws may not be clear on the responsibilities of intermediaries, so it is important for them to seek advice from the relevant government. In Malaysia, for example, there are strong penalties if unauthorized intermediaries pass materials to users that do not have authorization from the government to use them (Humphries *et al.*, 2021b). On the other hand, in the European Union countries, the onus is on the user to ensure that prior informed consent and mutually agreed terms were obtained by an intermediary when the resources were originally accessed (Humphries *et al.* 2021b), although the Register of Collections, provides a voluntary registry of collections to ensure ABS compliance (Yurkov *et al.*, 2019).

<u>How to determine who is entitled to prior informed consent and benefit sharing:</u>

- Because gene banks may be providers, users and intermediaries of AqGRs, with different obligations over the same genetic resources, ABS protocols might need to be created for each gene bank role.
- The ABS National Focal Point in each country (whose ABS law may be relevant to AqGR in a collection) should be able to provide advice on how to determine the provider responsible for negotiating prior informed consent and benefit sharing. If no answer is received, utilization of the AqGR should be discontinued.
- Few countries clarify the ABS obligations of intermediaries, which highlights the need for gene banks to develop specific ABS protocols at the repository, network and community level and cryopreservation pathway level.

Traceability and reporting requirements

Gene banks and other users of AqGR may have reporting and other requirements for sharing information with governments about ongoing uses of samples, information and knowledge. National ABS measures differ in the range of tools for tracing the chain of custody of genetic resources as they move from the place of origin through research, development and commercialization and between third parties. These tools include periodic reporting (on resource use and movements), material transfer agreements (which may transfer benefit sharing and reporting obligations to third parties) and internationally recognized certificates of compliance. Governments issue the latter as evidence that authorization procedures were followed, including details of the permit, subject matter, and mutually agreed terms, and post them on the ABS Clearing House website (https://absch.cbd.int/en/). These certificates are an important way of proving compliance with ABS measures, especially if the genetic resources are sent to countries that require demonstration of compliance with the provider country's ABS measures before they can be imported and used. Examples include countries of the European Union, Norway, Japan and the Republic of Korea (Humphries *et al.*, 2021b). Penalties for non-compliance with ABS measures vary from fines to imprisonment, depending on the national law (Humphries *et al.*, 2021b).

Other means of monitoring include checkpoints (often government export and patent offices) that collect information while genetic resource products pass through research and development pathways. This information can alert national authorities to possible non-compliance with ABS measures. Checkpoint communique information should be made publicly available by national authorities on the ABS Clearing House. Such measures highlight the importance of maintaining good records of the movements and use of samples and data held by gene banks and users of AqGR and traditional knowledge.

The scientific community also has practices that may contribute to traceability and transparency, including the use of globally unique identifiers. Identifiers attached to samples (e.g., OBIS database) or DSI (e.g. the accession number system of the International Nucleotide Sequence Database Collaboration)[13] may link data on the location of sampling, environmental data and subsequent processing to the sample/information record (Rogers *et al.*, 2021). These records can be linked to other identifiers such as Digital Object Identifiers (DOIs) for publications and patent databases. Countries may link their administrative ABS infrastructure to these databases, highlighting the need to promote interoperability between the gene bank's records and other databases.

Considerations for compliance with traceability and reporting requirements:

- Gene banks and other users of AqGR for research and development need to maintain good record-keeping practices that document the origin, access, biological characteristics, utilization and movement of every sample, including the generation of DSI and any use of TK.
- They need also to ensure their data management systems are as interoperable as possible with other databases because governments are increasingly requiring the use of identifiers and information on samples as they move through the research and development pathway (including subsequent users).
- Related to the concerns above is the need to develop suitable labels and metadata structures to classify, trace and recover information about AqGRs.

[13] www.insdc.org/

BOX 3

Examples of ABS laws that accommodate the special features of AqGR

Following are examples of countries with ABS laws that have exclusions (where certain subject matter and activities are not regulated) or examples of laws with simplified arrangements relevant to AqGR research and development.

Excluding AqGR subject matter or collections

- Exclusions for domesticated genetic resources (e.g. France)
- Exclusions for fisheries resources if they are governed under legislation other than ABS (e.g. Spain)
- Exclusions for entire gene bank collections (e.g. Australia where public *ex situ* collections in Commonwealth areas may be exempt if they meet ABS objectives)
- Exclusion of privately held genetic resources (e.g. Queensland, Australia)

Excluding aquaculture-related activities

- Aquaculture-related exclusions from activity scope e.g. "aquaculture or mariculture activities involving freshwater and marine species producing specimens for consumption" (South Africa)
- "collection of broodstock for aquaculture" except if carried out for research and development (Australia)

Simplified procedures for AqGR activities

- Simplified prior informed consent and mutually agreed terms procedures for:
    - non-commercial research such as breeding, taxonomic and conservation research (e.g. India); and
    - agrobiodiversity research (e.g. The Philippines).
- Exclusion from ABS for non-commercial research (e.g. Queensland, Australia).

References

Margo, B., Karger, E., Ruiz Muller, M., Perron-Welch, F. & Thambisetty, S. 2020. Fact-finding study on how domestic measures address benefit-sharing arising from commercial and non-commercial use of digital sequence information on genetic resources and address the use of digital sequence information on genetic resources for research and development as requested by decision 14/20 (paragraph 11 I from the Fourteenth Conference of the Parties to the Convention on Biological Diversity). 20 January 2020. CBD/DSI/AHTEG/2020/1/5.

COP (Conference of the Parties to the Convention on Biological Diversity). 2022. Digital Sequence Information on Genetic Resources: Draft Decision Submitted by the President, Fifteenth meeting, 18 December 2022, CBD/COP/15/L.30.

FAO. 2010. *The Second Report on the State of the World's Plant Genetic Resources for Food and Agriculture*. Rome.

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*. FAO, Rome. Available at: www.fao.org/documents/card/en/c/8afd52bc-f3ef-48fc-93be-8fbd44b0e327.

Girard, F., Hall, I. & Frison, C. 2022. *Biocultural Rights, Indigenous Peoples and Local Communities: Protecting Culture and the Environment*. Taylor and Francis.

Greer, D. & Harvey, B. 2004. *Blue Genes: Sharing and Conserving the World's Aquatic Biodiversity*. IDRC/Earthscan. 231 pp.

Humphries, F., Benzie, J.A.H., Lawson, C. & Morrison, C. 2021a. A review of the implications of access and benefit-sharing laws in key aquaculture-producing countries. *Reviews in Aquaculture*, 1–18. doi: 10.1111/raq.12532.

Humphries, F., Laird, S., Wynberg, S., Morrison, C., Lawson, C. & Kolesnikova, A. 2021b. Survey of access and benefit-sharing country measures accommodating the distinctive features of genetic resources for food and agriculture and associated traditional knowledge, FAO Background Study Paper 70. Rome, FAO on behalf of the Commission on Genetic Resources for Food and Agriculture. Available at: https://doi.org/10.4060/cb6525en.

Humphries, F., Lawson, C., Benzie, J.A.H., Morrison, C. 2024. Access and Benefit-sharing in Global Aquaculture: Genetic Resources, Digital Sequence Information, and Traditional Knowledge. Law Futures Centre, Griffith Law School, Griffith University, Australia; Aquatic Foods, WorldFish, Penang, Malaysia; and School of Biological, Earth and Environmental Sciences, University College Cork, Ireland.

ICC (Inuit Circumpolar Council). 2023. ABS and the North. Available at: www.inuitcircumpolar.com/icc-activities/environment-sustainable-development/convention-on-biological-diversity/abs-and-the-north/.

Laird, S., Wynberg, R., Rourke, M., Humphries, F., Muller, M.R. & Lawson, C. 2020. Rethink the expansion of access and benefit-sharing. *Science*, 367(6483): 1200–1202.

Lawson, C. 2012. *Regulating Genetic Resources: Access and Benefit Sharing in International Law*. Edward Elgar Publishing.

Oguamanam, C. 2018. *Genetic Resources, Justice and Reconciliation: Canada and Global Access and Benefit Sharing*. Cambridge University Press. doi: https://doi.org/10.1017/9781108557122.002.

Robinson, D. & Raven, M. 2017. Identifying and preventing biopiracy in Australia: Patent landscapes and legal geographies for plants with Indigenous Australian uses. *Australian Geographer*, 48(3): 311–331.

Rogers, A.D., Baco, A., Escobar-Briones, E., Currie, D., Gjerde, K., Gobin, J., Jaspars, M., Levin, L., Linse, K., Rabone, M., Ramirez-Llodra, E., Sellanes, J., Shank, T.M., Sink, K., Snelgrove, P., Taylor, M., Wagner, D. & Harden-Davies, H. 2021. Marine genetic resources in areas beyond national jurisdiction: Promoting marine scientific research and enabling equitable benefit sharing. *Frontiers in Marine Science*, 8: 667274. 1–22. doi: 10.3389/fmars.2021.667274.

Saitou, M. & Hayashi, K. 2021. Mammalian in vitro gametogenesis. *Science*, 374(6563). doi: 10.1126/science.aaz6830.

Scholz, A.H., Lange, M., Habekost, P., Oldham, P., Cancio, I., Cochrane, G. & Freitag, J. 2021. Myth-busting the provider-user relationship for digital sequence information. *GigaScience*, 10(2021), 1–8. https://doi.org/10.1093/gigascience/giab085.

Scholz, A.H., Freitag, J. & Lyal, C.H.C. 2022. Multilateral benefit-sharing from digital sequence information will support both science and biodiversity conservation. *Nature Communications*, 13(1086). https://doi.org/10.1038/s41467-022-28594-0.

Sonesson, A.K., Hallerman, E., Humphries, F., Hilsdorf, A.W.S., Leskien, D., Rosendal, K., Bartley, D., Hu, X., Garcia Gomez, R. & Mair, G.C. 2023. Sustainable management and improvement of genetic resources for aquaculture. *Journal of the World Aquaculture Society*, 54(2), 364–396.

UN General Assembly. 2023. Agreement under the United Nations Convention on the Law of the Sea on the Conservation and Sustainable Use of Marine Biological Diversity of Areas Beyond National Jurisdiction (A/Conf.232/2023/4). Available at: https://documents-dds-ny.un.org/doc/UNDOC/LTD/N23/177/28/PDF/N2317728.pdf.

Young, T.R. & Tvedt, M.W. 2017. *Drafting Successful Access and Benefit-Sharing Contracts*. Brill Nihhoff.

Yurkov, A., Püschner, H.M. & Hartman Scholz, A. 2019. DSMZ: The European Union's First Registered Collection under the Nagoya Protocol. *Microbiology Australia*, 40, 108–113. https://doi.org/10.1071/MA19030.

Chapter 9. Establishing a gene bank for aquatic genetic resources: physical structures and costs

Joachim Carolsfeld, World Fisheries Trust, Victoria, Canada
Michael Ross, Culture Collection of Algae and Protozoa (CCAP), The Scottish Association for Marine Science, Oban, United Kingdom
Terrence Tiersch, Aquaculture Germplasm and Genetic Resources Center (AGGRC), Louisiana State University Agricultural Center, Baton Rouge, Louisiana, United States of America

Introduction

Gene banks can range from a storage dewar in the corner of a research laboratory to a dedicated building with high-throughput processing and storage capabilities. The purpose and scope of the gene bank are key factors in determining the type of gene bank that will be developed, as they determine the development strategy (the most appropriate infrastructures, capital and operational costs, and business plan). In addition to the species of interest of the *in vitro* conservation, these will determine the processing and storage strategies that will be adopted. Table 5, summarizes the most common gene banking purposes, processing and storage strategies in relation to the gene bank processing capacity (throughput rate).

Table 5. Purposes, processing and storage strategies in relation to processing capacity of gene banks for aquatic species.

	Small Storage / Low Through-put	Medium Storage / High Through-put		Large Storage / Medium Through-put
Purpose	Protocol development – research lab and/or aquaculture facility based	Aquaculture production support (larger numbers and through-put); aquaculture facility based	Development of genetic lines for aquaculture or other purposes. Collection from field or cultivation facilities	Conservation of wild genetic material, consideration of eventual recovery of endangered species or population; collection often in the field
Processing Strategy	Collection of material close to processing facility	Build incorporated processing capacity or use mobile facility	Transport of living material to processing facility and/or use of mobile trucks; backpack approach in remote areas to minimise live transport	
Storage Strategy	Local storage in dewars	Establish reliable on-site storage capacity or (better) participate in a central storage facility (transport of preserved material)		Store material in reliable and established repository, consideration of long-term storage

The availability of funds and type (private or public) are also critical factors to consider in the establishment and long-term maintenance of a gene bank and helps to determine the processing and storage strategy that will be chosen. Most small-scale endeavors are at greater risk of failure over time, in particular for long-term storage. Large, well-funded central facilities that support comprehensive germplasm repository activities are more likely to survive over time as either public or private, because of a probable greater resilience with funding, staffing, and revenue generation. The capital, operational, and access costs of public

vs private facilities may vary significantly depending upon taxes, procurement criteria, their purpose and commercial/research success.

This chapter is complementary to Chapter 4 and Annex 7. Chapter 4 describes in detail the basic steps in a gene banking process and provides a framework for planning the material, human, and infrastructure needs. Annex 7 explains how process mapping can be a useful tool to outline cryopreservation pathways. Here, we summarize the critical requirements of gene banks for aquatic genetic resource (AqGR) and provide insights into associated costs.

A. Critical requirements at the different steps of gene banking

As discussed in Chapter 4, the key steps in an *ex situ in vitro* preservation pathway are represented by:

a) Collection of donor animals and plants
b) Collection of gametes and other cells and tissues of interest
c) Sample processing (eg dilution of extenders, addition of cryoprotectant, filling of cryocontainers, freezing)
d) Storage
e) Thawing and use
f) Transport (at various points of the pathway)
g) Data management (applies to all steps)

For each step, basic considerations are discussed and key requirements are listed below. Table 6 summarizes the main items required at each step organized by categories of infrastructure, staff, reagents and equipment, data management. Of note, *transport* can happen at various points of the process flow whilst *data management* requires collection of data related to each step of the process flow, so costs of these activities may be needed at more than one step. Requirements will also depend on the particular strategy of the gene bank and the chosen protocol.

a) Collection of animals or plants

The donor animals or plants may come from the field, a biological repository, aquaculture, or a combination of the above, depending on the gene bank purpose and the targeted taxa. At this step, it is critical that the gene bank ensures that its accessions are properly collected and used in compliance with the access and benefit-sharing (ABS) laws of the country from where the samples are collected (Chapter 8).

Sample collection may require a dedicated budget, for example for field collection trips. In such cases, costs of transportation, accommodation, meals, consumables, storage liquid nitrogen tank, etc. need to be considered (FAO, 2023). A careful planning of the collection step can help optimizing the activities and limit their costs: for example, field technicians can extend a single field mission over several days to cover several collection sites in a single trip.

Critical requirements to consider at this step include:

- Identification of sources and collection sites
- Permissions for use and ABS expenses and compliance.
- Travel and subsistence
- Human resources (including field technicians) and capacity training
- Handling and transportation of donor organisms
- Field collection equipment and material
-
- Data collection (see Chapter 10, Box 4 for the data that should be collected at this stage)

b) Collection of gametes or tissues

The collection of gametes, cells and tissues to be preserved may take placein the wild during collection of donor organisms; in aquaculture facilities; or at centralized processing facilities after transport of the donor organisms. Preservation of the material may likewise take place immediately after collection or after transportation to a more suitable processing site. Deterioration of the materials generally starts immediately after collection, but if effective transport protocols are applied, this deterioration can be offset by advantages of processing under improved conditions. Decisions on how to achieve the best net results are part of the gene banking strategy that depends on available practices, facilities and priorities, as determined by the gene bank's purpose.

Standardized, tested collection and processing protocols should be used, if available for the species or for other related species, and data on the chosen collection procedures (e.g., donor selection criteria, method of gamete collection, volume of collected gametes etc.) needs to be recorded in the gene bank database.

Critical requirements to consider in this step include:

- Appropriate facilities for handling of donor organisms and for material collection
- Anesthetics or other reagents for handling donors
- Containers and reagents to prepare materials for transport or preservation
- Qualified technical staff
- Data collection (see Chapter 10, Box 4: for the data that should be collected at this stage)

c) Processing

Processing is the core element of establishing effective gene banks and, in a cryopreservation process, includes those steps going from the dilution with extenders up to freezing. It is discussed in greater detail in Box 1, and in each of the taxa-specific chapters above (Chapter 5, 6 and 7). In general, processing requires clean laboratory space, or equivalent in the field, with standard equipment, consumables and some protocol-specific equipment. Processing details depend upon the chosen protocols, that may be species or taxonomic group specific, based upon research, standards and/or historical practice but also depends on other factors like the purpose of the gene bank and the type of processing facilities.

As mentioned above, deterioration of collected semen or other tissue generally starts as soon as it is collected. This is compensated for by preserving the material as soon after collection as possible and by developing processing protocols that optimize the quality of the preserved material. As optimal preservation generally requires refined laboratory conditions, and the stress of transporting donor organisms may also reduce the quality of collected material, a trade-off is often needed between "adequate" preservation of high-quality material at the collection site and transporting fresh material, with some deterioration, to where optimal preservation can take place. This will depend on the gene bank purpose and scope, the species of interest, and the collection situation. Strategies for cryopreservation have taken three main pathways to address the question of preserving sample quality from collection to processing: i) transport of live donor organisms closer to a central processing facility/specialized laboratory; ii) a "backpack" field method that brings processing capacity to the collection site of the organism; and iii) a self-contained mobile laboratory (a cargo trailer or a truck) that brings refined processing closer to the collection site (eg Childress et al., 2018) (see "transportation" discussion below").

On-site cryopreservation is a way to overcome these constraints: either with the "backpack" field method and the self-contained mobile laboratory are possible strategies for on-site cryopreservation. The "backpack" approach freezes samples directly in dry shippers carried to the collection site, with the advantage of immediate processing and freezing of high-quality gametes at remote sampling locations.

However, it is usually applied only for small-scale sample production (tens of samples per day) due to the limited size of the shipping dewars, and variability in freezing rates may compromise the quality of the preservation (Wayman and Tiersch, 2011).

Self-contained mobile laboratories, in a trailer or truck, are another approach to get quality processing closer to the collection site. Childress et al. (2018) describe in detail the design and functioning of a mobile laboratory and related investment costs. The trailer design described by these authors includes either standardized freezing over a liquid nitrogen pool (including 3D printed racks) or with a programmable freezer, microscope-based QA, and variable options of automation. The company Cryogenetics SA, similarly uses trucks designed as mobile laboratories to bring cryopreservation facilities close to the collection stie.

A key consideration for all gene banking activities is Quality Assurance (QA), particularly for goals that include long-term storage (Chapters 3 and 4). The investment in collection, processing, storage, and application can be substantial, it is therefore vitally important to also ensure that the samples are of high quality. On arrival to the processing facility, the samples should be checked for viability, and properly identified (including with supporting documentation of provenance) and characterized. For example, microscopic examination is used for checking for milt viability (ability to become mobile), or to validate the health and condition of algal and microbial samples, including checking for any signs of contamination.

Critical requirements to consider during sample processing include:

- Facility(s) to receive and process materials
- Research and testing of appropriate protocols
- Reagents and equipment, both general and protocol-specific
- Liquid nitrogen and associated containers for cryopreservation
- Trained technical staff with experience in the chosen protocols

Data collection (see Chapter 10, Box 4: for the data that should be collected at this stage)

d) Storage

Storage of preserved material is the main function of a gene bank repository. In the case of cryopreserved material, these consist of liquid nitrogen dewars or tanks, scaled to the quantity of material being stored. Key elements are the security and maintenance of the facility. Dedicated larger facilities are better at this, with specialized staff and purpose-specific equipment, such as controlled-temperature rooms with storage vats, temperature and oxygen monitors and alarms. Gene banking for conservation of endangered species or genetic material of particularly academic or commercial value, may require planning for decades of storage, with smaller facilities at greater risk of human, equipment, or funding fails or downfalls. Nevertheless, smaller purpose-specific gene banks are common and need to consider the risks associated with their facility. Ideally, to mitigate the risk of sample loss, at least the most valuable material is stored in two independent locations. This practice, however, is rarely followed, owing to the cost, but should be considered for particularly valuable samples.

Liquid nitrogen carries risks due to its extremely cold temperature, with serious burns resulting from skin contact. Too much nitrogen vapor can cause oxygen deprivation for the user and may result in asphyxiation, particularly if working in a small or enclosed environment. Appropriate safety considerations need to be in place. Alternatively, several algal gene banks make use of ultra-low temperature freezers for storage, but these have not been found to be adequately cold for long term storage of finfish or shellfish milt.

BOX 4

Liquid nitrogen handling

Liquid nitrogen is highly hazardous and requires careful handling. Its vapor can rapidly freeze the skin tissue and eye fluid, resulting in cold burns, frostbite, and permanent eye damage, even after a brief exposure. Therefore, the following measures should always be applied:

- Protective equipment, such as gloves, lab coats, and eye protection, must be weared at all times. To note that gloves should be loose-fitting so that they can be quickly removed if they come into contact with cryogenic liquid. To note that insulated gloves are not designed for direct immersion in cryogenic liquids, they only offer brief protection against accidental contact with the liquid.
- It should be avoided to wear shorts, sandals, or open-toed shoes.
- The use of purposely designed tongs to handle storage containers or straws is also strongly recommended as the extreme cold can alter the properties of materials, making standard tools unsafe for use.
- If liquid nitrogen contacts the skin, the area should be rinsed with cold water, treated with cold compresses, and a medical intervention should be sought.
- When liquid nitrogen vaporizes, it expands 695 times its original volume and lacks any detectable warning properties like odor or color. If enough nitrogen gas accumulates, there is a risk of oxygen depletion, which can lead to unconsciousness and in the most severe cases can be fatal. To prevent asphyxiation, liquid nitrogen should be handled only in properly ventilated areas.
- Whenever possible, liquid nitrogen should be transported in a well-ventilated cargo area rather than inside the passenger compartment. If transportation within the passenger area is unavoidable, at least one window must remain open to allow proper airflow and prevent oxygen displacement. Additionally, handlers should be aware of emergency procedures in case of accidental leaks or exposure.

References

https://ehs.research.uiowa.edu/liquid-nitrogen-handling

FAO. 2012. Cryoconservation of animal genetic resources. FAO Animal Production and Health Guidelines No. 12. Rome

Critical requirements of the storage component of the gene bank include:

- Personal safety equipment and routines
- Environmentally controlled and secure storage space, ranging from a corner of a lab to a dedicated room(s) in larger facilities.
- Liquid nitrogen supply or generator, and mechanisms to transfer equipment for liquid nitrogen (e.g., piping manifolds in larger facilities)
- Nitrogen dewars for sample storage, scaled to the size and purpose of the gene bank
    - 45 L nitrogen dewars are common in small facilities, but may range to several hundred L which are the core of larger gene banks
- Nitrogen dewars for storage of excess liquid nitrogen

- Temperature level and oxygen sensors and alarms
- Equipment and infrastructure to manage holdings
- Trained technical staff
- Data collection (see Chapter 10, Box 4: for the data that should be collected at this stage)

e) Thawing and use

The access by eventual users to stored material will depend upon the purpose and functionality of the gene bank. Public gene banks generally have protocols that allows access and use by qualified users of the general public (free or for a cost), whereas private gene banks will either have clients that own the stored material and/or will sell material to users.

Generally, preserved material is sent to the users in its frozen state, and thawed out or otherwise re-animated at the application site. Algal material, on the other hand, are more commonly re-animated at the gene bank and sent out as a live culture. Thawing or re-animation protocols vary between species and users, as described in Chapter 6. Whatever protocol is used for thawing, it needs to fit into a proven fertilization or other hatchery/production process, and effectiveness needs to be recorded. QA at this stage includes collecting data on the protocols used, motility and fertilization rates (or other measures of viability), and any other relevant information on development.

Critical requirements to consider during thawing and use of thawed samples include:

- Temperature controlled water bath for thawing (if needed)
- Thermistor to monitor temperature
- Equipment for fertilization/re-animation – specific to the type of organism and the culture facility
- Incubation equipment for the fertilized eggs or reconstituted material (as used in the culture facility)
- Microscope to monitor viability and cryopreservation success
- Technical staff involved (with expertise in shipping, thawing, and using of the preserved material)
- Data collection (see Chapter 10, Box 4: for the data that should be collected at this stage)

f) Transportation

Transportation can be required at various steps, including potential transport of the donor organisms, of the fresh collected materials, or of the preserved material. The decision for transport depends on the situation and species under consideration.

Transport of donor organisms

Transport of donor organisms may be required to get to locations that are better for collection of good material for preservation, depending on the species and situation. In general, the quality of gametes may deteriorate with the stress of transport, which needs to be taken into consideration in the choice of protocols. Low-stress transport methodologies will vary between species and taxa.

Transport of fresh materials to be preserved

Generally, the deterioration of collected materials starts immediately after the collection, influencing the quality of preservation. While protocols can slow this deterioration, there is a trade-off in the quality and quantity of material preserved between rapid field-based processing versus transportation to better equipped and controlled laboratory facilities.

Protocols for chilled transport or storage of finfish and shellfish semen or testes, before their cryopreservation at the processing facility, are relatively well developed for several species. These generally involve extenders that provide good *in vitro* conditions without triggering motility, though shorter storage/transport is also possible with undiluted semen. The use of sterile airtight and watertight containers is needed to prevent leakage and contamination, whilst the use of insulated packaging with ice packs or gel packs ensure that the temperature remains within the required range for samples requiring low temperatures. Samples can be protected against physical damage by using materials such as foam pellets or bubble wrap.

Transport of cryopreserved material

The transportation of cryopreserved material is required in the exchanges between gene banks and another user in the gene bank network. In the case of cryopreserved material, it is important to keep the preserved material at liquid nitrogen temperatures until it is thawed for use. Thawed material generally are only viable for a short period of time, depending on their nature (see below). Transportation thus requires the use of dry shippers. These shippers are insulated dewars that contain porous material cooled with liquid nitrogen but do not have free liquid nitrogen inside (if prepared correctly). This ensures safe transportation of material without the risks of liquid nitrogen spilling.

Critical requirements to consider during the transport of the genetic material include:

- Appropriate containers and protocols for transport, based on the species and established protocols:
    - Appropriate oxygenated transport tanks or other proven low-stress transport solutions
    - Appropriate containers, coolers and ice packs to maintain collected material fresh until processing
    - Dry shippers for transportation of cryopreserved materials
- Transportation arrangements, costs and import/export licences
- Trained human resources (field or lab technician)
- Data collection (see Chapter 10, Box 4: for the data that should be collected at this stage).

g) Data management

Data on the holdings in a gene bank are particularly important. Without usable data and adequate labelling, many years of storage can be wasted. This data should include origin, condition, and any genetic information of the source organism; collection and preservation protocols, any QA carried out on the samples, storage locations, eventual use, and results of its use. Box 4 in Annex 6 provides a comprehensive checklist of the critical information to collect at the various steps of a gene banking process. Smaller gene banks may use simple spreadsheets for data collection, whereas larger and more established gene banks have customized database softwares that may include searchable on-line presence. There are few off-the-shelf inventory management software available (e.g., FreezerPro). However, a gene bank may develop its own in-house database which is customized with regard to its specific needs and functioning.

Back-ups, storage of data, and cybersecurity is essential, as is monitoring and upgrading software so that it does not become obsolete.

Critical requirements for accurate data management include:

- Standardized data forms for each stage of gene banking
- Computers for data storage and management (with data backup processes in place)
- Database maintenance and security
- Database manager collecting data and managing the gene bank database
- Rules or regulations of access and rights to data (and samples) (also according to ABS, IPR etc.

Table 6. List of requirements by stage of the gene banking process. The main requirements are identified by item category. Some items can be needed at more than one stage, also depending on the strategy of the gene bank and the protocol adopted.

Item category	Item	Stage of the gene banking process					
		Collection	**Transport**	**Processing**	**Storage**	**Thawing & use**	**Data management**
Infrastructure	Trailer or truck		X	X			
	Facility for cell and tissue collection and handling		X	X	X		
	Clean facilities to receive and process materials			X			
	Storage facility (if separate from cells and tissues collection facility)			X	X		
	Culture facility (e.g. for cells and tissues culturing)					X	
Equipment and reagents	Reagents	X	X	X		X	
	Coolers boxes and ice packs		X	X			
	Dry shippers		X	X			
	Microscope			X		X	
	Sperm counting instruments			X			
	Temperature bath for equilibration			X		X	
	Freezing containers			X	X		

	Freezing apparatus			X			
	Thermistor to monitor temperatures			X	X	X	
	Liquid nitrogen			X	X		
	Liquid nitrogen storage dewar				X		
	Nitrogen dewars or tubs for specimen storage				X		
	Nitrogen dewars for extra nitrogen				X		
	Temperature and oxygen monitors			X	X		
	Liquid nitrogen generator (or liquid nitrogen source)				X		
	Personal protective gear			X	X	X	
	Transfer pump				X		
	Liquid nitrogen circulating pump				X		
	-80 °C freezer (algae only)				X		

	Temperature control bath			X		X	X
	Thermistor to monitor temperature			X	X	X	X
	Equipment for fertilization					X	
	Incubation equipment					X	
Permits	Collection permits & Access Sharing agreement	X					
	Shipping costs and permits		X				
Data management							
	Computers and software						X
	Sample datasheets	X	X	X	X	X	X
Key staff	Gene bank manager	X	X	X	X	X	X
	Field technician(s)	X	X	X	X	X	X
	Laboratory technician(s)	X	X	X	X	X	X
	Database manager	X	X	X	X	X	X

Costs

The costs of a gene bank can vary for different countries and situations. In addition, costs can fluctuate from one year to another. This makes it impossible to estimate costs in a fashion that is applicable to all gene banking contexts and countries. As a result, studies on the costs of AqGR gene banks are very limited. Caffey and Tiersch, (2000) and Childress et al.(2018) provide such an analysis for a mobile laboratory for cryopreservation of finfish or oysters sperm in the United States of America in 2016-17 (Tables 7 and 8). While the actual costs are specific to the United States of Aerica and outdated, the tables provide a starting framework for planning other cryopreservation facilities.

Table 7. Average prices and related expected life for the main equipment used for cryopreservation of aquatic species in a mobile laboratory (source: Childress et al., 2018).

Equipment	Average price (USD)	Useful life (Years)
Liquid nitrogen freezing tank (120L)	3,158	10
Liquid nitrogen generator (120L/d)	126,000-200,000	15
Storage dewar (35 L, high capacity)	1,574	15
Roller base for storage dewars	242	15
Low-level alarms (storage dewars, 115V)	949	10
Shipping Dewar (4.1 L, spill proof)	1,018	15
Case for shipping dewar	490	10
LN transfer hose	271	15
Phase Separator	90	10
Thermometer (digital, hand-held, -73 to 260°C)	258	10
Pipettor (1-10)	367	10
Pipettor (10-100)	367	10
Pipettor (100-1,000)	367	10
Water bath (10L, temperature to 100°C)	579	10
Analytical balance (1,000 - 3,000g max, 0.01 g readability)	1,038	10
Portable balance (3,000 - 4,000g max, 0.1 g readability)	666	10
Data logger (hand-held, 5 inputs)	157	10
Vapor pressure osmometer (0-2,000 mOsmol/kg)	3,132	10
Microscope (dark field)	4,981	15
Controlled freezer	34,722	10
Automated packager (1 straw)	36,490	10
Automated packager (4 straws)	73,774	10
Straw printer	36,490	10
Manual sealer	2,365	10
Mackler	783	10
Air vacuum (20" Hg)	266	10
Tent (10' X 10')	99	10

Table (6')	76	10
Trailer	2,933	10
Generator (5500 watts or more)	584	10
Supply case	83	10
Serological pipetting device 10 mL	46	10
Serological pipetting device 25 mL	55	10
Laboratory table	474	15
Shelving unit	57	10
High-capacity shipping dewar	1,584	15
Utility cart	143	-
Moving dolly	19	-
Cryo gloves	200	-

Table 8. Average prices and related unit prices for the main supplies and reagents used for cryopreservation of aquatic species (source: Childress et al., 2018).

Supplies	Average price (USD)	Unit price (USD)
Straws (0.5 mL)	0.06	0.06
Goblets (200)	39.03	0.20
Daisy Goblets (5)	44	8.73
Canes	23.25	0.23
Sealing powder (PVC, 454 g)	19	0.04
Ingredients for HBSS (ACS grade) 500 g each		
NaCl	29	0.06
KCL	40	0.08
$CaCl_2*2H_2O$	41	0.08
$MgSo_4*7H_2O$	61	0.12
Na_2HPO_4	50	0.10
KH_2PO_4	39	0.08
Na_2HCO_3	21	0.04
$C_6H_{12}O_6$	30	0.06
Cryoprotectants 500 mL each		
Dimethyl sulfoxide (DMSO)	40	0.08
n,n-dimethyl acetamide (DMA)	23	0.05
Methanol	14	0.03
Glycerol	44	0.09
Liquid Nitrogen Rental	65	-
Cryovials	223	0.45
Centrifuge tubes (15 mL) case of 500	148	0.30
Centrifuge tubes (50 mL) case of 500	253	0.51

Microcentrifuge tubes (1.5 mL) pack of 500	19	0.04
Pipettor tips (1-10uL) pack of 1,000	33	0.03
Pipettor tips (20-200uL) pack of 1,000	23	0.02
Pipettor tips (100-1000uL) pack of 1,000	27	0.03
Serological pipet (1 mL) pack of 1,000	218	0.22
Serological pipet (5 mL) pack of 500	216	0.43
Serological pipet (10 mL) pack of 500	232	0.46
Serological pipet (25 mL) pack of 200	215	1.08
Nalgene bottels (1L) case of 24	204	8.50
Type T thermocouple (5 pack)	56	11.27
Safety goggles	14	13.57
12" Tweezers	9	9.00
Syringe (1 mL - 100) pack of 100	59	0.59
Syringe (3 mL - 100) pack of 100	33	0.33
Sterile filters (.22 um) pack of 50	171	3.42
Exam Gloves	25	0.25
Kimwipe (60 boxes, 280 wipes per box)	180	0.01
MRS1 0.5-mL Disposable Needle (Box of 60)	50	0.84
MRS1 0.25-mL Disposable Needle (Box of 100)	60	0.60
Microcentrifuge tubes (5 mL) pack of 250	70	0.28
Ziploc storage bags (quart) box of 48	4.31	0.09

References

Caffey, R. H., and T. R. Tiersch. 2007. Cost analysis for integrating cryopreservation into an existing fish hatchery. First published: 03 April 2007. Available at: https://doi.org/10.1111/j.1749-7345.2000.tb00697.x.

Childress, W. C., R. H. Caffey, and T. R. Tiersch. 2018. Design and cost analysis of a self-contained mobile laboratory for aquatic species cryopreservation. Journal of the World Aquaculture Society, 49: 805–826.

FAO. 2012. Cryoconservation of animal genetic resources. FAO Animal Production and Health Guidelines No. 12. Rome, FAO.

Wayman, W. R., and T. R. Tiersch. 2011. Research methods for cryopreservation of sperm. In: Cryopreservation in aquatic species. Second edition, edited by T. R. Tiersch and C. C. Green, pp. 672–683. Baton Rouge, Louisiana, USA, World Aquaculture Society.

Chapter 10. Future directions

Terrence Tiersch, Aquaculture Germplasm and Genetic Resources Center (AGGRC), Louisiana State University Agricultural Center, Baton Rouge, Louisiana, United States of America
Joachim Carolsfeld, World Fisheries Trust, Victoria, Canada

Introduction

For reasons already identified in this FAO document, it is becoming increasingly necessary to develop repositories to protect the genetic resources of aquatic species around the world. The lack of resources and capability to safeguard aquatic genetic resources for food and agriculture (AqGR) is a looming crisis that threatens the livelihood and food security of a large percentage of humanity and imperils the existence of thousands of aquatic organisms. Basic research can still play an important role in addressing this global problem, but there should be serious consideration to ensuring that the work can lead to application (Tiersch and Green, 2011). Looking inward, within the field, there is a significant opportunity to address reproducibility of outcomes, standardization of approaches and reporting, and generalization of process pathways. Looking outward, into other fields, there is opportunity for multidisciplinary and interdisciplinary collaboration to scale up production capability, develop efficient high-throughput systems, establish quality management programs, and develop aggregate throughput through open hardware. Fortunately, these needs are not independent or mutually exclusive, and advances made in addressing one can be used for others, especially if they can be viewed as simultaneous interacting factors that can be addressed holistically rather than as separate, perhaps conflicting, solutions to a roster of individual problems. For example, a checklist/worksheet approach has been proposed that would simultaneously address problems with reproducibility and standardization (Torres and Tiersch, 2018). This approach can be used at the level of scientific journals as a checklist for reviewers, thus encouraging broad acceptance across the research community while standardizing the way to protocol development (Box 4).

BOX 4

Proposed checklist for data collection and publications on cryopreservation and gene banking

Terrence Tiersch

The checklist below would help improving the reproducibility and standardization of reporting of research studies on cryopreservation and gene banking. This constitutes a guideline to the factors that should be recorded during the work, as they may have an impact on the success of the cryopreservation and should be reported out to allow for comparisons with the work of others. For simplicity, major activities were arranged chronologically in three groupings, although other arrangements could be developed. A checklist such as this could be employed by journals that publish cryopreservation results, and it would probably best be implemented in two phases (indicated in brackets) to allow gradual changes in practices. For example, *Phase 1* could include data and practices required in every publication to be phased in within a 1–2 year period, and *Phase 2* could include data and practices required in every publication within a 5-year period. The following list is intended only to stimulate discussion. Terms requiring specific definition in publications are indicated in bold.

A. Source, housing, conditioning, and transport

- [1] Specify **farmed type** or wild stock and source of organisms
- [1] Specify culture conditions, including water quality, **photoperiod**, and stocking density
- [1] Provide description (e.g., water quality), and GPS[14] coordinates of the collection site
- [1] Provide descriptive statistics for the body size, **condition factor**, estimated age, **reproductive stage**
- [1] Specify if the collection took place during the breeding period and describe **secondary sex characteristics**
- [1] Provide information describing reproductive condition (e.g., **sperm volume** and **GSI**[15])
- [1] Specify conditioning regime, including diet, photoperiod, or hormone treatments
- [2] Specify disease screening information for organisms and populations
- [1] Specify how long organisms were in transit, the water quality and changes during transport

B. Sperm collection, freezing, and cryogenic storage

- [1] Provide **male selection criteria**
- [1] Specify method of sperm collection (e.g., were the fish dissected or stripped?)
- [1] Specify volume of collected sperm
- [1] Provide protocol and data on **initial sperm quality analyses**
- [1] Specify composition, osmotic pressure, and pH of any extenders or buffers
- [1] Provide **sample quality selection criteria**
- [1] Specify if sperm samples were **pooled** or not at collection
- [1] Specify **sample dilution ratios**
- [1] Provide protocol for sperm concentration estimation
- [1] Provide **initial** and **adjusted sperm concentrations**
- [1] Specify storage method and storage duration if freezing is not immediate
- [2] Provide details about sample transport (e.g., transit time and temperature at arrival)
- [1] Specify if samples were pooled before freezing
- [1] Provide cryoprotectant **final concentration**
- [1] Specify cryoprotectant addition method
- [1] Provide protocol and data on **pre-equilibration** sperm quality analyses
- [1] Specify and define **equilibration time** (after addition of cryoprotectant, before freezing)
- [2] Specify packaging container (e.g., catalog number), sample labeling method, sample loading volume, and container sealing method
- [1] Provide protocol and data on **post-equilibration** sperm quality analyses
- [1] Provide standardized **thermal mass value** (number of samples frozen per batch)
- [1] Specify placement of containers in the freezing chamber
- [1] Provide freezing protocol when using dry ice
- [1] Provide controlled-rate freezer manufacturer and model
- [1] Provide **definition of cooling rate**, including **freezing curve** (specified time vs. temperature), and sample, chamber and programmed cooling rates
- [1] Specify freezing termination steps
- [1] Specify methods for sample sorting, storage, and storage duration
- [1] Specify protocol for handling of frozen samples

[14] GPS: global positioning system
[15] GSI: gonadosomatic index

- [2] Specify disease screening information for frozen samples
- [1] Specify transport method for frozen samples

C. Egg collection, thawing, and use

- [2] Provide information on conditioning regime, health status, and reproductive condition of females[1] Specify egg collection method
- [1] Specify **egg storage** duration and conditions
- [1] Specify if eggs were **pooled**
- [2] Provide information on biosecurity precautions for fertilization trials
- [1] Specify time intervals before and after thawing and thawing method
- [1] Specify post-thaw centrifugation method, if used
- [1] Specify if thawed samples were **pooled**
- [1] Specify **interval before fertilization** (i.e., transfer time of samples after thawing before gamete activation)
- [1] Provide fertilization protocol, **total fertilization volume**, and **sperm-to-egg ratio**
- [1] Specify final **working concentration** of sperm (e.g., number of motile sperm per milliliter)
- [1] Provide protocol for water hardening step
- [2] Provide data on toxicity evaluation of cryoprotectants to gametes and early life stages
- [2] Provide protocol and data on **post-thaw sperm quality analyses**
- [2] Provide **egg quality assessment** data (and **control treatments**)
- [1] Specify experimental design
- [1] Provide definitions of f**ertilization** and **hatching**

GPS = global positioning system; GSI = gonadosomatic index

References

Torres, L., Tiersch, T.R. Addressing Reproducibility in Cryopreservation, and Considerations Necessary for Commercialization and Community Development in Support of Genetic Resources of Aquatic Species. J World Aquac Soc. 2018 Aug;49(4):644-663. doi: 10.1111/jwas.12541. Epub 2018 Jun 28. PMID: 30467453; PMCID: PMC6241537.

Generalization

Another important approach for the future will be to expand and test mechanisms for generalization. There is good evidence and experience that effective conservation protocols vary between species, or between larger taxonomic groups. However, the physiological basis of this diversity remains largely unknown, even for the better studied human spermatozoa (Ozimic *et al.*, 2023), and protocols tend to be developed on an empirical basis. As such, patterns of biological diversity are masked by the diversity of researcher preferences and historical practice. The pervasive lack of standardization in protocols and reporting greatly limits evaluation and direct comparison of research results (Tiersch *et al.*, 2007, Yang *et al.*, 2010). Improved standardization for methods and reporting, as described above, should help generalization (consolidation) of protocols for a variety of species. It is possible to rapidly develop practical approaches for previously unstudied species. Several recent publications test protocols in different species, while still empirical, are reporting much of the above matrix of details (e.g., Horvath *et al.*, 2003, 2010; Maulida *et al.*, 2021). Service providers like Cryogenetics are adapting proven protocols to a variety of species with best available knowledge (Annex 13). More generalized protocol guidelines may thus be on the horizon, given continuity of these trends and their consolidation.

Interdisciplinary collaboration

The different cryopreservation solutions need to be applied and generalized to avoid unnecessary species-by-species research. In addition, many of the needs for the development of a repository can be addressed using tools from other disciplines like industrial engineering (Hu *et al.*, 2013, 2015). Industrial engineering can provide powerful tools specifically designed to solve problems related to scaling up, high-throughput systems, process efficiency, and quality management (Bodenstein et al., 2023). These tools are well-established and are in practice throughout manufacturing and other industries.

Other engineering fields can be brought in as well to make standardized cryopreservation widely available through open hardware. This can involve the creation of open scientific hardware to support cryopreservation and repository development, as well as the establishment and expansion of open-source environments, communities, and networks (Liu *et al.*, 2021). The goal is to provide innovative solutions to real challenges in repository development, especially those at the community-level, that would otherwise be difficult to solve by traditional biological research.

The feasibility of using existing livestock cryopreservation facilities for aquatic species has been reviewed (Caffey and Tiersch 2000a, Lang *et al.*, 2003, Dong *et al.*, 2005b, Dong *et al.*, 2007b) with consideration for adaption of equipment (e.g., Hu *et al.*, 2011). Cryogenic equipment specific to aquatic resources is limited in availability but the conditions for freezing and using AqGR can be different from those used for terrestrial material. Custom solutions have been developed, though these may be difficult to reproduce. The use of 3-D printers with open-source designs can help make such specific solutions more readily available in standardized form (e.g., Tiersch and Monroe, 2016; Hu *et al.*, 2017).

A particularly important additional challenge is the lack of database models for AqGR repositories. While existing repositories have generally built their own custom databases, none are available for new repositories to use in their development. Collaboration between repositories and with computer science are important in facilitating such development, and a global library of holdings would be very beneficial.

Enabling the transition from research to repositories

The past and current emphasis in *ex situ in vitro* research of aquatic species has been on traditional academic endpoints such as publication of peer-reviewed articles, and this consequently has focused on details that often do not have direct relevance to levels beyond the research on protocols, such as the development of working repositories. The pervasive lack of standardization in protocols and reporting also greatly limits evaluation and direct comparison of research results (Tiersch *et al.*, 2007, Yang *et al.*, 2010). If the attention is placed at higher operational levels from the beginning, it is possible to rapidly develop practical approaches for previously unstudied species. With planning and practice, significant progress towards workable protocols can be made within a week with a two-person team. This work would be guided by considerations such as the form and availability of biological material, the context of the application, and the availability of resources by the intended users. Such a translational research approach could be informed by the history and practices of established livestock industries.

Another key challenge is the lack of consistent funding and policy support. Support for the establishment of gene banks is often difficult as it requires long-term investment in a safety measure that may never be

used. Increased public appreciation of the potential and importance of gene banks should be part of the strategy to ensure financial and policy support. In this sense, enhancing science popularization and public display of gene banks is crucial for raising awareness about biodiversity conservation and the value of genetic resources. For instance, the Animal Germplasm Resources Information Network (A-GRIN) established by United States Department of Agriculture (USDA) serves as an online portal offering information about agricultural genetic resources (including AqGR) managed by the USDA's Agricultural Research Service, making data accessible to both researchers and the general public. While the primary role of germplasm banks is the conservation and provision of genetic resources for research and breeding, there is a clear and growing need to enhance science popularization and public display. By doing so, these institutions can foster greater public understanding and support for biodiversity conservation efforts.

References

Bodenstein, S., Nahmens, I., Callam, B. & Tiersch, T.R. 2023. Simulation analysis of high-throughput oyster cryopreservation at three scales of production. *Aquaculture International*. Published online: 7 May 2023. https://doi.org/10.1007/s10499-023-01139-y

Hu, E., Liao, T.W. & Tiersch, T.R. 2013. A quality assurance initiative for commercial-scale production in high-throughput cryopreservation of blue catfish sperm. *Cryobiology*, 67: 214–224.

Hu, E., Liao, T.W. & Tiersch, T.R. 2015. Simulation modeling of high-throughput cryopreservation of aquatic germplasm: a case study of blue catfish sperm processing. *Aquaculture Research*, 46: 432–445.

Liu, Y., Monroe, W.T., Belgodere, J., Choi, J.W., Gutierrez, M.T. & Tiersch, T.R. 2022. The emerging role of open technologies for community-based improvement of cryopreservation and quality management for repository development in aquatic species. *Animal Reproduction Science*, 246: 106871. https://doi.org/10.1016/j.anireprosci.2021.106871

Tiersch, T.R. & Green, C.C., eds. 2011. *Cryopreservation in Aquatic Species: A Comprehensive Overview of Current Practices, Programmatic Development and Future Directions for Cryopreservation of Gametes, Embryos and Larvae of Aquatic Species*. 2nd edition. Baton Rouge, Louisiana, USA, World Aquaculture Society, Advances in World Aquaculture. 1003 pp.

Torres, L. & Tiersch, T.R. 2018. Addressing reproducibility in cryopreservation, and considerations necessary for commercialization and community development in support of genetic resources of aquatic species. *Journal of the World Aquaculture Society*, 49(4): 644–663. https://doi.org/10.1111/jwas.12541

Annex 1
Representative protocols for finfish milt cryopreservation

Elsa Cabrita, Center for Marine Science (CCMAR), University of Algarve, Faro, Portugal
Francisca Felix, Center for Marine Science (CCMAR), University of Algarve, Faro, Portugal
José Beirão, Nord University, Bodo, Norway

Introduction

There are many protocols developed for cryopreservation of finfish milt, many of them reviewed in Cabrita et al., (2009); Martínez-Páramo et al., (2017); and Tiersch and Green (2011). This Annex provides protocols for some key finfish species used for aquaculture production and in which cryopreservation has a role in their production.

Protocols for cryopreservation of freshwater finfish species

Cryopreservation of sperm from rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*)

(Nynca et al. 2017., Ciereszko et al., 2014; Judycka et al., 2018, 2019)

1. Prepare the extender media containing 0.15 M glucose and 7.5 percent methanol as cryoprotectants.
2. Maintain the extender media on ice.
3. Strip sperm from mature fish avoiding urine contamination.
4. Dilute sperm 1:5 in extender solution or adjust to sperm concentration (0.5–1 x10^9 cells/ml for rainbow trout or 4 x10^9 cells/ml for Atlantic salmon) and fill 0.5 ml straws during the 15 min equilibration time. Straw filling can be done one by one manually or using larger automatic filling systems.
5. Fill the bottom of a styrofoam box with liquid nitrogen where straws will be frozen.
6. After the equilibration time, place the straws on a floating rack[16] 3 cm above the liquid nitrogen for 5 min. If using 0.25 ml straws, reduce the liquid nitrogen vapour exposure time to 3 min.
7. Samples are thawed in a 40ºC water bath for 5–10 seconds, depending on the type of straws used. Larger straws are thawed for longer.

This protocol has also been used for masu salmon (*Oncorhynchus masou*) with some small adaptations (Fujimoto *et al.*, 2022).

[16] These floating trays are generally a raft consisting of a metal or polystyrene rack mounted on Styrofoam blocks of a protocol-specific height that floats on a liquid nitrogen pool in the bottom of a Styrofoam cooler. the milt on the tray in nitrogen vapours. As described in Appendix 6, this tray unit can also be 3-D printed. Babiak *et al.* (2008) report that a tray 4 cm above the surface of liquid nitrogen is approximately at a temperature of -80ºC.

Cryopreservation of sperm from sturgeon species: sterlet (*Acipenser ruthenus*), siberian sturgeon (*A. baerii*), Russian sturgeon (*A. gueldenstaedtii*) and beluga (*Huso huso*)

(Nascimento et al. 2021; Judynka et al., 2015; Fopp-Bayat et al., 2023; Igna et al., 2022)

1. Prepare the extender media at pH 8, as follows:
   a. for *Acipenser ruthenus:* 23.4 mM sucrose, 0.25 mM KCl, 30 mM Tris-HCl;
   b. *Acipenser baerii and Huso huso:* 100mM glucose; *and*
   c. *Acipenser gueldenstaedtii:* 30 mM sucrose, 1 mM KCl, 25 mM Tris.
2. Add 10-15 percent methanol to the extender. There are also good results using 10 percent DMSO.
3. Strip sperm from mature fish. Hormonal induction may be necessary, therefore: for *A. ruthenus* use 4 mg/Kg BW carp pituitary; and for *A. baerii*, *A. gueldenstaedtii* and *H. huso* use a LHRHa injection dose varying from 0.03 mg/Kg BW (*A. baerii* and *H. huso*) and 20 µg/Kg BW for Russian sturgeon. Sperm can be collected between 20-36 h after hormonal injection.
4. For *Acipenser* species, dilute sperm 1:1 in the extender solution whilst for *H. huso* dilute 1:2. Leave sperm in contact with the extender for 10 min (equilibrium time). In some species such as *A. baerii*, the equilibrium time can be extended until 30 min and the sperm stored in 0.25 ml straws. For other species, 0.5 ml straws are used.
5. Fill the bottom of a styrofoam box with liquid nitrogen where straws will be frozen on a rack suspended 3-4 cm above the liquid nitrogen surface for 5–10 min. Beluga sperm has been frozen in a programmable freezer with a freezing rate of 0.4ºC/min from 0ºC to 4ºC followed by 32ºC/min to -160ºC.
6. Samples are thawed in a 35- 40ºC water bath for 6-10 s.

Cryopreservation of sperm from catfish species includes protocols for European catfish (*Silurus glanis*), African catfish (*Clarias gariepinus*), and blue catfish (*Ictalurus furcatus*)

An extensive review can be consulted in the book chapter Viveiros (2011) and in Boker et al., (2010, 2019); Hu et al., (2011); Horvath and Urbanyi,(2000); Kovács et al., (2010)

1. For the African and European catfish, prepare an extender media containing 6 percent fructose and 20 percent and 10 percent methanol, respectively. For African catfish, 10 percent DMSO and 6 percent glucose can also produce good results.
2. For the blue catfish, Hanks balanced salt solution (300 mOsm/Kg) is used as extender adding 10 percent methanol.
3. For sperm collection, fish are usually sacrificed to remove the testis after anesthesia overdose or by a sharp blow to the head. Testis are then sliced and squeezed in a mesh bag or passed through sequential mesh strainers (1 mm to 200 µm), collecting exuded sperm either in petri dishes or tubes, according to the recovered volume. Sperm hand-stripping is rarely used due to the difficulty in obtaining sperm with this method in the catfish. Hormonal induction of 4 mg/Kg BW of carp pituitary or 10–15 mg GnRHa plus 2.5–3 mg metoclopramide 10–12 hours before collection can be used to obtain higher sperm amounts, though hand stripping remains challenging.
4. Dilute sperm 1:1 in the extender solution or to a sperm concentration of 1 $x10^9$ cells /ml, and allow for equilibration, while filling the cryostraws of vials.
5. Sperm is frozen in 0.5 ml straws for blue catfish and African catfish, though higher volumes of 4-5ml straws and 10 ml cryotubes are also used for the European catfish.

6. Freezing is carried out in nitrogen vapour on a rack 3 cm above liquid nitrogen in a styrofoam cooler for 3 to 7 min depending on the type of cryo container, or in a programmable freezer at 45ºC/min.
7. Thawing is carried out in a 40ºC water bath for 13 to 35 seconds, depending on the type of straws used. The larger 10 ml cryotubes require up to 4 min to thaw.

Similar protocols for Channel catfish (*Ictalurus punctatus*) are also described in the references.

Cryopreservation of sperm from Common carp (Cyprinus carpio)

Bernath et al., (2016)

The large number of different protocols and the lack of comparable procedural information in many of them make difficult to select the most efficient method (Bernath *et al.*, 2016). The present protocol was developed by Bernath *et al.* (2016), to standardize the procedures for this species.

1. Prepare the extender containing 200 mM glucose, 40 mM KCl, 30 mM Tris, (pH 8) or Hanks Balanced Salt Solution (HBSS)[17], both containing 10% methanol.
2. To strip the fish, 2.5 mg/Kg BW of carp pituitary can be used to induce hormonally the sperm release 24–48 h post injection.
3. Before sperm collection, clean and dry the genital pore to prevent sperm activation by contamination from water or urine. Sperm is hand-stripped into 10 ml tubes.
4. Dilute sperm 1:9 in the extender solution, though up to a 1:20 dilution can be used to increase the volume and still maintain sperm functionality. Sperm is equilibrated in the extender for up to 60 min. During the equilibration time, 0.5 ml straws are filled and placed in a rack.
5. Freeze samples in either nitrogen vapor for 3 minutes (on a rack 3 cm above liquid nitrogen surface in a Styrofoam cooler) or in a programmable freezer with a 56ºC/min freezing rate.
6. Samples are thawed for 13s in a water bath of 40ºC.

Cryopreservation of sperm from Indian carp (*Labeo rohita*) and orangefin labeo (*Labeo calbasu*)

Routray et al., (2003, 2006, 2020)

1. Prepare an extender-of 750 mg NaCl, 20 mg KCl, 20 mg $CaCl_2$, 20 mg $NaHCO_3$ per 100 ml water; (osmolality of 280–300 mOsm/kg) combined with 10 mg glucose (pH 7.2) and 15–20 percent DMSO.
2. To ensure adequate milt, the fish is injected intraperitoneally with 0.2 mL/kg of Ovaprim (Salmon GnRH+ Domperidone) 5 hours prior to sperm collection.
3. Milt is stripped from the fish into a collection test-tube, cleaning the genital pore beforehand to prevent contamination by water or urine.
4. Dilute sperm 1:3 to 1:4 in the extender solution. The equilibrium time must be 30–45 min at low temperatures of 4–10ºC.

[17] Calculation of composition of different volumes available at: www.aatbio.com/resources/buffer-preparations-and-recipes/hbss-hanks-balanced-salt-solution; also available commercially.

5. During the equilibration time, 0.25–0.5 mL straws or 5 mL visotubes[18] should be filled and sealed, with poly-vinyl alcohol (PVA) powder as per manufacturer's instructions. Immediately after, straws are placed in a floating rack over liquid nitrogen for 5 min, while visotubes are frozen in a programmable freezer at a rate of -15ºC/min to -60ºC before being transferred to liquid nitrogen.
6. To thaw the samples, place them in a 38 ± 1ºC water bath for 8-10 s (straws) or 50-70 s (visotubes).

Protocols for cryopreservation of marine finfish species

For marine finfish, sperm cryopreservation protocols have been developed for several species, but applications have been limited mostly to species with artificial fertilization. Protocols are reported here for turbot (*Scophthalmus maximus)* and halibut (*Hippoglossus hippoglossus*), two commercial species (Suquet *et al.*, 2009; Babiak, 2009) where sperm cryopreservation has been used in genetic programs for specific crosses and to produce all-female fish (Cabrita, personal communication).

Cryopreservation of sperm from turbot (*Scophthalmus maximus*)
(Suquet et al., 2009)

1. Prepare a simplified Mounib extender containing 100 mM $KHCO_3$, 125 mM sucrose, 6.5 mM reduced glutathione, 10 mg/ml bovine serum albumin (BSA) and 10 percent DMSO.
2. Sperm collection is made by stripping ripe males after the urinary bladder has been cleared out by gentle abdominal pressure and the genital papilla dried using absorbent paper. Milt can be collected using a 1 mL syringe.
3. Dilute sperm 1:2 in extender solution and fill 0.25 ml straws. These are frozen on a rack 6.5 cm above the surface of liquid nitrogen for 15 min.
4. Plunge samples into liquid nitrogen.
5. Samples are thawed in a water bath at 30ºC for 5 s.

This protocol has been used in research and for commercial production to perform specific crosses. For commercial purposes, the use of larger volume cryovials is a more practical way of handling sperm during thawing and fertilization.

Cryopreservation of sperm from halibut (*Hippoglossus hippoglossus*)
(Babiak et al., 2008; Babiak,, 2009)

1. Prepare an extender containing 70 mM NaCl, 1.5 mM KCl, 2.7 mM $CaCl_2$, 25 mM $NaHCO_3$, 6.1 mM MgCl, 200 mM glucose, 10 mg/ml bovine serum albumin (BSA) and 10 percent v/v DMSO. Alternatively, 10 percent v/v methanol or dimethylacetamide (DMA) can be used instead of DMSO as cryoprotectants.
2. Sperm is collected by stripping where 5–100 ml can be easily recovered from each male, especially during the beginning and middle of the reproductive season. Milt has high viscosity at the end of

[18] Triangular, polygonal or round coloured-coded tubes designed to fit together in a larger goblet for storage. Designed to hold straws but are also used themselves for freezing of higher volumes of milt. Sealed with sealing powder once filled.

the reproductive season and low motility. After collection, always maintain sperm tubes in crushed ice.

3. Dilute sperm 1:3 in the extender solution and fill straws or cryotubes (0.5 ml up to 5 ml). These are frozen on a floating rack 4 cm above the surface of liquid nitrogen for 5-15 min, depending on using straws or cryovials, respectively. No equilibration in the extender solution is needed.
4. The floating rack should be maintained 4 cm from the surface of the liquid nitrogen.
5. Thaw samples in a water bath set at 40ºC for 3 s (straws) and 10 s (bigger cryovials).
6. Dilute the thawed sperm in Hanks Balanced Salt Solution (HBSS) from 1:4 to 1:10 to dilute the cryoprotectant.
7. Sperm is ready for fertilization for up to 2 h after thawing.

This protocol has been used in research and commercial production, in particular for the use of all-female sperm.

References

Asturiano, J.F., Cabrita, E., Horváth, Á. 2017. Progress, challenges and perspectives on fish gamete cryopreservation: a mini-review. *Gen. Comp. Endocrinol.* 245, 69–76.

Babiak, I. 2009. Cryopreservation of Atlantic halibut sperm. *Hippoglossus hippoglossus*. In: Methods in Reproductive Aquaculture: Marine and Freshwater species, Cabrita E, Robles V, Herráez MP (eds.), Taylor and Francis group, CRC press, Boca Raton 433–436.

Babiak, I, Bolla, S., and Ottesen, O. 2008. Suitable methods for cryopreservation of semen from Atlantic halibut, *Hippoglossus hippoglossus* L. *Aquaculture International* 16:561–572. DOI 10.1007/s10499-008-9167-9.

Beirão, J., Boulais, M., Gallego, V., O'Brien, J.K., Peixoto, S., Robeck, T.R., Cabrita E. 2019. Sperm handling in aquatic animals for artificial reproduction. *Theriogenology* 133, 161–178.

Beirão, J., Lewis, J., Purchase, C.F. 2015. Spermatozoa ultrastructure of two osmerid fishes in the context of their family (Teleostei: Osmeriformes: Osmeridae). *Journal of Applied Icthyology* 31, 28–33.

Bernáth, G., Żarski, D., Kása, E., Staszny, Á., Várkonyi, L., Kollár, T., Hegyi, Á., Bokor, Z., Urbányi, B., Horváth, Á. 2016. Improvement of common carp (*Cyprinus carpio*) sperm cryopreservation using a programmable freezer. *General and Comparative Endocrinology*, 237, 78–88.

Billard, R., Cosson, J., Noveiri, S. B., & Pourkazemi, M. 2004. Cryopreservation and short-term storage of sturgeon sperm, a review. *Aquaculture*, 236(1-4), 1–9.

Blaxter, J.H.S. 1953. Sperm storage and cross-fertilization of spring and autumn spawning herring. *Nature* 26, 1189–1190.

Bokor, Z., Bernáth, G., Várkonyi, L., Molnár, J., Láng, Z. L., Tarnai-Király, Z., Solymosi, E., & Urbányi, B. 2019. The applicability of large-scale sperm cryopreservation in wels catfish (*Silurus glanis*) optimized for hatchery practice. *Aquaculture*, 506, 337–340.

Bokor, Z., Urbányi, B., Horváth, L., & Horváth, Á. 2010. Commercial-scale cryopreservation of wels catfish (*Silurus glanis*) semen. *Aquaculture Research*, 41(10), 1549–1551.

Boryshpolets, S., Kholodnyy, V., Cosson, J., Dzyuba, B. 2020. Fish sperm quality evaluation after cryopreservation. In: Betsy, J., Kumar, S. (Eds.), *Cryopreservation of fish gametes*, Springer, Singapore, pp. 117–133.

Cabrita, E., Engrola, S., Conceição, L.E.C., Pousão-Ferreira, P., Dinis, M.T. 2009. Successful cryopreservation of sperm from sex-reversed dusky grouper, *Epinephelus marginatus*. *Aquaculture* 287, 152–157.

Cabrita, E., Horváth, A., Marinović, Z., Asturiano, J.F. 2022. Technologies and strategies for ex situ conservation of aquatic organisms: the role of cryopreservation in long-term management. In: Monzón, I., Fernandes, J. (Eds.), *Cellular And Molecular Approaches In Fish Biology*, Springer, Berlin, pp. 1–48.

Cabrita, E., Ma, S., Diogo, P., Martínez-Páramo, S., Sarasquete, C., Dinis, M.T. 2011. The influence of certain aminoacids and vitamins on post-thaw fish sperm motility, viability and DNA fragmentation. *Anim. Reprod. Sci.* 125 (1–4), 189–195.

Cabrita, E., Martínez-Páramo, S., Gavaia, P.J., Riesco, M.F., Valcarce, D.G., Sarasquete, C., Herráez, M.P., Robles, V. 2014. Factors enhancing fish sperm quality and emerging tools for sperm analysis. *Aquaculture* 432, 389–401.

Cabrita, E., Pacchiarini, T., Fatsini, F., Sarasquete, C., Herráez, M.P. 2023. Post-thaw quality assessment of testicular fragments as a source of spermatogonial cells for surrogate production in the flatfish *Solea senegalensis*. *Fish Physiology and Biochemistry*. https://doi.org/10.1007/s10695-023-01232-2

Cabrita, E., Robles, V., Cuñado, S., Wallace, J.C., Sarasquete, C., Herráez, M.P. 2005. Evaluation of gilthead sea bream, *Sparus aurata*, sperm quality after cryopreservation in 5ml macrotubes. *Cryobiology* 50 (3), 273–284.

Cabrita, E., Sarasquete, C., Martínez-Páramo, S., Robles, V., Beirão, J., Pérez-Cerezales, S., Herráez, M.P. 2010. Cryopreservation of fish sperm: applications and perspectives. *Journal of Applied Ichthyology* 26, 623–635.

Chatterjee, A., Saha, D., Niemann, H., Gryshkov, O., Glasmacher, B., Hofmann, N. 2017. Effects of cryopreservation on the epigenetic profile of cells. *Cryobiology*, 74, 1–7.

Chenais, N., Depince, A., Le Bail, P.-1 Y., Labbe, C. 2014. Fin cell cryopreservation and fish reconstruction by nuclear transfer stand as promising technologies for preservation of fish genetic resources. *Aquacult. Int.* 22, 63–76.

Ciereszko, A., Dietrich, G. J., Nynca, J., Dobosz, S., & Zalewski, T. 2014. Cryopreservation of rainbow trout semen using a glucose-methanol extender. *Aquaculture*, 420, 275–281.

Cosson, J. 2019. Fish sperm physiology: structure, factors regulating motility, and motility evaluation. *IntechOpen London*.

Depincé, A., Gabory, A., Dziewulska, K., Le Bail, P. Y., Jammes, H., & Labbé, C. 2019. DNA methylation stability in fish spermatozoa upon external constraint: Impact of fish hormonal stimulation and sperm cryopreservation. *Molecular Reproduction and Development*, 87(1), 124–134.

Diogo, P., Martins, G., Quinzico, I., Nogueira, R., Gavaia, P.J., Cabrita, E. 2018. Electric ultrafreezer (-150 °C) as an alternative for zebrafish sperm cryopreservation and storage. *Fish Physiol. Biochem.*, 1–13.

Emel'yanova, N.G., Pavlov, D.A. 2020. Spermatozoa morphology of Goatfishes (Mullidae): *Upeneus sulphureus*. *Journal of Ichthyology* 60, 71–78.

FAO. 2019. The State of the World's Aquatic Genetic Resources for Food and Agriculture. FAO Commission on Genetic Resources for Food and Agriculture assessments. Rome.

FAO. 2022. The State of World Fisheries and Aquaculture 2022. Towards Blue Transformation. Rome, FAO. https://doi.org/10.4060/cc0461en

FAO. 2023. Database of Farmed types in Aquaculture. In: Food and Agriculture Organization of the United Nations [online]. Rome. Database version 1-2023. www.fao.org/fishery/aquagris/home

Félix, F., Oliveira, C.C.V., Cabrita, E. 2021. Antioxidants in fish sperm and the potential role of melatonin. *Antioxidants*. 10, 36.

Fitzpatrick, J.L. 2020. Sperm competition and fertilization mode in fishes. *Phil. Trans. R. Soc. B* 375: 20200074. http://dx.doi.org/10.1098/rstb.2020.0074

Fopp-Bayat, D., Nitkiewicz, A., Sarosiek, B., & Kowalski, R. K. 2023. The successful production of "sterbel" hybrids using beluga (*Huso huso*) cryopreserved sperm. *Animal Reproduction Science*, 249, 107197.

Franěk, R., Marinović, Z., Lujić, J., Urbányi, B., Fučíková, M., Kašpar, V., Pšenička, M., Horváth, Á. 2019a. Cryopreservation and transplantation of common carp spermatogonia. *PLoS One* 14 (4), e0205481.

Franěk, R., Pšenička, M. 2020. Cryopreservation of germ stem cells in fish. In: Betsy, J., Kumar, S. (eds.). *Cryopreservation of Fish Gametes*. Springer, Singapore.

Franěk, R., Tichopád, T., Steinbach, C., Xie, X., Lujić, J., Marinović, Z., Horváth, Á., Kašpar, V., Pšenička, M. 2019b. Preservation of female genetic resources of common carp through oogonial stem cell manipulation. *Cryobiology* 87, 78–85.

Gallego, V., Asturiano, J.F. 2019. Fish sperm motility assessment as a tool for aquaculture research, a historical approach. *Rev. Aquac.* 11, 697–724.

Horvath, A., Urbányi, B. 2000. The effect of cryoprotectants on the motility and fertilizing capacity of cryopreserved African catfish *Clarias gariepinus* (Burchell 1822) sperm. *Aquaculture Research*, 31(3), 317-324.

Hu, E., Yang, H., Tiersch, T.R. 2011. High-throughput cryopreservation of spermatozoa of blue catfish (*Ictalurus furcatus*): establishment of an approach for commercial-scale processing. *Cryobiology*, 62(1), 74–82.

Igna, V., Telea, A., Florea, T., Popp, R., Grozea, A. 2022. Evaluation of Russian sturgeon (*Acipenser gueldenstaedtii*) semen quality and semen cryopreservation. *Animals*, 12(16), 2153.

Jawahar, K.T.P., Betsy, J. 2020. Cryopreservation of fish gametes: An overview. In: Betsy, J., Kumar, S. (Eds.), *Cryopreservation of fish gametes*, Springer, Singapore, pp. 151–175.

Judycka, S., Nynca, J., Ciereszko, A. 2019. Opportunities and challenges related to the implementation of sperm cryopreservation into breeding of salmonid fishes. *Theriogenology*, 132, 12–21.

Judycka, S., Nynca, J., Liszewska, E., Dobosz, S., Grudniewska, J., Ciereszko, A. 2018. Optimal sperm concentration in straws and final glucose concentration in extender are crucial for improving the cryopreservation protocol of salmonid spermatozoa. *Aquaculture*, 486, 90–97.

Judycka, S., Szczepkowski, M., Ciereszko, A., & Dietrich, G.J. 2015. New extender for cryopreservation of Siberian sturgeon (*Acipenser baerii*) semen. *Cryobiology*, 70(2), 184–189.

Judycka, S., Zarski, D., Dietrich, M.A., Palinska-Zarska, K., Karol, H., Ciereszko, A. 2019. Standardized cryopreservation protocol of European perch (*Perca fluviatilis*) semen allows to obtain high fertilization rates with the use of frozen/thawed semen. *Aquaculture* 498, 208-2016.

Khosla, K., Kangas, J., Liu, Y., Zhan, L., Daly, J., Hagedorn, M., Bischof, J. 2020. Cryopreservation and Laser Nanowarming of Zebrafish Embryos Followed by Hatching and Spawning. *Adv Biosyst.* 2020 Nov; 4(11): e2000138.

Kobayashi, T., Takeuchi, Y., Takeuchi, T., Yoshizaki, G. 2007. Generation of viable fish from cryopreserved primordial germ cells. *Mol. Reprod. Dev.* 74, 207–213.

Kovács, É., Müller, T., Márián, T., Krasznai, Z., Urbányi, B., & Horváth, Á. 2010. Quality of cryopreserved African catfish sperm following post-thaw storage. *Journal of Applied Ichthyology*, 26(5), 737–741.

Kowalski, R.K., Cejko, B.I. 2019. Sperm quality in fish: determinants and affecting factors. *Theriogenology* 135, 94–108.

Labbé, C., Robles, V., Herráez, M.P. 2013. Cryopreservation of gametes for aquaculture and alternative cell sources for genome preservation. In: Allan, G., Burnell, G. (Eds.), *Advances in Aquaculture Hatchery Technology*. Woodhead Publishing, pp. 76–116. ISSN: 0213-3911.

Lee, S., Yoshizaki, G. 2016. Successful cryopreservation of spermatogonia in critically endangered Manchurian trout (*Brachymystax lenok*). *Cryobiology* 72, 165–168.

Lujić, J., Marinović, Z., Sušnik Bajec, S., Djurdjevič, I., Kása, E., Urbányi, B., Horváth, Á. 2017. First successful vitrification of salmonid ovarian tissue. *Cryobiology* 76, 154-157.

Marcantonini, G., Bartolini, D., Zatini, L., Costa, S., Passerini, M., Rende, M., Luca, G., Basta, G., Murdolo, G., Calafiore, R., Galli, F. 2022. Natural cryoprotective and cytoprotective agents in cryopreservation: a focus on melatonin. *Molecules* 27.

Marinović, Z., Li, Q., Lujić, J., Iwasaki, Y., Csenki, Z., Urbányi, B., Yoshizaki, G., Horváth, Á. 2019. Preservation of zebrafish genetic resources through testis cryopreservation and spermatogonia transplantation. *Sci. Rep.* 9, 13861.

Marques, L.S., Mikich, A.B., Leandro, G., Laura, A.S., Daniel, M., Zhang, T., Streit Jr, D.P. 2015. Viability of zebrafish (*Danio rerio*) ovarian follicles after vitrification in a metal container. *Cryobiology* 71, 1–7.

Martínez-Páramo, S., Horváth, Á., Labbé, C., Zhang, T., Robles, V., Herráez, P., Suquet, M., Adams, S., Viveiros, A., Tiersch, T.R., Cabrita, E. 2017. Cryobanking of aquatic species. *Aquaculture* 472, 156–177.

Mattei, X. 1991. Spermatozoon ultrastructure and its systematic implications in fishes. *Canadian Journal of Zoology* 69 (12), 3038–3055.

Nascimento, J.P., Horokhovatskyi, Y., Kholodnyy, V., Rodina, M., Dzyuba, V., Stechkina, T., Boryshpolets, S., & Dzyuba, B. 2021. Optimization of sterlet sperm concentration for cryopreservation. *Aquaculture*, 540, 736682.

Nynca, J., Judycka, S., Liszewska, E., Dobosz, S., & Ciereszko, A. 2017. Standardization of spermatozoa concentration for cryopreservation of rainbow trout semen using a glucose-methanol extender. *Aquaculture*, 477, 23–27.

Nynca, J., Judycka, S., Liszewska, E., Dobosz, S., Ciereszko, A. 2017. Standardization of spermatozoa concentration for cryopreservation of rainbow trout semen using a glucose-methanol extender. *Aquaculture* 477, 23–27.

Paredes, E. 2016. Biobanking of a marine invertebrate model organism: the sea urchin. *Journal of Marine Science Engineering* 4 (1), 7.

Riesco, M.F., Martínez-Pastor, F., Chereguini, O., Robles, V. 2012. Evaluation of zebrafish (*Danio rerio*) PGCs viability and DNA damage using different cryopreservation protocols. *Theriogenology* 77, 122–130.

Riesco, M.F., Robles, V. 2014. Cryopreservation Causes Genetic and Epigenetic Changes in Zebrafish Genital Ridges. *PLOS ONE* 9(1).

Robles, V., Riesco, M.F., Psenicka, M., Saito, T., Valcarce, D.G., Cabrita, E., Herráez, M.P. 2017. Biology of teleost primordial germ cells (PGCs) and spermatogonia: Biotechnological applications. *Aquaculture* 472, 4–20.

Routray, P. 2020. Cryopreservation and Short Duration Storage of Germ Cells and Male Gametes of Freshwater Fish. In: *Cryopreservation of Fish Gametes*. Springer. Betsy, J. and Kumar, S. (Eds.), pp 177–186.

Routray, P., Choudhary, A.K., Dash, S.N., Verma, D.K., Dash, C., Swain, P., Jena, J.K., Gupta, S. D. and & Sarangi, N. 2006. Cryopreservation of dead fish spermatozoa several hours after death of Indian major carp, *Labeo rohita* and its successful utilization in fish production. *Aquaculture*, 261(4), 1204–1211.

Sandoval-Vargas, L., Mauricio, S.J., González, J.R., Figueroa, E.V., Cabrita, E., Valdebenito, I.I. 2020. Oxidative stress and use of antioxidants in fish semen cryopreservation. *Rev. Aquac.* 13, 365–387.

Šćekić, I., Marinović, Z., Lujić, J., Müller, T., Kitanović, N., Urbányi, B., Horváth, Á. 2020. A novel strategy for conservation of European eel (*Anguilla anguilla*) genetic resources: cryopreservation of ovarian stem cells. *Cryobiology* 95, 151–156.

Suquet, M., Chereguini, O., Fauvel, C. 2009. Cryopreservation of sperm from turbot (*Psetta maxima*) In: *Methods in Reproductive Aquaculture: Marine and Freshwater species*, Cabrita E, Robles V, Herráez MP (eds), Taylor and Francis group, CRC press, Boca Raton 463–468.

Tiersch, T.R., Green, C.C. (eds.). 2011. *Cryopreservation in Aquatic Species*, second Edition. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Torres, L., Liu, Y., Guitreau, A., Yang, H., Tiersch, T.R. 2017. Challenges in development of sperm repositories for biomedical fishes: quality control in small-bodied species. *Zebrafish* 14, 552e60.

Tsai, S., Rawson, D.M., Zhang, T. 2009. Studies on chilling sensitivity of early stage zebrafish (*Danio rerio*) ovarian follicles. *Cryobiology* 58, 279–286.

Tsai, S., Rawson, D.M., Zhang, T. 2010. Development of in vitro culture method for early stage zebrafish (*Danio rerio*) ovarian follicles for use in cryopreservation studies. *Theriogenology* 74, 290–303.

Viveiros, A.T.M. 2011. Current Status of Sperm Cryopreservation in Siluriform Catfishes. In: *Cryopreservation in Aquatic Species*, 2nd Edition. T R. Tiersch and C.C. Green, eds. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 387–397.

Xin, M., Niksirat, H., Shaliutina-Kolešová, A., Siddique, M.A.M., Sterba, J., Boryshpolets, S., Linhart, O. 2019. Molecular and subcellular cryoinjury of fish spermatozoa and approaches to improve cryopreservation. *Rev. Aquac.* 12, 909–924.

Yoshizaki, G., Fujinuma, K., Iwasaki, Y., Okutsu, T., Shikina, S., Yazawa, R., Takeuchi, Y. 2011. Spermatogonial transplantation in fish: a novel method for the preservation of genetic resources. *Comp. Biochem. Phys. D* 6, 55–61.

Yoshizaki, G., Lee, S. 2018. Production of live fish derived from frozen germ cells via germ cell transplantation. *Stem Cell Res.* 29, 103–110.

Yoshizaki, G., Yazawa, R. 2019. Application of surrogate broodstock technology in Aquaculture. *Fish. Sci.* 85, 429–437.

Annex 2
Cryopreservation protocols for shellfish bivalve species

Serean L. Adams, Cawthron Institute, Nelson, New Zealand
Julien Vignier, Cawthron Institute, Nelson, New Zealand

Introduction

There are many studies investigating cryopreservation of different shellfish species (see Hassan *et al.*, 2015; Liu *et al.*, 2015; Martínez-Páramo *et al.*, 2017, Paredes *et al.*, 2019, Guo and Weng, 2020; Diwan *et al.*, 2020; Morales-Uno and Paniagua-Chavez, 2020; Aquino *et al.*, 2021; and Yang and Huo, 2022 for extensive reviews). Here we provide protocols for some key shellfish species that are being used in cryobanking but please also refer to the original publications for full details.

Cryopreservation of sperm of Pacific oyster (*Crassostrea gigas*)

Adams et al. (2004) and Adams et al. (2008)

1. Prepare a stock solution of trehalose by adding 15.2 g of trehalose to 40 mL of distilled water (0.8 M trehalose).
2. Prepare a working cryoprotectant solution by diluting 30 mL of the 0.8 M trehalose solution with 7.8 mL of distilled water and 2.2 mL of dimethyl sulphoxide (DMSO).
3. Cool the working cryoprotectant solution on ice.
4. Remove testes from mature oysters and rinse in clean seawater.
5. Strip sperm from the testes by lacerating the gonad wall and gently scraping the exuded milt into a container.
6. Dilute sperm 1:10 gradually with cryoprotectant solution (for example in 4 steps of equal volume over a period of 1-2 min), to avoid osmotic injury to sperm.
7. Load the diluted sperm into 4.5 mL cryovials and attach to aluminum canes. Allow 15–30 min for equilibration.
8. Place the canes directly into a methanol dry ice bath and, after 10 minutes, transfer the canes directly to liquid nitrogen. Alternatively, a polystyrene frame, 3 cm in thickness can be made, with a stainless-steel rack to support the samples. This is placed in a styrofoam cooler with a small amount of liquid nitrogen in the bottom: i.e., samples are approximately 3 cm above the liquid nitrogen, bathed in its vapour. Canes with the cryovials are placed on the frame and left for 10 min before plunging them in liquid nitrogen. The frame should also be chilled before use or placed in the vapour before adding the canes.
9. Samples are thawed by placing in a covered water bath at room temperature until the ice has melted. A lid is used as a precaution for exploding cryovials: Nalgene has warned that liquid nitrogen may become entrapped in cryovials if incorrectly sealed and can explode during thawing.

Using this method, fertilization rates greater than 50 percent have been achieved, with larval yields averaging 37 percent ± 7 percent. 30 x more of the frozen sperm is used for these results, compared with the use of unfrozen sperm. In the case it is more desirable that the sperm is frozen at a high concentration and to use straws rather than canes for the storage, the method described above has been adapted to meet these conditions (J. Vignier pers. comm.), as follows:

1. Prepare a stock solution of trehalose by adding 15.2 g of trehalose to 17 mL of distilled water (1.5 M trehalose).
2. Prepare working cryoprotectant solution by combining 9 mL of the 1.5 M trehalose solution and 1 mL of DMSO.
3. Cool the working cryoprotectant solution on ice.
4. Strip the sperm from mature male oysters by lacerating the gonad wall and gently scraping it into a container.
8. Dilute the sperm 1:1 gradually with cryoprotectant solution (for example in 4 equal volume steps over 1–2 minutes), to avoid osmotic injury to the sperm.
5. Aspirate the sperm into 0.5 mL plastic straws and seal with polyvinyl chloride (PVC) powder. Allow 15–30 minutes for the sperm to equilibrate with the cryoprotectant solution.
6. Place the 0.5 mL straws on a polystyrene frame 3 cm in thickness and with a stainless-steel rack attached (rack should be maintained on ice prior to use), floating in a styrofoam cooler on liquid nitrogen as described above. After 10 min, samples can be plunged into liquid nitrogen for storage.
7. Samples are thawed by placing in a water bath at 20 °C until the ice has melted.

Cryopreservation of eggs of Pacific oyster

Tervit et al., 2005

1. Prepare a working cryoprotectant solution of 20 percent ethylene glycol in distilled water.
2. Obtain eggs by stripping ripe oyster females into water as described for milt above. Adjust the density of eggs to 2 million mL^{-1} by diluting with seawater.
3. Dilute the egg suspension 1:1 with the cryoprotectant solution at room temperature (23°C) gradually (e.g., in steps) to avoid osmotic injury (see above).
4. Load the eggs into 0.25 mL straws and seal with PVC powder.
5. Approximately 20 min after the start of cryoprotectant addition, load straws into a controlled rate freezer programmed to hold at 0°C for 5 min, then cool at 1 °C min^{-1} to – 10 °C, hold for 5 min, then cool at 0.3 °C min^{-1} to – 35 °C, then plunge into liquid nitrogen (seed straws manually with ice if necessary).
6. For the thawing, place the straws into a water bath at 28 °C until the ice visibly melts.

Post-thaw fertilization of cryopreserved oocytes using this method is highly variable ranging from 1–75 percent.

Cryopreservation of late trochophore (20 h post-fertilization) or D-stage larvae (24 hours post-fertilization) Pacific oyster (*Crassostrea gigas*)

Labbé et al. (2018)

1. Prepare a working cryoprotectant solution of 20 percent ethylene glycol +2 percent polyvinyl pyrollidone+ 0.4 M sucrose in distilled water and cool on ice.
2. Dilute concentrated collected larvae (60,000 larvae ml^{-1}) 1:1 with the cryoprotectant solution and then equilibrate for 20 min at 4 °C.
3. Transfer larvae into 0.5 mL straw and seal with PVC.
4. Load straws into a programmable freezer programmed to hold the samples at 0°C for 5 min, then cool at 2.5 °C min^{-1} to – 10 °C, hold for 5 min, then 0.3 °C min^{-1} to − 20°C, then 2.5 °C min^{-1} to – 35 °C, then plunge into liquid nitrogen.
5. Thaw at 37 °C for 10 s and then on-grow.

The survival rates after 12 days of development post-thaw under commercial hatchery conditions were 9.4 percent ± 4.5 percent. Similar protocols used on oyster and mussels resulted in 50 percent survival to 24 hours (D-stage larvae), indicating delayed mortality is high. Nevertheless, as the number of larvae is very high, even a small survival rate of 9 percent is commercially interesting. The higher survival to D-stage larvae is also interesting as early larval food for finfish (Delbare and Dhert, 1996), and was marketed as "Trochofeed" in the late 1990s.

Cryopreservation of sperm of Eastern oyster (*Crassostrea virginica*)

Yang et al. (2012) and Yang et al. (2021)

1. Prepare Ca-free Hank's balanced salt solution(HBSS) osmolality of 650 mOsmol L^{-1} (see footnote for Hank's recipe, leaving Ca out).[19]
2. Prepare a working cryoprotectant solution of 20 percent DMSO v/v in Ca-free Hank's balanced salt solution.
3. Collect the sperm by putting macerated testes into the Ca-free Hanks' balanced salt solution and filtering, adjusting for sperm concentration. Dilute about 1:1 with the above cryoprotectant solution to yield a final concentration of between 10^8–10^9 sperm mL^{-1}.
4. Load sperm into 0.5 mL straws and seal. In the 2021 study, an automatic filling station was used, with ultrasonic sealing. Smaller scale adaptation could use manual filling and sealing with PVC powder.
5. Place straws into a programmable freezer that is programmed to cool from 5 to -80 °C at 10–15 °C min^{-1} followed by storage in liquid nitrogen.
6. Thaw straws in a 50°C water bath for 6 sec.

This protocol was designed for streamlined high throughput application in a hatchery. Post-thaw fertilization rates ranging from 18–95 percent have been reported (Yang *et al*., 2012 and 2021), with the

[19] See AAT webpage for calculator of Hank's Balanced Salt Solution contents of different volumes; calcium is left out for the oyster application (www.aatbio.com/resources/buffer-preparations-and-recipes/hbss-hanks-balanced-salt-solution). The solution is also available commercially ready-made.

lower values likely due to the inclusion of immature milt. Nevertheless, due to the large numbers of gametes, even low fertilization rates can support significant production when greater quantities of frozen milt is used (in comparison with protocols with fresh milt).

References

Chao, N.-H., **Lin, T.T.**, **Chen, Y.-J.**, and **Hsu, H.-W.** 1995. Cryopreservation of late embryos and early larvae of oyster and hard clam. In: Larvi'95 - Fish & Shellfish Larviculture Symposium. Lavens, P., E. Jaspers and I. Roelandts (Eds.). European Aquaculture Society, Special Publication No. 24, Gent, Belgium, p 46.

Delbare, D. & **Dhert, P.** 1996. Cladocerans, nematodes, and trochophora larvae. IN: Lavens, P. and Sorgeloos, P. (eds) Manual on the Production and Use of Live Food for Aquaculture. FAO FISHERIES TECHNICAL PAPER 361.

Labbé, C., **Haffreay, P.**, **Mingat, C.**, **Quittet, B.**, **Diss, B.**, **Tervit, H.R.**, **Adams, S.L.**, **Rimond, F.**, and **Suquet, M.** 2018. Cryopreservation of Pacific oyster (Crassostrea gigas) larvae: Revisiting the practical limitations and scaling up the procedure for application to hatchery. *Aquaculture* 488: 227-234.

Annex 3
Cryopreservation protocols of algae

Cecilia Rad-Menéndez, Culture Collection of Algae and Protozoa (CCAP), The Scottish Association for Marine Science, Oban, United Kingdom
Michael Ross, Culture Collection of Algae and Protozoa (CCAP), The Scottish Association for Marine Science, Oban, United Kingdom

Protocol for cryopreservation of microalgae

The following protocol is a standard microalgae cryopreservation method regularly utilized by the Culture Collection of Algae and Protozoa (CCAP).[20] This method has been successfully adopted by CCAP to cryopreserve a variety of microalgae, including strains of interest for aquaculture such as strains of the following microalgae genus: *Dunaliella*, *Tisochrysis*, *Nannochloropsis*, *Tetraselmis* and *Chaetoceros*.

Cultures selected for cryopreservation are recommended to be in a healthy and dense state, and in a late log-phase, or early stationary phase. All culture handling steps should be conducted in a laminar flow cabinet using aseptic techniques to avoid contamination.

1. Prepare the desired volume of 20 percent (*v/v*) DMSO solution in the appropriate culture sterile medium and filter/sterilize the cryoprotectant solution into a sterile glass vessel.
2. Aseptically transfer the desired volume of the dense culture of interest into the glass vessel containing the sterilized cryoprotectant solution to make up a final concentration of 10 percent (*v/v*) DMSO. Seal the vessel and invert twice to ensure thorough mixing.
3. Decant 1ml aliquots into cryovials and incubate for 10–15 min at the right temperature for the chosen culture (approximately the same temperature used to grow the culture) to allow the mixture to equilibrate.
4. Programme the programmable freezer to start with the temperature at which the culture is maintained; cool at -1°C min^{-1} to -40°C; hold at -40°C for 10 min.
5. Start the programme to allow the system to stabilize at the start temperature, then transfer the cryovials to the cooling chamber of the programmable cooler and initiate the cooling.
6. After the end of the programme, rapidly transfer the cryovials to a container filled with liquid nitrogen taking appropriate safety precautions. It is essential that the cryovials are not allowed to warm up prior to plunging into liquid nitrogen.
7. The samples to be stored should be transferred to the cryostat or ultra-cold freezer in the liquid nitrogen filled container.
8. To recover the cultures, thaw the vials by placing them in a pre-heated water-bath (40°C) until the last ice crystal has just melted.
9. Once thawed, transfer the 1ml of culture into a sterile culture vessel containing >20ml of an appropriate sterile culture medium.
10. Cover in aluminum foil (to ensure that cells are not subjected to light levels likely to induce photo-oxidative stress during the recovery phase) and incubate at standard culturing temperature for the

[20] www.ccap.ac.uk/wp-content/uploads/2020/06/KB_Controlled_rate_cooling_cryo.pdf

organism for 24 hours. After 24 hours fold back ½ of the foil, and after a further 24–96 hours remove all the foil covering.

11. The viability can be simply assessed by culture growth using cell counts and visual appearance of the culture. Specific vital staining methods can be employed, as they can demonstrate active metabolic/enzymatic activity and the presence of an intact cell membrane (Day and DeVille, 1995).
12. Cryopreserved cultures can take several weeks to recover normal growth. After an appropriate period (2–8 weeks, depending on the strain), a normal culture should be obtained.

Protocols for cryopreservation of macroalgae

Several studies have focused on the development of protocols for the cryopreservation of macroalgae, a comprehensive review by Yang *et al.* (2021) addresses the different methodologies and protocols published so far for green, red, and brown macroalgae, with several of these species important for aquaculture. Some protocols from those published studies are reported here for facilitation, but it is recommended to refer to the original publications for full explanation and details.

Cryopreservation of gametophytes of the sugar kelp (*Saccharina latissima*)
Visch et al., 2019

1. Fragment gametophytic thalli into lengths of around 2 mm prior to cryopreservation.
2. Prepare the desired volume of 5 percent (*v/v*) DMSO solution or a mixed of D-sorbitol (9 percent *v/v*) together with 10 percent DMSO (*v/v*) in the appropriate culture sterile medium and filter/sterilize the cryoprotectant solution into a sterile glass vessel. Both cryoprotectants were proven to work successfully for this species.
3. Aseptically transfer 1 ml aliquots of the cryoprotectant solution to sterile cryovials and cool to 10°C before transferring *S. latissima* gametophyte fragments into the vials.
4. Transfer *S. latissima* gametophytes into the cooled cryovials containing the cryoprotectant and incubate for 15–30 min at 10°C to allow the mixture to equilibrate.
5. Programme the controlled cooler: start at 10°C; cool at -1°C min^{-1} to -40°C; hold at -40°C for 10 min.
6. Start the programme to allow the system to stabilize at the start temperature and once reaching the start temperature, transfer the cryovials to the cooling chamber of the programmable cooler and initiate the cooling.
7. After the end of the programme, rapidly transfer the cryovials to a container filled with liquid nitrogen taking appropriate safety precautions. It is essential that the cryovials are not allowed to warm up prior to plunging into liquid nitrogen.
8. Samples for storage should be transferred to the cryostat or ultra-cold freezer in the liquid nitrogen filled container.
9. To recover cultures, thaw vials by placing then in a pre-heated water-bath (40°C) until the last ice crystal has just melted.
10. Once thawed, transfer samples to a Petri dish and wash with 10mL of fresh sterile medium using standard aseptic techniques and incubate in the dark at 8°C for 24h.
11. Transfer the samples to a new vessel and wash with fresh medium before exposure to standard culture conditions. Change the medium regularly with additional washing steps if bacterial growth is observed.

An antibiotic mixture added to the culture medium was found to be positive for recovery and increased viability, Visch *et al*., 2019.

12. Viability can be simply assessed by culture growth, and visual appearance of the culture. In the reference study of this protocol, viability was visually estimated as the proportion of brown coloured cells (viable cells) of the total number of gametophytic cells within a sample.
13. *S. latissima* gametophytes can take a long time to recover to normal growth levels after cryopreservation. In this study, viability was assessed over a period of 7 weeks. Moreover, morphological normal sporophytes were observed to develop from cryopreserved vegetative gametophytic cells, which was directly related to viability.

Cryopreservation of gametophytic thalli of Dark green nori (*Ulva prolifera*) *Lee and Nam, 2016*

1. Fragment gametophytic thalli into lengths of less than 2 mm prior to cryopreservation.
2. Prepare the desired concentration of cryoprotectant. In this study, different cryoprotectants were tested, as well as a combination of them. The most successful cryoprotectant was 20 percent glycerol (*v/v*). The most successful combination was 10 percent glycerol (*v/v*), 5 percent DMSO (*v/v*) and 5 percent proline (*v/v*).
3. Add the chosen cryoprotectant slowly (over 15 min to avoid changes in osmotic pressure) into a cryovial containing an equal volume of thalli suspension and incubate for 45 min at room temperature to equilibrate.
4. Programme the controlled cooler: start temperature at room temperature; cool at -1°C $min.^{-1}$ to -40°C; hold at -40°C for 5 min.
5. Start the programme to allow the system to stabilize at the start temperature and once reaching the start temperature, transfer the cryovials to the cooling chamber of the programmable cooler and initiate the cooling.
6. After the end of the programme, rapidly transfer the cryovials to a container filled with liquid nitrogen taking appropriate safety precautions. It is essential that the cryovials are not allowed to warm up prior to plunging into liquid nitrogen.
7. Samples for storage should be transferred to the cryostat or ultra-cold freezer in the liquid nitrogen filled container.
8. To recover cultures, thaw vials by placing them in a pre-heated water-bath (40°C) and carefully shack until the last ice crystal has just melted.
9. In order to remove any residual cryoprotectants, transfer the thawed thalli to 30 mL of ice-chilled sterilized seawater for 30 min.
10. Transfer the samples to a new vessel and wash with fresh medium before exposure to standard culture conditions.
11. Viability can be simply assessed by culture growth, and visual appearance of the culture. In the reference study of this protocol, viability was investigated using vital staining, please refer to the original paper for further details.

Please note that there are several protocols developed for the cryopreservation of different germplasm of *Ulva* species: zoospores (Taylor and Fletcher, 1999a), gametophytic thalli (Kono *et al.*, 1997; Lee and Nam, 2016) and apical tips (Lalrinsanga *et al.*, 2009).

Cryopreservation of the conchocelis of nori *Neopyropia yezoensis* *Kuwano et al., 1993*

1. Fragment the conchocelis colonies into short fragments prior to cryopreservation.
2. Prepare the desired concentration of the cryoprotectant. In the reference study of this protocol, a solution of 10 percent DMSO (*v/v*) and 0.5 M sorbitol in 50 percent seawater was the most successful.
3. Add conchocelis fragments to 0.75 ml of a basal solution composed of 0.5 M sorbitol and 0.01 M N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) in seawater (36.2 percent) diluted with distilled water to 50 percent (pH 8.0). Place the conchocelis suspension in cryovials and cool in an ice bath.
4. Gradually, over a period of 15 min, add an equal volume of the ice-cooled basal solution containing twice the final concentration of DMSO to the cooled suspension, and incubate for 45 min to equilibrate.
5. Programme the controlled cooler: start temperature at -20ºC; cool at 0.1-1°C min from -1 to -80ºC.
6. Start the programme to allow the system to stabilize at the start temperature and once reaching the start temperature, transfer the cryovials to the cooling chamber of the programmable cooler and initiate the cooling.
7. After the end of the programme, rapidly transfer the cryovials to a container filled with liquid nitrogen taking appropriate safety precautions. It is essential that the cryovials are not allowed to warm up prior to plunging into liquid nitrogen.
8. Samples for storage should be transferred to the cryostat or ultra-cold freezer in the liquid nitrogen filled container.
9. To recover the cultures, thaw the conchocelis suspension quickly by agitation of the vial in a water bath at 40°C until the last ice crystal has melted.
10. In order to remove any residual cryoprotectants, wash cryoprotectants by gradual dilution with seawater.
11. Transfer the samples to a new vessel and wash with fresh medium before exposure to standard culture conditions.
12. Viability can be simply assessed by culture growth, and visual appearance of the culture. In the study, the viability was investigated using vital staining but it is recommended to refer to the original paper for further details.

Several other protocols have been developed for the cryopreservation of different germplasm of *Porphyra, Neoporphyra and Neopyropia* species, particularly for the conchocelis (Kuwano *et al*., 1993; Zhou *et al*., 2007) and the gametophytes (Choi *et al.*, 2013).

References

Kuwano, K., Aruga, Y. & Saga, N. 1993. Cryopreservation of the conchocelis of the marine alga *Porphyra yezoensis* Ueda (*Rhodophyta*) in liquid nitrogen. *Plant Science*, 94: 215–225.

Lee, Y.N. & Nam, K.W. 2016. Cryopreservation of gametophytic thalli of *Ulva prolifera* (Ulvales, Chlorophyta) from Korea. *Journal of Applied Phycology*, 28(2): 1207–1213. Available at: https://doi.org/10.1007/s10811-015-0621-2.

Visch, W., Burson, A., & Broch, O.J. 2019. Cryopreservation of gametophytes of the sugar kelp (*Saccharina latissima*). *Journal of Applied Phycology*, 31(4): 2451–2460. Available at: https://doi.org/10.1007/s10811-019-01801-5.

Annex 4
Artemia: *ex situ in vitro* gene banking of naturally preserved embryos

Gonzalo Gajardo, Laboratory of Genetics, Aquaculture and Biodiversity, University of Los Lagos, Osorno,
Chile
Patrick Sorgeloos, Artemia Reference Center, Ghent University, Belgium

Introduction

Many branchiopod crustaceans live in temporary aquatic environments and, in sub-optimal conditions, can produce a dehydrated, dormant (diapaused) embryos, so-called 'cysts', in resilient shells to survive the unfavourable periods. The cyst shell is primarily composed of lipoproteins, mucopolysaccharides, and other compounds (Fryer, 1996), that enable the diapaused gastrula embryo to remain inactivated but viable for years.

Brine shrimp species of the genus *Artemia* are branchiopods found in hypersaline lakes and lagoons on all continents (except Antarctica), mainly in arid and semi-arid areas. Due to the size of the larvae, ease of culture, and storage capacity of the cysts, *Artemia* have become worldwide the principal cultured live food for feeding aquaculture species, particularly the larvae of a wide range of finfish and crustaceans. This has turned the exploitation of *Artemia* into a multi-billion-dollar business (Sorgeloos and Roubach, 2021). Storage of the cysts, primarily in standard freezers (Clegg and Trotman, 2002), is a form of *ex situ in vitro* conservation that makes use of natural preservation processes.

The bulk of *Artemia* cyst production is collected from the wild or man-made salt ponds. The cysts are produced by females seasonally and float. The harvesting consists of skimming the cysts off the water surface. Processing consists of sequential steps of cleaning, mechanical sieving, brine washing/dehydration and drying, combined with one or more storage periods in specific conditions of temperature and water content. The final storage and distribution is generally in vacuum sealed cans, best stored in a freezer at -4-20 °C. The business of Artemia cysts production is quite competitive, and processing protocols are largely proprietary, as they influence subsequent hatching success. Detailed descriptions on culture, harvest, and use of Artemia cysts are provided in FAO (2024).

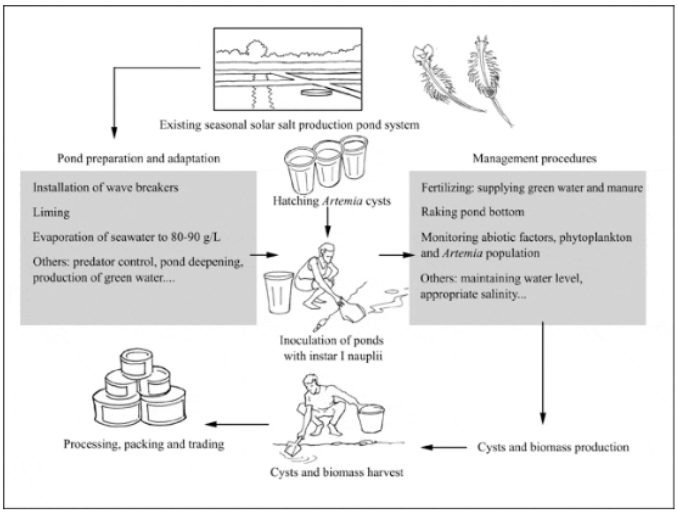


Figure 6. Overview of Artemia culture for cyst production (FAO, 2024)

Use in the hatcheries consists of rehydrating the cysts at low salinities with aeration until the hatching of the cyst shell into a free-swimming larval stage called nauplius and eventual separation of the nauplius from the shell as sometimes the hatching membrane can remain attached to the shell (FAO, 2024). The nauplii may be nutritionally enhanced (Merchie, 1996), either with a fatty-acid coating like Selco (INVE Aquaculture) or with a variety of microscopic (e.g., microalgae) or microencapsulated feed (FAO, 2024) and used as fish feed. *Artemia franciscana* is the main Artemia species being used as a universal live feed. The Great Salt Lake, in the United States of America, where the Artemia business started in the 1960s, is currently still the epicentre of the *A. franciscana* business. *Artemia franciscana* cysts are collected from the wild, in a tightly regulated but competitive fishery (Roubach & Sorgeloos, 2021. However, with an increasing market, cyst shortage, and escalating prices, *A. franciscana* has been introduced to several developing countries (Brazil, China, Kenya, Thailand, and Vietnam) since the 1980s for local cyst production. From these, they have been distributed to many others (including, Bangladesh, Cambodia, India, Myanmar, Sri Lanka and the United Arab Emirates). This has included culture in salt ponds, where the *Artemia* also enhances salt production through the control of microalgae and crystal nucleation.

The introduction of *A. franciscana* to new environments has resulted in locally adapted strains. For example, a successful incipient "farm type" is the Vin Chau *A. franciscana* strain in Vietnam, originated from San Francisco Bay cysts in 1986, that is characterized by high thermal tolerance (>40° C) (De Vos *et al.*, 2021). A multidisciplinary study of the Vin Chau farmed type is under way with different partners of the International Artemia Aquaculture Consortium[21] to better understand the evolution of special characteristics such as the tolerance to higher temperatures, and how to create other farmed types of value to aquaculture.

There are nine recognized *Artemia* bisexual species, as well as an undetermined number of parthenogenic[22] populations that are not yet assigned to any species (Van Stappen *et al.*, 2024). These populations have a variety of distinguishing characteristics but are threatened by changing habitat as well as competition from introduced *A. fransiscana.* Several institutes have established Artemia cyst gene banks for research and preservation of natural populations and emerging new farm types. The largest of these collections are the Artemia Reference Centers (Belgium), the Instituto de Acuicultura de Torre de la Sal (Spain) and the Asia Regional Artemia Reference Center (China) (Box 2). In addition to being a repository of Artemia genetic diversity, all these collections have a research focus.

Wild genetic *Artemia spp*. resources need monitoring and protection. They represent the source of novel traits and genetic variation for breeding programs that may support future mariculture, as well as supporting local ecosystems that are changing because of climate change and other anthropic impacts. For these reasons, continued genetic characterization of unique traits in species, populations and emerging farm types is critical. A good example of initiatives for the conservation and characterization of the Artemia genetic resources is the Asian Regional Artemia Reference Center (Box 2).

[21] https://artemia.info/
[22] Viable eggs are produced by females in these populations without fertilization.

References

Baert, P., Bosteels, T., and Sorgeloos, P. 1996. 4.5. Pond production. In: *Manual on the Production and Use of Live Food for Aquaculture*. FAO FISHERIES TECHNICAL PAPER 361. www.fao.org/3/w3732e/w3732e0q.htm#b74.5.7.%20Harvesting%20and%20processing%20techniques

Clegg, J.S. and Trotman, C.N.A. 2002. Physiological and biochemical aspects of *Artemia* ecology. In Th.J. Abatzopoulos, J.A. Beardmore, J.S. Clegg and P. Sorgeloos (eds.), *Artemia: Basic and Applied Biology*. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 129–170.

De Vos, S., Rombauts, S., Coussements, L. et al. 2021. The genome of the extremophile *Artemia* provides insight into strategies to cope with extreme environments. *BMC Genomics* (2021) 22:635. https://doi.org/10.1186/s12864-021-07937-z

FAO 2023. *Artemia spp.* Cultured Aquatic Species Information Programme. Text by Van Stappen, G. Fisheries and Aquaculture Division [online]. Rome. Updated 2012-02-28 [Cited Monday, June 12th 2023]. www.fao.org/fishery/en/culturedspecies/artemia_spp/en

Fryer, G. 1996. Diapause, a potent force in the evolution of freshwater crustaceans. *Hydrobiologia* 320: 1–14.

Lavens, P. & Sorgeloos, P. (eds). *Manual on the Production and Use of Live Food for Aquaculture*. FAO FISHERIES TECHNICAL PAPER 361. www.fao.org/3/w3732e/w3732e00.htm#Contents

Merchie, G. 1996. 4.3. Use of nauplii and meta-nauplii. IN: Patrick Lavens, P & Sorgeloos, P. (eds). *Manual on the Production and Use of Live Food for Aquaculture*. FAO FISHERIES TECHNICAL PAPER 361. www.fao.org/3/w3732e/w3732e0o.htm#b4-4.3.4.%20Enrichment%20with%20nutrients

Sorgeloos, P. and Roubach, R. 2021. Past, present, and future scenarios for SDG-aligned brine shrimp *Artemia* aquaculture. *FAO Aquaculture News* 63: 55–56.

Van Stappen, G., Sorgeloos, P. and Rombaut, G. (eds). 2024. *Manual on the Production and Use of Brine Shrimp Artemia in Aquaculture*. FAO Fisheries Technical Paper, in press.

Annex 5
Cryopreservation methods and repository development for corals and other cnidarians

Jack C. Koch, Aquatic Germplasm and Genetic Resources Center (AGGRC), Louisiana State University Agricultural Center, Baton, United States of America
Anastazia T. Banaszak , Instituto de Ciencias del Mar y Limnología, Universidad Nacional Autónoma de México, Puerto Morelos, Mexico
Jonathan Daly, Taronga Conservation Society, Sydney, Australia
Virginia M. Weis, Department of Integrative Biology, Oregon State University, Corvallis, Oregon, United States of America
Mary Hagedorn, Smithsonian Conservation Biology Institute, Front Royal, Virginia, United States of America; Hawai'i Institute of Marine Biology (HIMB), University of Hawai'I, Mānoa, Hawaii, United States of America
Terrence R. Tiersch, Aquatic Germplasm and Genetic Resources Center (AGGRC), School of Renewable Natural Resources, Louisiana State University Agricultural Center, Baton Rouge, LA 70820, USA

Introduction

There is an urgent need for research, conservation, and restoration solutions for corals and coral reefs that are degrading as a result of climate change and other disturbances. Coral reef ecosystem services are valued at USD12 trillion per year (Hoegh-Guldberg and Ormond, 2018) and support the livelihoods and major protein needs of close to a billion people, many in Small Island Developing States (SIDS) or low to middle income nations (Sing Wong, Vrontos and Taylor, 2022). Coral reef ecosystem services support Sustainable Development Goals 2, 3, 8, 12, 13, and 14 (United Nations Department for Economic and Social Affairs, 2021). These services include the provision of habitat for commercially harvested fish and millions of other marine species, coastal barriers against storms, food resources, recreation and tourism opportunities, and potential for bioprospecting for pharmaceuticals (Hughes *et al.*, 2017a).

Coral reefs are among the most diverse and productive ecosystems in the world (Knowlton *et al.*, 2010). In total, they support an estimated 25 percent of all marine life (Knowlton *et al.*, 2010; Reaka-Kudla, 1997). The success of reef-building corals is based on an intimate symbiotic relationship with microalgae (family Symbiodiniaceae) and other microorganisms (Kingdoms Bacteria, Archaea, and Fungi) that allow coral animals to secrete the calcium carbonate skeletons of reefs. Even small changes in the environment can cause a breakdown of this symbiosis, observed as coral bleaching or disease and often resulting in coral death and reef degradation (Kleypas *et al.*, 2021; van Woesik *et al.*, 2022). Coral bleaching and disease are increasing due to sea surface temperature increases, pollution, eutrophication, overfishing, ocean acidification, and other anthropogenic stressors (Hughes *et al.*, 2018; Lesser, 2011). Corals have limited ability to adapt to environmental stressors, and their limits have been exceeded in recent years, with mass coral bleaching events in 2002, 2006, 2016, 2017, 2020, and 2022 (Eakin, Sweatman and Brainard, 2019; Hughes *et al.*, 2017b).

The consequences of rapid climate change and reef declines have motivated the coral research community to focus the attention on cryopreservation as a tool to preserve current genetic diversity, including climate-robust variants, and to buy time to prolong the development of action plans aiming at reducing threats to corals and reef ecosystems. This appendix summarizes the current state of coral and, more broadly, cnidarian cryopreservation, identifies cryopreservation and repository constraints, highlights the need for infrastructure and technology, and proposes integration of repositories into local and international management plans.

As scientists and communities rush to understand the complex relationships among corals and the changing climate, the creation and implementation of new strategies, policies, and technologies will be imperative for protecting valuable reef ecosystems (Kleypas *et al.*, 2021; Knowlton *et al.*, 2021; Vardi *et al.*, 2020). Cryopreservation has emerged as a crucial technology for corals and their symbiotic partners (Hagedorn *et al.*, 2012, 2021; Hagedorn and Carter, 2016; Liu *et al.*, 2019; National Academies of Sciences, Engineering, and Medicine *et al.*, 2019; Vardi *et al.*, 2020), and there is a rapidly growing need not only for more research but also for a global repository infrastructure or network to equitably share resources.

Current state of cnidarian cryopreservation

Efforts to develop cryopreservation protocols for corals and their symbionts have been ongoing since 2006 (Hagedorn *et al.*, 2006), and a recent review on coral cryopreservation protocols was published by Bouwmeester *et al.*, 2022.

Several cryopreservation protocols for different types of coral and symbiont materials have been developed and are being implemented in laboratories and on coral reefs around the world (Daly *et al.*, 2018; Hagedorn *et al.*, 2012, 2021; Hagedorn and Carter, 2015; Zuchowicz *et al.*, 2021). As of April 2022, these protocols have been used to successfully cryopreserve sperm from at least 48 species of coral.[23] Some of this sperm has been used in assisted migration and selective breeding experiments (Daly *et al.*, 2022; Hagedorn *et al.*, 2021), and there has been progress in developing methods that allow more efficient cryopreservation of coral sperm in the field using three-dimensional (3-D) printed open hardware cooling devices (see Appendix 6 and Zuchowicz *et al.*, 2021). The species studied are from the Caribbean, Hawaii, Puerto Rico, French Polynesia, the Gulf of Mexico, the Great Barrier Reef, and the Red Sea. Larvae from one coral species produced with cryopreserved sperm were shown to develop and settle, suggesting that coral cryopreservation can be successful, and its application could be eventually scaled up (Hagedorn *et al.*, 2017).

Cryopreservation of coral gametes is constrained by the short spawning periods (minutes to hours each year) typical of most coral species, and an incomplete understanding of coral spawning cues and timing. Unfortunately, artificial induced reproduction of corals is generally not yet possible, and it is not known when most of the coral species spawn during the year. Coral eggs have not been successfully cryopreserved, largely because they are not tolerant of mechanical damage and have properties, such as a high surface-area-to-volume ratio, high lipid content, and chill sensitivity that make them difficult to cryopreserve. Recent efforts have thus focused on sperm and are expanding to include larvae (Daly *et al.*, 2018), fragments, somatic cells (Toh *et al.*, 2022), and microalgal symbionts (Hagedorn and Carter, 2015; Kihika *et al.*, 2022b, 2022a). Coral fragment cryopreservation has the advantage of access to materials year-round. These efforts to cryopreserve non-gametic coral material exploit new cryopreservation approaches, for

[23] https://nationalzoo.si.edu/center-for-species-survival/coral-species-cryopreserved-global-collaborators

example the use of ultra-fast cooling (vitrification) and ultra-fast warming using gold nano-rods (Daly *et al.*, 2018). This protocol is not yet practical for field use and will require further investigation and significant scaling up. However, it could be useful for preserving current genetic diversity of hard-to-freeze tissues or life stages.

More broadly, new protocols are available for other cnidarians (e.g., sea anemones and hydroids), many of which are used as model systems to study some aspects of coral biology (Fricano *et al.*, 2020; Huene *et al.*, 2022; Puntin Giulia *et al.*, 2022). Although there are differences between the reproductive biology of corals and other cnidarians, this broad group of cnidarian cryopreservation protocols have already increased our understanding of the response of cell assemblages to extreme cooling, something that needs to happen if future cryopreservation of aquatic genetic material is to rest on a solid scientific foundation.

Repositories

Several coral cryopreservation repositories exist today, notably at the Taronga Conservation Society (Australia), the Smithsonian Institution (United States), the United States Department of Agriculture National Animal Germplasm Program (USDA-NAGP, United States of America), the King Abdullah University for Science and Technology (KAUST, Saudi Arabia), the Caribbean Research and Management of Biodiversity Foundation (CARMABI Foundation, Country of Curaçao), and the Universidad Nacional Autónoma de México (Mexico). Although some of these repositories have samples from around the world, all are fundamentally the result of local efforts, therefore there is a need for a global cnidarian genetic resource network to connect and coordinate.

In addition to repositories for cryopreserved material, there are several living or *ex situ in vivo* coral repositories holding regionally representative populations of coral colonies within aquarium networks. The Coral Biobank Alliance[24] has a number of partners including the Great Barrier Reef Legacy Foundation (Australia), the World Coral Conservatory (Monaco), the Smithsonian Institution and Taronga Conservation Society Australia Partnership (United States of America and Australia), the National Oceanographic and Atmospheric Association (NOAA, United States of America), the Mote Marine Laboratory International Genebank (United States of America), and the Association of Zoos and Aquariums-Florida Coral Rescue (United States of America). These partners hold hundreds of cultured coral species along with some cryopreserved samples.

The national gene banks and the Coral Biobank Alliance provide excellent starting points for a global network that will need international buy-in, support, and cooperation. Network activities could be coordinated by "hub" repositories that specialize in connecting research with community and commercial applications and can offer regional support for smaller research repositories. Regional hub repositories should be embedded in national or state government agencies, ideally as part of biodiversity management strategies, to ensure long-term management and funding. At present, only four of the repositories holding coral samples meet this requirement, particularly: the Taronga CryoDiversity Bank (Australia), the USDA-NAGP (United States), the Smithsonian Institution (United States of America), and the Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (Mexico). All these repositories currently hold coral samples along with other priority species (e.g., agriculture or wildlife species) and would need additional support to expand coral gene banking significantly beyond current capacity.

[24] https://nationalzoo.si.edu/center-for-species-survival/coral-biobank-alliance

Constraints

Most coral reefs are located close to SIDS or low-to-middle income nations where cryopreservation may not be a long-term priority and where equipment, reliable electricity and liquid nitrogen may be scarce. Overall, the major constraints to creating and maintaining coral repositories include:

1. Lack of long-term financing, particularly because equipment can be expensive and requires extra maintenance due to the humid, salty conditions.
2. Scarcity of experts trained and experienced in cryopreservation, coral reproduction, and coral spawn collection.
3. General lack of government policy regarding the use of cryopreserved material for restoration and conservation.
4. The long lead times required for obtaining permits for collection, importation, and exportation of biological material for exchanges or donations.
5. The limits to coral spawn collection: for some well-known species, these reproductive events only take place once or twice a year. For lesser-known species, life history information is sorely lacking. The latter are currently not included in any repository.
6. The ever-increasing difficulty of obtaining good quality, genetically variable samples due to the high rate of coral stress and loss, requiring more effort and time to obtain healthy coral for sampling.
7. Access to reliable repository facilities with long-term support and back-up facilities. At a minimum this will require development of regional repository facilities to support local efforts, along with new relationships, policies, and agreements between existing facilities that can act as back-ups for each other. On a larger scale, construction of a global cnidarian genetic resource that would be a back-up and distribution hub for multiple facilities around the world could alleviate the burdens and space constraints of maintaining back-ups between more local facilities.
8. Remoteness and susceptibility of the existing repository facilities to natural disasters.
9. Corals are tropical organisms. Tropical environments tend to be hot and humid which complicates the cryopreservation process. Many locations do not have the facilities to support even short-term (i.e., weeks) maintenance of reproductive coral colonies *ex situ*, because of the lack of infrastructure (such as buildings for protection from weather), and cannot provide a suitable environment for cryopreservation (i.e., air temperature in laboratory facilities where work is performed), water tables to maintain stable temperatures (i.e., water temperature in *ex situ* facilities), pumps to bring fresh seawater into facilities, and stable electricity.
10. Many small airlines will not fly dry shippers, so it can be impossible to transport frozen material out of the country even if all the correct permits are in place.

Opportunities

Opportunities for advancing cnidarian cryopreservation repositories include building international infrastructure, increasing access to funding, and sharing of knowledge and resources. Here we highlight opportunities for open technology, training programs, and adding cnidarian model organisms to repositories.

Open technology

Open technology refers to accessible devices that can be fabricated by a wide range of community members (Liu *et al.*, 2021 and Appendix 6). Customization is a major advantage of open technology as it incorporates off-the-shelf electronic parts, consumer-fabricated electronics, and 3-D printed parts, all of which can be

modified to suit user needs (Liu *et al.*, 2021). Open technology could assist in enabling reproducibility and standardizing protocols across user groups while streamlining cryopreservation in research and commercial applications in the laboratory and in the field.

Currently at least three open technology devices (Childress, Liu and Tiersch, 2021; Hu, Childress and Tiersch, 2017; Zuchowicz *et al.*, 2021) are being used in coral cryopreservation, mainly for controlled-rate cooling of sperm. These devices arose from collaborations between biologists and engineers and have been used to freeze coral sperm in laboratories and on beaches, remote islands, and boats at sea. Several other open devices have been developed to assist with husbandry and cryopreservation of other cnidarians as well (e.g., Huene *et al.*, 2022). Open technology is a rapidly growing field that requires attention, direction, and resources especially for cnidarian cryopreservation repositories. Opportunities for expanding the use of open technology devices in cnidarian cryopreservation include addressing additional steps in cryopreservation pathways, driving aggregate throughput, and increasing the scale of operation and the accessibility of cryopreservation to more user communities (Liu *et al.*, 2021).

Training courses

Researchers at the Smithsonian Institution have created a coral cryopreservation training course with multiple modules addressing topics from coral biology to sample collection and a cryopreservation protocol.[25] This resource includes a list of corals for which sperm has been banked and a short section on the biology and cryopreservation of coral microalgal symbionts. The importance of generating and updating training resources like this cannot be understated, and requires trained instructors, adequate funding, and media management and distribution.

Model systems

Recent advances in off-cycle coral spawning have increased opportunities to collect spawn at different times of the year (Craggs *et al.*, 2017, 2020). Model systems like the non-symbiotic hydroid *Hydractinia symbiolongicarpus*, and two sea anemone species, the non-symbiotic *Nematostella vectensis* and the symbiotic *Exaiptasia diaphana*, allow for rapid advancement in cryopreservation techniques by freeing researchers from the limitations of rare coral spawns. These model systems are cultured by hundreds of research groups worldwide and their chief benefit is robust reproduction in a laboratory setting (*Hydractinia, Nematostella,* and *Exaiptasia* spawn eggs and sperm daily, weekly, and bi-weekly respectively). Development of germplasm repositories for these model species would benefit their study and allow for the freezing of clonal lines.

Beyond the repository

Repositories should not exist on their own. A goal of cnidarian cryopreservation repositories should be to develop functional collections that can be integrated and coordinated with local reef management and restoration. Integration of repositories with regional community members (e.g., reef managers, research and restoration programs, and traditional owners) enables the prioritization of species and populations for cryopreservation and provides routes to utilize cryopreserved material. These integration efforts should expand to include international partnerships and permit agreements, and promote policies on research, transportation of samples, and sharing of samples, equipment, and knowledge.

[25] https://nationalzoo.si.edu/center-for-species-survival/coral-reproduction-and-cryopreservation

The restoration of functional coral populations will require the deployment of juvenile corals on a large scale, a feat that has not yet been achieved and will require creative minds. This is especially true for large reef systems that include hundreds of species, and reefs extending across large geographic ranges. The use of cryopreserved material as a tool for production of juvenile corals for deployment may take different forms depending on the scale required and capacity available.

Most coral species are broadcast spawners that produce millions of larvae with a naturally high initial rate of attrition (Graham, Baird and Connolly, 2008). They thus rely on relatively few colonies surviving to adulthood to continue the species. Replicating this reproductive strategy using cryopreserved gametes is challenging given the relatively small volumes of gametes (usually milliliters) that are cryopreserved using existing technologies. Approaches are needed to integrate cryopreservation into large-scale restoration programs that aim to increase the recruitment of juvenile corals in reef systems. Fortunately, existing cryopreservation and biobanking technologies for coral, particularly for sperm, are suitable for genetic management of aquaculture broodstock (i.e., <100 adult colonies), which could in turn produce large numbers of juvenile corals for reef deployment. Significant upscaling will be required if this objective is agreed upon, by using cryopreserved material to produce juvenile corals that can be used to seed natural areas. As indicated above, this is constrained by limitations of coral reproduction and growth even with fast-maturing corals such as *Acropora*, and a lack of permitting required to place cultured corals on reefs. This process – from laboratory-scale coral cryopreservation technologies, to aquaculture, to reef-scale application – will require ongoing support.

Conclusions

Time is growing short for continued availability of high-grade coral germplasm suitable for cryopreservation. Climate change is producing ever-increasing cycles of warming and incidence of marine heatwaves, leading to devastating bleaching events that either kill or severely stress corals, and thus reduce their reproduction – sometimes for years. Collaboration and participation in a global cnidarian genetic resource network and in funding existing repositories that hold coral samples is key to supporting conservation and restoration efforts that cryopreservation can help to reinforce. Partnerships among cryobiologists, coral experts, engineers, conservation organizations, and policymakers will be critical in developing and implementing the new tools needed to collect and preserve the genetic diversity of all corals and prevent species extinctions.

References

Bouwmeester, J., Daly, J., Zuchowicz, N. & Hagedorn, M. 2022. Cryopreservation to conserve genetic diversity of reef-building corals. In: M.J.H. van Oppen & M. Aranda Lastra, eds. *Coral Reef Conservation and Restoration in the Omics Age*. Springer Nature.

Childress, W.M., Liu, Y. & Tiersch, T.R. 2021. Design, alpha testing, and beta testing of a 3-D printed open-hardware portable cryopreservation device for aquatic species. *Journal of Applied Aquaculture*: 1–24.

Craggs, J., Guest, J., Davis, M. & Sweet, M. 2020. Completing the life cycle of a broadcast spawning coral in a closed mesocosm. *Invertebrate Reproduction & Development*, 64(3): 244–247.

Craggs, J., Guest, J.R., Davis, M., Simmons, J., Dashti, E. & Sweet, M. 2017. Inducing broadcast coral spawning ex situ: Closed system mesocosm design and husbandry protocol. *Ecology and Evolution*, 7(24): 11066–11078.

Daly, J., Hobbs, R.J., Zuchowicz, N., O'Brien, J.K., Bouwmeester, J., Bay, L., Quigley, K. & Hagedorn, M. 2022. Cryopreservation can assist gene flow on the Great Barrier Reef. *Coral Reefs*, 41(2): 455–462.

Daly, J., Zuchowicz, N., Nuñez Lendo, C.I., Khosla, K., Lager, C., Henley, E.M., Bischof, J. et al. 2018. Successful cryopreservation of coral larvae using vitrification and laser warming. *Scientific Reports*, 8(1): 15714.

Eakin, C.M., Sweatman, H.P.A. & Brainard, R.E. 2019. The 2014–2017 global-scale coral bleaching event: insights and impacts. *Coral Reefs*, 38(4): 539–545.

Fricano, C., Röttinger, E., Furla, P. & Barnay-Verdier, S. 2020. Cnidarian cell cryopreservation: A powerful tool for cultivation and functional assays. *Cells*, 9(12). https://doi.org/10.3390/cells9122541

Graham, E.M., Baird, A.H. & Connolly, S.R. 2008. Survival dynamics of scleractinian coral larvae and implications for dispersal. *Coral Reefs*, 27(3): 529–539.

Hagedorn, M., Carter, V., Martorana, K., Paresa, M.K., Acker, J., Baums, I.B., Borneman, E. et al. 2012. Preserving and using germplasm and dissociated embryonic cells for conserving Caribbean and Pacific coral. *PloS One*, 7(3): e33354.

Hagedorn, M. & Carter, V.L. 2015. Seasonal preservation success of the marine dinoflagellate coral symbiont, *Symbiodinium* sp. *PloS One*, 10(9): e0136358.

Hagedorn, M. & Carter, V.L. 2016. Cryobiology: principles, species conservation and benefits for coral reefs. *Reproduction, Fertility, and Development*, 28(8): 1049–1060.

Hagedorn, M., Carter, V.L., Henley, E.M., van Oppen, M.J.H., Hobbs, R. & Spindler, R.E. 2017. Producing coral offspring with cryopreserved sperm: A tool for coral reef restoration. *Scientific Reports*, 7(1): 14432.

Hagedorn, M., Carter, V.L., Steyn, R.A., Krupp, D., Leong, J.C., Lang, R.P. & Tiersch, T.R. 2006. Preliminary studies of sperm cryopreservation in the mushroom coral, *Fungia scutaria*. *Cryobiology*, 52(3): 454–458.

Hagedorn, M., Page, C.A., O'Neil, K.L., Flores, D.M., Tichy, L., Conn, T., Chamberland, V.F. et al. 2021. Assisted gene flow using cryopreserved sperm in critically endangered coral. *Proceedings of the National Academy of Sciences of the United States of America*, 118(38).

https://doi.org/10.1073/pnas.2110559118

Hoegh-Guldberg, O. & Ormond, R. 2018. Consensus statement on coral bleaching climate change. *International Coral Reef Society*.

Hu, E., Childress, W. & Tiersch, T.R. 2017. 3-D printing provides a novel approach for standardization and reproducibility of freezing devices. *Cryobiology*, 76: 34–40.

Huene, A.L., Koch, J.C., Arregui, L., Liu, Y., Nicotra, M.L., Weis, V.M. & Tiersch, T.R. 2022. Cryopreservation of *Hydractinia symbiolongicarpus* sperm to support community-based repository development for preservation of genetic resources. *Animals*, 12(19). https://doi.org/10.3390/ani12192537

Hughes, T.P., Anderson, K.D., Connolly, S.R., Heron, S.F., Kerry, J.T., Lough, J.M., Baird, A.H. et al. 2018. Spatial and temporal patterns of mass bleaching of corals in the Anthropocene. *Science*, 359(6371): 80–83.

Hughes, T.P., Barnes, M.L., Bellwood, D.R., Cinner, J.E., Cumming, G.S., Jackson, J.B.C., Kleypas, J. et al. 2017a. Coral reefs in the Anthropocene. *Nature*, 546(7656): 82–90.

Hughes, T.P., Kerry, J.T., Álvarez-Noriega, M., Álvarez-Romero, J.G., Anderson, K.D., Baird, A.H., Babcock, R.C. et al. 2017b. Global warming and recurrent mass bleaching of corals. *Nature*, 543(7645): 373–377.

Kihika, J.K., Wood, S.A., Rhodes, L., Smith, K.F., Miller, M.R., Pochon, X., Thompson, L. et al. 2022a. Cryopreservation of six *Symbiodiniaceae* genera and assessment of fatty acid profiles in response to increased salinity treatments. *Scientific Reports*, 12(1): 12408.

Kihika, J.K., Wood, S.A., Rhodes, L., Smith, K.F., Thompson, L., Challenger, S. & Ryan, K.G. 2022b. Cryoprotectant treatment tests on three morphologically diverse marine dinoflagellates and the cryopreservation of *Breviolum* sp. (*Symbiodiniaceae*). *Scientific Reports*, 12(1): 646.

Kleypas, J., Allemand, D., Anthony, K., Baker, A.C., Beck, M.W., Hale, L.Z., Hilmi, N. et al. 2021. Designing a blueprint for coral reef survival. *Biological Conservation*, 257: 109107.

Knowlton, N., Brainard, R.E., Fisher, R., Moews, M., Plaisance, L. & Caley, M.J. 2010. Coral reef biodiversity. In: A.D. McIntyre, ed. *Life in the World's Oceans*, pp. 65–78.

Knowlton, N., Grottoli, A.G., Kleypas, J., Obura, D., Corcoran, E., de Goeij, J.M., Felis, T. et al. 2021. Rebuilding coral reefs: A decadal grand challenge. *International Coral Reef Society* and *Future Earth Coasts*. http://coralreefs.org/wp-content/uploads/2021/07/ICRS_2021_Policy_Brief_high_resol.pdf

Lesser, M.P. 2011. Coral bleaching: Causes and mechanisms. In: Z. Dubinsky & N. Stambler, eds. *Coral Reefs: An Ecosystem in Transition*, pp. 405–419. Dordrecht, Springer Netherlands.

Liu, Y., Blackburn, H., Taylor, S.S. & Tiersch, T.R. 2019. Development of germplasm repositories to assist conservation of endangered fishes: Examples from small-bodied livebearing fishes. *Theriogenology*, 135: 138–151.

Liu, Y., Monroe, T.W., Belgodere, J.A., Choi, J.-W., Teresa Gutierrez-Wing, M. & Tiersch, T.R. 2021. The emerging role of open technologies for community-based improvement of cryopreservation and quality management for repository development in aquatic species. *Animal Reproduction Science*: 106871.

National Academies of Sciences, Engineering, and Medicine, Division on Earth and Life Studies, Board on Life Sciences, Ocean Studies Board & Committee on Interventions to Increase the Resilience of Coral Reefs. 2019. *A decision framework for interventions to increase the persistence and resilience of coral reefs*. National Academies Press.

Puntin, G., Sweet, M., Fraune, S., Medina, M., Sharp, K., Weis, V.M. & Ziegler, M. 2022. Harnessing the power of model organisms to unravel microbial functions in the coral holobiont. *Microbiology and Molecular Biology Reviews: MMBR*, 0(0): e00053-22.

Reaka-Kudla, M.L. 1997. The global biodiversity of coral reefs: a comparison with rain forests. *Biodiversity II: Understanding and Protecting Our Biological Resources*, 2: 551.

Sing Wong, A., Vrontos, S. & Taylor, M.L. 2022. An assessment of people living by coral reefs over space and time. *Global Change Biology*. https://doi.org/10.1111/gcb.16391

Toh, E.-C., Liu, K.-L., Tsai, S. & Lin, C. 2022. Cryopreservation and cryobanking of cells from 100 coral species. *Cells*, 11(17): 2668.

United Nations Department for Economic and Social Affairs. 2021. *The Sustainable Development Goals Report 2021*. United Nations Fund for Population Activities.

Vardi, T., Rankin, T., Oliver, T., Moulding, A.L., Parrish, F., Moore, T., Enochs, I.C., Viehman, T.S. & Koss, J.L. 2020. NOAA Action Plan on Coral Interventions. NOAA Tech. Memo. NMFS-F/SPO-208. https://doi.org/10.25923/weyp-fy32

Van Woesik, R., Shlesinger, T., Grottoli, A.G., Toonen, R.J., Vega Thurber, R., Warner, M.E., Marie Hulver, A. et al. 2022. Coral-bleaching responses to climate change across biological scales. *Global Change Biology*. https://doi.org/10.1111/gcb.16192

Zuchowicz, N., Daly, J., Lager, C., Williamson, O. & Hagedorn, M. 2021. Freezing on the beach: A robust coral sperm cryopreservation design. *Cryobiology*, 101: 135–139.

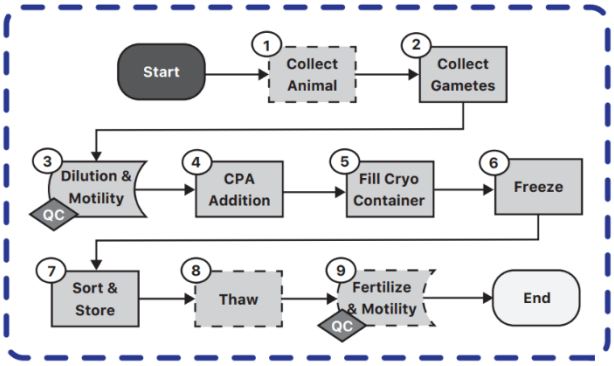
Annex 6
Process mapping in establishing AqGR gene banks

Sarah Bodenstein, Aquaculture Germplasm and Genetic Resources Center (AGGRC), Louisiana State University Agricultural Center, Baton Rouge, Louisiana, United States of America
Terrence Tiersch, Aquaculture Germplasm and Genetic Resources Center (AGGRC), Louisiana State University Agricultural Center, Baton Rouge, Louisiana, United States of America

Introduction

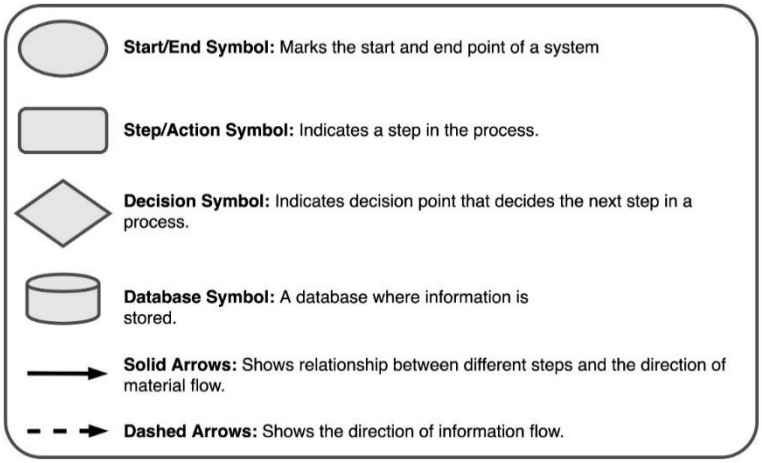
For a repository development in aquatic gene banking to be successful, tools from the field of industrial engineering can be used to understand the interactions at community level and the needs the repository should meet. Such tools are not currently in general use. One of these tools is *process flow mapping*; commonly used in industrial engineering to illustrate all steps in a specific process (Renger *et al.*, 2016). It consists of diagrams displaying the sequence of activities (steps) used to produce a product and allow users to track how materials flow through the process (Damelio, 2011). Process flow diagrams are often applied in commercial-scale factory settings to identify and eliminate wastes and bottlenecks (Damelio, 2011; Diego Fernando and Rivera Cadavid, 2007). These diagrams have also been used in agriculture, with studies tracking the production cycle of farmed products (Apaiah and Hendrix, 2005). In the context of building a germplasm repository network for aquatic species, process flow diagrams can for example be used to outline a cryopreservation pathway. The example illustrated in Figure 7 shows a typical pathway-level process for cryoconservation of sperm of aquatic organisms.

Figure 7. A pathway-level process flow diagram outlining all steps, in sequence, of a generalized cryopreservation process. Boxes with solid lines indicate steps in the cryopreservation pathway that take place on the same day that samples are frozen. Boxes with dashed lines indicate steps in the cryopreservation pathway that can take place in the days before or after freezing. Of special importance are the numbered steps that can be timed. The diamonds indicate quality control (QC) steps, and the arrows indicate the flow of materials through the steps. The dashed outline surrounding the diagram serves to indicate where this pathway resides in the following two higher organizational levels (repository and network).



After the sequence has been confirmed, the process flow diagram can be created by visually representing each step on the diagram. Process flow diagrams can be drawn or created on software such as draw.io (JGraph, United Kingdom). In general, oval shapes indicate the start and end of the entire process, rectangles represent individual steps, and diamonds represent decision points (Damelio, 2011; Figure 8). Arrows connect steps and show the flow of material and information. After the process flow map has been created, it can be studied to understand the process, distinguish between steps that "add value" to the end product and those that do not (waste), and plan improvements (Damelio, 2011). Process flow diagrams can be used to generate quantitative data as well when used in combination with another common industrial engineering tool, discrete-event simulation (DES) modeling.

Figure 8. The meaning of some common symbols used in process flow diagrams.



Well-constructed process flow diagrams can be further used to develop simulation models. Discrete-event simulation (DES) models emulate processes by modeling all the steps of a process throughout time (Allen *et al.*, 2015; Gittins *et al.*, 2020; Schriber *et al.*, 2013). Logic rules can also be applied to make simulations reflecting "real world" processes (Schriber *et al.*, 2013). A process flow diagram provides the basic structure of a DES model and data collection via time studies allows the model to simulate the amount of time required to complete the process. The resources that are used (labor, costs, supplies) in the process can also be inserted into the model (Allen *et al.*, 2015). By using DES models, users can capture the complexity of commercial-scale, production systems and inform decisions made about those systems (Gittins *et al.*, 2020).

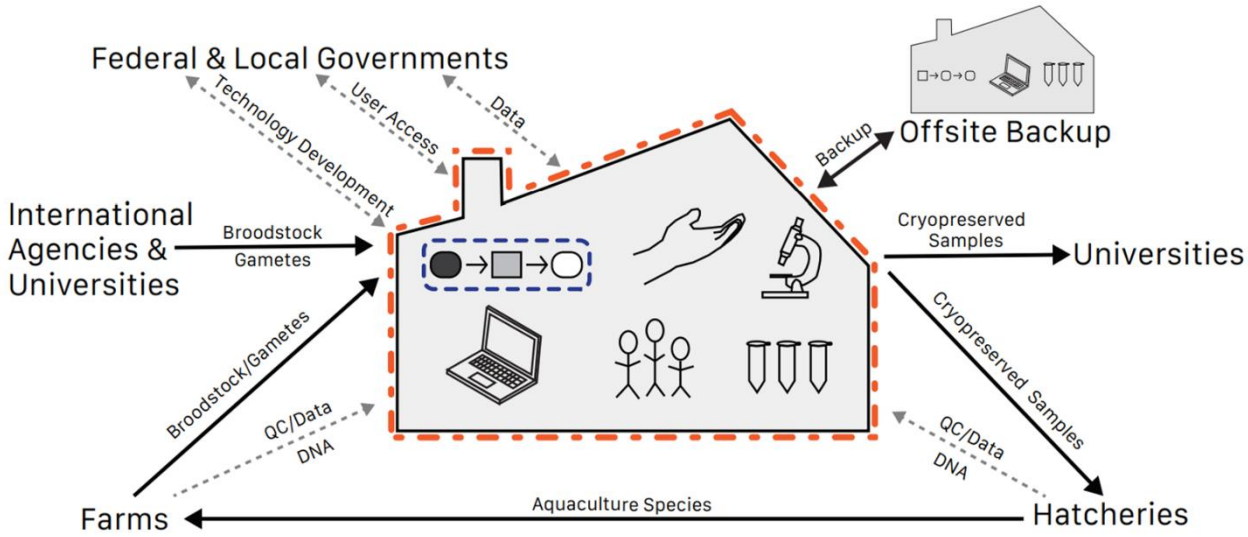
To construct a DES model, the process in question must be defined and outlined, which can be accomplished as described above with a process flow diagram. The process diagram is recreated in a DES modeling software, such as Simio (Simio LLC, Sewickley, PA), to form the basis of the model. Data on how long each step takes must then be collected and put into the model. It is common for data to be collected through repeated time studies where an operator performs the process and an observer records time per step. In addition, costs such as labor, supplies, and equipment would be incorporated. Models can be used to estimate the time and resources necessary to process a specific number of samples of a certain quality. Discrete-event simulation modeling has already been applied to the field of aquatic species cryopreservation to assist in facility planning and repository development (Hu *et al.*, 2015). Process flow diagrams and simulation modeling are valuable tools that can facilitate the development of high-throughput

cryopreservation pathways within germplasm repositories and to facilitate interactions with their communities.

While process flow diagrams are appropriate to understand processes such as cryopreservation, a different type of diagram addressing the community interaction and network levels should be used when outlining higher level organization of a repository system. *Relationship diagrams* depict the interactions between different members of a system and the flow of materials and information among those members (Damelio, 2011). These diagrams are used to display what resources (inputs) a facility uses and what products (outputs) the facility produces, as well as how those inputs and outputs move throughout the network. These diagrams can be used to highlight how each community member contributes to the repository network and how the repository facility must operate to meet the needs of its community.

To create a relationship diagram for the *Community Interaction Level*, all activities and components involved at a repository facility must be identified. For example, for a repository that serves catfish farmers, activities would include cryopreservation, spawning, raising the fish and cleaning tanks, and the components needed would be personnel, equipment, and supplies. The components should be represented inside the repository building icon in the center of the diagram. Next, the list of activities can be used to identify all the groups the repository interacts with, such as farmers, suppliers, and regulatory bodies. These groups would form the repository community and should be placed around the repository on the diagram. The type of repository model (central facility, mobile laboratory, or aggregate throughput) should be identified so that the diagram can depict how the repository and community interact. The repository should be connected (via arrows) to each community member by the materials and information they exchange. For example, the repository would receive live fish and germplasm samples from farmers. Finally, after all interactions have been outlined on the diagram, quantitative information can be added, such as how many frozen samples the repository distributes to farms each year (Figure 9).

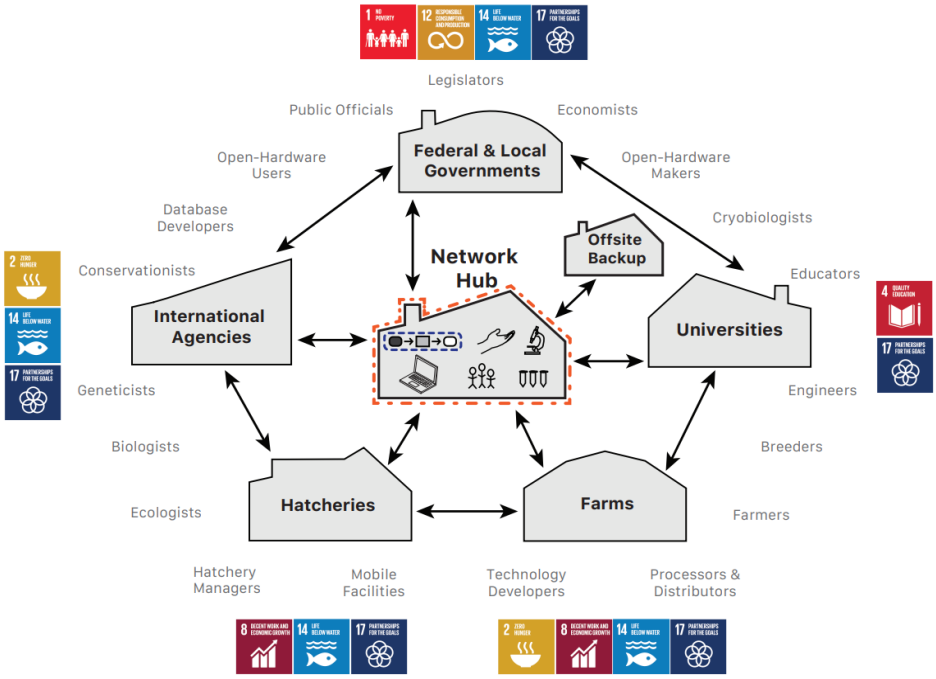
Figure 9. A community-interaction relationship diagram that encompasses all activities at a central repository facility (building outlined with dashed line) and the immediate interactions between the central repository and its surrounding community. The symbols within the repository represent six key repository components, from left to right: the dashed line surrounding a pathway diagram represents the cryopreservation pathway; the open hand represents community outreach programs; the microscope represents specialized equipment; the laptop computer represents a database and data management; the people represent repository personnel; and the microcentrifuge tubes represent supplies. The solid arrows indicate the flow of materials and the dashed arrows indicate the flow of information within the system.).



Relationship diagrams can also be used to portray interactions at the network level. Once all the members of the network have been identified, they can be placed on the diagram to display how they interact with the repository and with each other. This diagram would focus on the highest level of organization and highlights how each member contributes to the network and highlights its overall goals. To decide on goals and directions, network members should discuss commonalities and how each member can contribute. Relationship diagrams at the community interaction and network levels can inform decisions at the pathway level. For example, a repository operator may decide to use a particular freezing container because it is the easiest to work with, even though it is expensive. The operator could be unaware, however, of the number of members in the repository network that would request samples, making it impossible for the repository to operate cost-effectively. By first examining the relationships at the community interaction and network levels, informed decisions can be made to ensure cryopreservation pathways can meet community needs from the beginning.

Relationship diagrams at the community and network levels can also be used for DES modeling. Costs and profits, the different processes that take place at a repository, the resources exchanged between the repository and the community and scheduling constraints can all be incorporated. With models such as this, repositories can be planned like a business and integrated into current systems in a strategic manner highlighting also as each member of the repository network can contribute, directly or indirectly, to the sustainable development goals (SDGs) (Figure 10).

Figure 10. A network-level relationship diagram that displays a central repository facility (the Network Hub) and the surrounding communities that interacts with the repository and with each other to form a Repository Network. Two-way, solid arrows represent the exchange of materials and information. The lighter, grey words surrounding the Network are examples of professionals that could be involved within Network community members. Square symbols around the outside of the Network indicate how each member's involvement with the repository network could align with particular FAO Sustainable Development Goals (SDGs). The SDGs listed are: 1) No Poverty, 2) Zero Hunger, 4) Quality Education, 8) Decent Work and Economic Growth, 12) Responsible Consumption and Production, 14) Life Below Water, and 17) Partnerships for the Goals. Full definitions of each SDG can be found at www.fao.org/sustainable-development-goals/goals/goal-1/en/



References

Allen, M., Spencer, A., Gibson, A., Matthews, J., Allwood, A., Prosser, S., & Pitt, M. 2015. Right cot, right place, right time: Improving the design and organisation of neonatal care networks–a computer simulation study. *Health Services Delivery Research*, 3(9). https://doi.org/10.3310/hsdr03200

Apaiah, R.K., & Hendrix, E.M.T. 2005. Design of a supply chain network for pea-based novel protein foods. *Journal of Food Engineering*, 70(3), 383–391. https://doi.org/10.1016/j.jfoodeng.2004.02.043

Damelio, R. 2011. *The Basics of Process Mapping*, 2nd Edition. CRC Press.

Diego Fernando, M. D., & Rivera Cadavid, L. 2007. Lean manufacturing measurement: The relationship between lean activities and lean metrics. *Estudios Gerenciales*, 23(105), 69–83. https://doi.org/10.1016/S0123-5923(07)70026-8

Gittins, P., McElwee, G., & Tipi, N. 2020. Discrete event simulation in livestock management. *Journal of Rural Studies*, 78, 387–398. https://doi.org/10.1016/j.jrurstud.2020.06.039

Hu, E., Liao, T.W., & Tiersch, T.R. 2015. Simulation modelling of high-throughput cryopreservation of aquatic germplasm: A case study of blue catfish sperm processing. *Aquaculture Research*, 46(2), 432–445. https://doi.org/10.1111/are.12192

Renger, R., McPherson, M., Kontz-Bartels, T., & Becker, K.L. 2016. Process Flow Mapping for Systems Improvement: Lessons Learned. *Canadian Journal of Program Evaluation*, 31(1), Article 1. https://journalhosting.ucalgary.ca/index.php/cjpe/article/view/31038

Schriber, T.J., Brunner, D.T., & Smith, J.S. (2013). Inside discrete-event simulation software: How it works and why it matters. 2013 Winter Simulations Conference (WSC), 424–438. https://doi.org/10.1109/WSC.2013.6721439

Yang, H., Huo, Y., Yee, J.C., Rikard, S., Walton, W.C., & Saillant, E. 2021. Sperm Repository for a Breeding Program of the Eastern Oyster Crassostrea virginica: Sample Collection, Processing, Cryopreservation, and Data Management Plan. *Animals*, 11(10), 2836. https://doi.org/10.3390/ani11102836

Yang, H., & Tiersch, T.R. 2009. Current status of sperm cryopreservation in biomedical research fish models: Zebrafish, medaka, and Xiphophorus. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, 149(2), 224–232. https://doi.org/10.1016/j.cbpc.2008.07.005

Annex 7
The Aquatic Germplasm and Genetic Resources Center: a working model of development for repository for aquatic genetic resources

Terrence Tiersch, Aquaculture Germplasm and Genetic Resources Center (AGGRC), Louisiana State University Agricultural Center, Baton Rouge, Louisiana, United States of America
Yue Liu, Aquaculture Germplasm and Genetic Resources Center (AGGRC), Louisiana State University Agricultural Center, Baton Rouge, Louisiana, United States of America
Sarah Bodenstein, Aquaculture Germplasm and Genetic Resources Center (AGGRC), Louisiana State University Agricultural Center, Baton Rouge, Louisiana, United States of America

Introduction

The Aquatic Germplasm and Genetic Resources Center (AGGRC)[26] of the Louisiana State University (LSU) Agricultural Center in Baton Rouge, Louisiana, was created to serve as an interdisciplinary resource center to support germplasm repository development for aquatic species. In 1947, a 4,200 ft^2 building was built adjacent to LSU, surrounded by agricultural lands next to the Mississippi River levee that housed the LSU Dairy Improvement Center (DIC). In the following decades additions to the DIC expanded its size (to 24,000 ft^2), and additional facilities were constructed to support activities to improve the genetics and milk yield of dairy cattle. In the mid-1990s aquaculture and dairy scientists began to work together at that facility to pioneer the freezing of fish sperm by use of established industrial approaches developed for semen of dairy bulls (Lang *et al.*, 2003, Dong *et al.*, 2005). This work in cryopreservation provided the foundation for shifting from laboratory protocols to development of programs and germplasm repositories. Despite the huge difference in genotype, the dairy sector provided a valuable model for finfish and shellfish because it is a large, mature global industry based on protecting, shipping, and selling valuable breeds in the form of frozen semen. Those are all activities that will be important for eventual application of similar technology to aquatic species.

This focus on applying the results of research in the real world was intended to help multiple stakeholders. For example, fish farmers, just like ranchers or crop farmers, need genetic improvement to ensure that they can produce a high-quality product. Maintaining valuable broodstock in fish ponds is expensive and risky. Freezing their gametes offers a way to preserve valuable genetic resources and more easily use them in selective breeding programs. The genetic diversity of wild stocks and species can also be protected by cryopreservation. Based on such needs the AGGRC was created in 2015 at the former DIC to continue the legacy of germplasm protection by converting the facility from dairy bulls to work with finfish, shellfish, and other aquatic species. The DIC facility became available at that time because, over the decades, the focus of dairy breeding shifted from university research farms to the private sector, and the Dairy Science faculty retired or moved their work onto the LSU campus.

[26] https://aggrc.com/index.php

The work performed at the AGGRC spans more than three decades of research on hundreds of species of finfish and shellfish, with the intention of transitioning genetic improvement and high-throughput gene banking into real-world application. This included integrated development, for example, of strategies for thermal management of catfish broodstock ponds to control and expand the spawning period (Pawiroredjo, 2008), the use of ultrasound to increase the efficiency of hormone injection for collection of high-quality eggs (Guitreau *et al.*, 2012, Novelo and Tiersch 2016), and machine learning approaches (Graham *et al.*, 2022). Technological capability has been developed including more than eighty 3-D printers of various types and configurations (Tiersch and Monroe 2016, Zuchowicz *et al.*, 2022) in multiple locations (e.g., training and prototyping, batch fabrication), and eight animal holding facilities serving multiple research laboratories. The mission of the AGGRC is to continue such efforts and to assist the development of germplasm repositories for aquatic species through interdisciplinary approaches that encompass four main programs: basic and applied research, interdisciplinary technology development, commercial-scale application, and outreach.

In summary, the AGGRC continues a 75-year legacy of addressing real-world problems while moving into the future, and a large part of its mission is to serve as an inspiration for developing other similar facilities and interactions around the world. The AGGRC has emerged as a unique interdisciplinary facility with interlocking programs designed to bring new approaches to large, global problems. Facilities such as this can create fresh opportunities and support the growth of lucrative markets and industries for protecting and distributing the genetic resources of aquatic species.

References

Bodenstein, S., Nahmens, I., Callam, B. and Tiersch, T.R. Simulation analysis of high-throughput oyster cryopreservation at three scales of production. *Aquaculture International*. Published online 07/05/2023. https://doi.org/10.1007/s10499-023-01139-y

Dong, Q., Eudeline, B., Huang, C., Allen, S.K., and Tiersch, T.R. 2005. Commercial-scale sperm cryopreservation of diploid and tetraploid Pacific oysters, *Crassostrea gigas*. *Cryobiology* 50:1-16.

Graham, C., Shamkhalichenar, H., Browning, V., Byrd, V., Liu, Y., Gutierrez-Wing, M.T., Novelo, N., Choi, J.W., and Tiersch, T.R. 2022. A practical evaluation of machine learning for classification of ultrasound images of ovarian development in channel catfish (*Ictalurus punctatus*). *Aquaculture* 552:738039. https://doi.org/10.1016/j.aquaculture.2022.738039 PMC8920069.

Guitreau, A.M., Eilts, B.E., Novelo, N.D., and Tiersch, T.R. 2012. Fish handling and ultrasound procedures for viewing the ovary of submersed, non-anesthetized, unrestrained channel catfish. *North American Journal of Aquaculture* 74:182-187.

Hu, E., Liao, T.W., and Tiersch, T.R. 2013. A quality assurance initiative for commercial-scale production in high-throughput cryopreservation of blue catfish sperm. *Cryobiology* 67:214-224.

Hu, E., Liao, T.W., and Tiersch, T.R. 2015. Simulation modeling of high-throughput cryopreservation of aquatic germplasm: a case study of blue catfish sperm processing. *Aquaculture Research* 46:432-445.

Huene, A., Koch, J., Arregui, L., Liu, Y., Nicotra, M., Weis, V. and Tiersch, T.R. 2022. Cryopreservation of *Hydractinia symbiolongicarpus* sperm to support community-based repository development for preservation of genetic resources. *Animals* 12, 2537. https://doi.org/10.3390/ani12192537 PMC9559378.

Lang, R.P., Riley, K.L., Chandler, J.E., and Tiersch, T.R. 2003. The use of dairy protocols for sperm cryopreservation of blue catfish *Ictalurus furcatus*. *Journal of the World Aquaculture Society* 34:66-75.

Liu, Y., Taylor, S., Blackburn, H. and Tiersch, T.R. 2019. Development of germplasm repositories to assist conservation of endangered fishes: Examples from small-bodied livebearing fishes. *Theriogenology* 135:138-151.

Novelo, N., and Tiersch, T.R. 2016. Development and evaluation of an ultrasound imaging reproductive index based on the ovarian cycle of channel catfish *Ictalurus punctatus*. *Journal of the World Aquaculture Society* 47:526–537.

Pawiroredjo, P., Lamoureux, J., Hall, S., and Tiersch, T.R. 2008. Degree-days as a tool to determine the heating requirement for channel catfish spawning in earthen ponds. *North American Journal of Aquaculture* 70:328-337.

Shirley, C.A., Colvin, M.E., Tiersch, T.R. and Allen, P.J. 2020. A generalized approach for sperm cryopreservation in the genus *Poxomis*: Sperm cryopreservation and fertilization efficiency of black-stripe black crappie, *Poxomis nigromaculatus*. *Journal of the World Aquaculture Society* 52:405-417.

Tiersch, T.R. 2011. Process pathways for cryopreservation research, application and commercialization. In: *Tiersch, T.R. and Green, C.C.*, (editors), *Cryopreservation in Aquatic Species*, 2nd edition. World Aquaculture Society, Baton Rouge, Louisiana. Pp. 646–671.

Tiersch, T.R., and Monroe, W.T. 2016. Three-dimensional printing with polylactic acid (PLA) thermoplastic offers new opportunities for cryobiology. *Cryobiology* 73:396-398. http://dx.doi.org/10.1016/j.cryobiol.2016.10.005 PMC5116258.

Torres, L. and Tiersch, T.R. 2018. Addressing reproducibility in cryopreservation, and considerations necessary for commercialization and community development in support of genetic resources of aquatic species. *Journal of the World Aquaculture Society* 49(4):644-663. https://doi.org/10.1111/jwas.12541.

Zuchowicz, N., Belgodere, J., Liu, Y., Semmes, I., Monroe, W.T., and Tiersch, T.R. 2022. Low-cost resin 3-D printing for rapid prototyping of microdevices: Opportunities for supporting aquatic germplasm repositories. *Fishes* 7, 49:1-12. https://doi.org/10.3390/fishes7010049 PMC9838218

Annex 8
Conservation of aquatic species under the National Animal Germplasm Program

Harvey D. Blackburn, National Animal Germplasm Program, Agricultural Research Service, United States Department of Agriculture (USDA), Fort Collins, Colorado, United States of America

Introduction

The United States Department of Agriculture (USDA) is developing a substantial gene bank of cryopreserved germplasm and tissues of aquatic species, as part of the National Animal Germplasm Program (NAGP). The NAGP, established in 1999, operates a gene bank for genetic resources for food and agriculture (livestock, aquatic, and insect genetic resources) that interacts with a wide range of researchers, producers and other industry representatives. It has three major technical objectives (Figure 11):

- Collect information (e.g., phenotypic parameters, management system, GIS coordinates etc.) on the genetic resources stored in the gene bank.
- Use the germplasm and live populations to conduct analysis on the genetic diversity.
- Conducting research on cryobiology to develop cryopreservation protocols for materials of interest to the gene bank.

The inclusion of aquatic organisms started as the NAGP was established in 1999. Industry and research partners help to collect samples, which are cryopreserved either before or after coming to the gene bank, depending on the species, partner expertise and logistical considerations.

The goal of the collection is to build a reserve of genetically distinct populations that are of agronomic, research, conservation and cultural importance. To date, the collection of aquatic organisms contains samples from 65 species or populations representing over 9,300 animals (Table 8). The main species constituting groups of interest to aquaculture for which samples are gene banked include Salmonidae, Ictaluridae, and Ostreidae, with samples from both wild and cultured individuals to have been collected.

The collection of physical samples is underpinned by a comprehensive database, the Animal Genetic Resources Information Network (Animal-GRIN).[27] The database strives to capture information not only about the samples and where they are stored but also on phenotypic, genotypic, environmental and management information. Animal-GRIN development and maintenance is a collaborative effort involving Agriculture and Agri-Foods Canada, the Brazilian Agricultural Research Corporation (EMBRAPA), and NAGP. These joint experiences have demonstrated that substantial databases are critical infrastructure for gene banks because they not only provide a mechanism for managing collections, but they also allow researchers, producers or the public to access the collection.

While the main goal of the collection is to provide genetic security, it is beneficial to use the collection for other purposes such as research projects, introducing new or lost alleles to a population, and genomic analysis. Samples can be used by industry or research community. For example, samples of rainbow trout

[27] https://agrin.ars.usda.gov/main_webpage_dev/ars?language=EN&record_source=US

(*Oncorhynchus mykiss*) were used by state agencies in a breeding program to increase resistance to whirling disease and Eastern oyster (*Crassostrea virginica*) samples have been genotyped to assess the genetic variability of oyster populations living in the Louisiana Gulf Coast.

Key lessons learned in the process include:

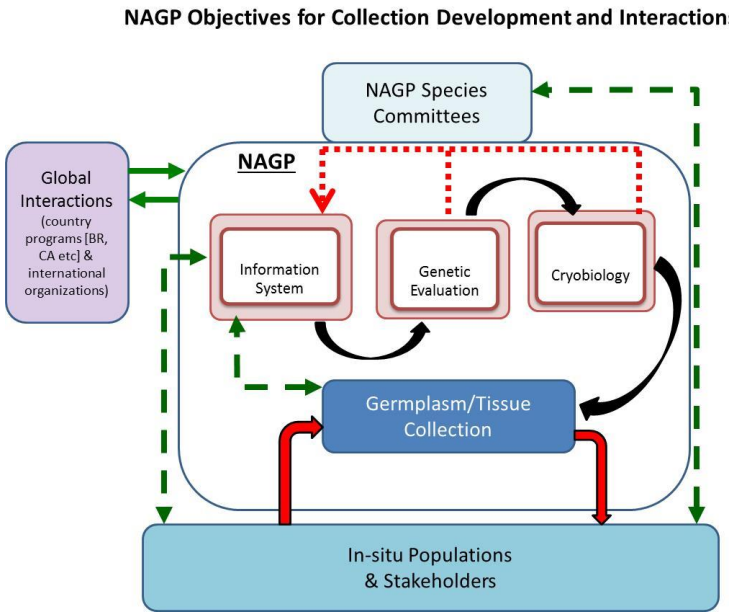
- A gene bank needs the **agility** to adapt to technology changes and the **flexibility** to meet different scopes.
- The importance of well curated and comprehensive **databases** must not be underestimated.
- **Proactivity** - Make sure the gene bank is used and appropriate KPIs recorded. Don't wait for the worst-case scenario to happen. When gene banks are not used, their value and return on investment is questioned.
- **Exercise caution** - Collect extra germplasm where possible, so new ways to use or operate the collection can be explored.
- **Resilience** – Collect samples for a population temporally rather than all in a short period of time. This will help build functionality into the collection.

Table 8. Summary of the USDA NAGP aquatic species collection (as of September 2022)*

Group	Species/populations	Number of animals sampled	Number of samples obtained
Aquatic freshwater fish	46	8,730	116,771
Aquatic Invertebrates	13	627	12,671
Aquatic marine fish	6	15	823
Total	65	9,372	130,265

* For a complete description of the collection, see Animal-GRIN at:
https://agrin.ars.usda.gov/main_webpage_dev/ars?language=EN&record_source=US

Figure 11. NAGP scheme illustrating the interconnectedness among information, genetic evaluation, and cryobiology of samples within the gene bank as germplasm is added to the collection. Black arrows represent connections between program areas; solid red= germplasm.



Annex 9
Conservation of endangered Brazilian fish species with in vitro germ cell conservation and transplant at CEPTA/ICMBio

José Augusto Senhorini – CEPTA/ICMBio – Centro Nacional de Pesquisa e Conservação da Biodiversidade Aquática Continental, Pirassununga, Brazil
Lucia Suarez Lopez – CICESE – Ensenada Center for Scientific and Higher Education, Ensenada, Mexico
Amanda Pereira dos Santos Silva – USP-University of São Paulo**,** São Paulo, Brazil; CEPTA/ICMBio
Gabriella Braga Carvalho – USP-University of São Paulo**,** São Paulo, Brazil; CEPTA/ICMBio, CEPTA/ICMBio - Centro Nacional de Pesquisa e Conservação da Biodiversidade Aquática Continental, Pirassununga, Brazil
George Shigueki Yasui- USP-University of São Paulo**,** São Paulo, Brazil; CEPTA/ICMBio, CEPTA/ICMBio – Centro Nacional de Pesquisa e Conservação da Biodiversidade Aquática Continental, Pirassununga, Brazil

Introduction

The National Center for Research and Conservation of Aquatic Biodiversity (CEPTA) is part of the Chico Mendez Institute for Conservation of Biodiversity (ICMBio) of the Brazilian Ministry of Environment and Climate Change (MMA).[28]

ICMBio is one of the three institutes of the MMA.[29] It is responsible for federally designated Conservation Areas of the country (Unidades de Conservação), including parks, ecological reserves, research stations, etc. CEPTA is the research station responsible for aquatic biodiversity. It works on aspects of management of aquatic biodiversity, including conservation and recovery of aquatic species at risk of extinction.

CEPTA now houses one of the largest finfish gene banks in Latin America. *In vivo* and *in vitro* collections have been established for endangered species including piracanjuba (*Brycon orbignyanus*), and the mottled catfish (bagre-sapo) (*Pseudopimelodus mangurus*) (Arashiro *et al.*, 2020) as well as for model species such as the yellow-tail tetra (*Astyanax altiparanae*) (Yasui *et al.*, 2022), mandi amarelo catfish (*Pimelodus maculatus*) (Arashiro *et al.*, 2018), curimbatá (*Prochilodus lineatus*) (Coelho *et al.*, 2019) and matrinchã *Brycon amazonicus* (Silva *et al.*, 2017). The tetra, curimbatá, and matrinchã are also aquaculture species.

Although cryopreservation of spermatozoa is relatively easy to achieve, it is not currently the main focus at CEPTA/ICMBio, considering the difficulty to fully restoring populations and their gene pool only from milt (due to the still limited success of eggs cryopreservation in aquatic animals). The institute, in collaboration with the University of São Paulo, have focused on the preservation of spermatogonial and oogonial stem cells, and primordial germ cells (PGCs). Both stem germ cells lineages may be transplanted

[28] www.icmbio.gov.br/cepta/
[29] The three institutes of MMA are: Instituto Brasileiro do Meio Ambiente e dos Recursos Renováveis – IBAMA; Instituto Chico Mendes de Conservação da Biodiversidade – ICMBio; and Instituto de Pesquisas Jardim Botânico do Rio de Janeiro – JBRJ

into a sterile host (Bertolini *et al.*, 2019), which then act as a surrogate and produce gametes of the donor species (Coelho *et al.*, 2019). Two model organisms have been used for the transplantation: the catfish *Pimelodus maculatus* (Bertolini *et al.*, 2019) and the banded astyanax (*Astyanax altiparanae*) (Yasui *et al.*, 2022). Both species are being used as hosts for stem germ cells from endangered siluriforms and characiforms, respectively, the two fish families that comprise more than 50 percent of neotropical species.

However, in the case of the banded astyanax , the cryopreservation of "X" spermatozoa from sex-reversed fish is also being performed for future production of monosex females for its sustainable aquaculture. The culture of monosex female fish of this and other species is interesting both to optimize the culture performance and reduce reproduction in the wild of potential escapees, considering the introduction of alien species is the second major cause of extinction of native species in Brazil (ICMBio, 2018). Most of the advanced techniques mentioned above were developed first in the yellowtail tetra, which has become a laboratory model or developing technologies in other related species (Yasui *et al.*, 2022). Ongoing research also includes cryopreservation of stem germ cells through vitrification and slow cooling and nuclear transplantation from embryonic cells.

These model systems and frozen gene bank at CEPTA will continue to be used, in collaboration with the University of São Paulo, in the development of alternative processes for recovery of species at extreme risk of extinction.

References

Arashiro, D.R., Yasui, G.S., Ninhaus-Silveira, A., Veríssimo-Silveira, R., & Foresti, F. 2018. Spermatogenesis, stem cell niche, and germ cell transplantation in *Pimelodus maculatus* (*Siluriformes: Pimelodidae*): A model Neotropical catfish for biotechnological studies. *Animal Reproduction Science*, 192: 171–182.

Arashiro, D.R., Yasui, G.S., Ninhaus-Silveira, A., Veríssimo-Silveira, R., & Foresti, F. 2020. Gene banking and conservation of neotropical fish: advances and perspectives in *Brycon orbignyanus*. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 30: 854–867.

Bertolini, F., Yasui, G.S., Miliorini, A.B., Veríssimo-Silveira, R., & Foresti, F. 2019. Cryopreservation and transplantation of germ cells in neotropical fish: developing biotechnological tools for conservation. *Journal of Fish Biology*, 94(4): 612–624.

Coelho, J.S., Yasui, G.S., Senhorini, J.A., & Foresti, F. 2019. Cryopreservation and germ cell transplantation of *Prochilodus lineatus*: Development of biotechnological tools for genetic conservation in Neotropical fish species. *Theriogenology*, 123: 158–167.

ICMBio. 2018. *Plano de Ação Nacional para Conservação de Espécies Ameaçadas de Extinção: Espécies Aquáticas Continentais*. Brasília, Brazil, Instituto Chico Mendes de Conservação da Biodiversidade (ICMBio).

Silva, A.M., Yasui, G.S., Senhorini, J.A., & Foresti, F. 2017. Gamete cryopreservation and germ cell transplantation in *Brycon amazonicus*: Developing strategies for genetic conservation and breeding programs. *Aquaculture Research*, 48: 2948–2959.

Yasui, G.S., Arashiro, D.R., Ninhaus-Silveira, A., Veríssimo-Silveira, R., & Foresti, F. 2022. The banded astyanax (*Astyanax altiparanae*) as a model organism for biotechnological studies in Neotropical fish species: Gene banking and stem cell technologies. *Aquaculture Reports*, 25: 101226.

References

Arashiro, D.R., Yasui, G.S., Calado, L.L., do Nascimento, N.F., do Santos, S.C.A., Shiguemoto, G.F., et al. 2020. Capturing, induced spawning, and first feeding of wild-caught Pseudopimelodus mangurus, an endangered catfish species. *Lat. Am. J. Aquat. Res.* 48.

Arashiro, D.R., Yasui, G.S., Calado, L.L., do Nascimento, N.F., dos Santos, M.P., do Santos, S.C.A., et al. 2018. Synchronizing developmental stages in Neotropical catfishes for application in germ cell transplantation. *Zygote*, 1–14.

Bertolini, R.M., Arashiro, D.R., Senhorini, J.A., Santos, S.A., Siqueira-Silva, D., Rocha, N.R.A., et al. 2019. Strategies for aquaculture and conservation of Neotropical catfishes based on the production of triploid Pimelodus maculatus. *Aquac. Int.* 1, 10.

Coelho, G.C.Z., Yo, I.S., Mira-López, T.M., Monzani, P.S., Arashiro, D.R., Fujimoto, T., et al. 2019. Preparing a fish embryo (Prochilodus lineatus) for staging, chorion removal and PGC traceability. *Int. J. Dev. Biol.* 63, 57–65. doi:10.1387/ijdb.180348gc.

ICMBio. 2018. "Livro Vermelho da Fauna Brasileira Ameaçada de Extinção - Peixes," in *Livro Vermelho da Fauna Brasileira Ameaçada de Extinção*, ed. I. C. M. de C. da Biodiversidade (Brasília: Instituto Chico Mendes de Conservação da Biodiversidade).

Raycon, R.F., Garcia, R.R.F., Streit, D.P. Jr, Cabrita, E., and Godoy, L.C. 2016. Semen Cryopreservation in Brazilian Freshwater Fish:Advances and Main Challenges. **IN:** Marco-Jiménez, F. and Akdemir, H. (eds.) Cryopreservation of Eukaryotes, IntechOpen. pp. 55-73. http://dx.doi.org/10.5772/65037

Silva, R.C., dos Santos, M.P., Senhorini, J.A., Paes, M. do C.F., Valentin, F.N., Fujimoto, T., et al. 2017. The effect of temperature on the initial development of Brycon amazonicus Spix & Agassiz, 1829 as tool for micromanipulation of embryos. *Zygote*, 1–15.

Yasui, G.S., Nascimento, N.F., Pereira-Santos, M., Santos-Silva, A., Coelho, G.Z., Visintin, J.A., et al. 2022. Establishing a model fish for Neotropical region: the case of the yellowtail tetra Astyanax altiparanae in advanced biotechnology Title. *Front. Genet.* 1, 1–15.

Annex 10
Aquatic genetic resource management in Brazil at EMBRAPA

Luciana Cristine Vasques Villela, Embrapa Fisheries and Aquaculture, Palmas, Brazil
Samuel Rezende Paiva, Embrapa Genetic Resources and Biotechnologies, Brasilia, Brazil

Background

The Brazilian Agricultural Research Corporation (EMBRAPA),[30] established in 1973, is one of the largest agricultural research corporations of the world. It has a mandate of generating knowledge and technology for Brazilian agriculture, including aquaculture. It has had an animal genetic resources conservation program since 1983, with both *ex situ* and *in vivo* components. The program started with endangered, locally adapted livestock breeds, but now includes all the main commercial livestock breeds as well as some other Brazilian native species with economical potential, such as stingless bees and freshwater fishes. The cryopreserved collection includes semen, embryos, and fibroblasts as well as DNA, hair, blood samples and other tissues (e.g., fish fins). All the *in situ* and *ex situ* collections can be accessed through the EMPRABA database.[31] Table 9 shows the general quantitative data for aquaculture regarding the whole collection.

Current and future efforts of the Brazilian *ex situ* collection include (but it is not limited to) establishing a process to fast-track legal agreements for the exchange of biological material which could bring greater interest from farmers, research institutions and industry. In addition, we are collaborating and monitoring the development of international quality standards (ISO) for biobanks for genetic resources., Improvements to gene bank management and utilization are also underway. One of the major changes was to broaden the scope of the collection. Initially focused on conservation of rare, local, or national breeds, the effort has now expanded to the cryopreservation of priority farmed types, for selective breeding (including native fish species tambaqui, caranha, piraracu and surubim). The goal of this work is improved food security contribution of the national aquaculture production system, as these species serve primarily a national market. A second major change was the implementation of a routine procedure to genotype all semen samples in the repository with available tools such as, low, medium density single nucleotide polymorphism (SNP) chips or a custom set of SNP markers. This is a dynamic process, with the genotyping of cryopreserved samples still underway. All of the genetic data are available on-line through the genomic module of Animal GRIN.[32] All the changes will be monitored and evaluated to adapt the strategy and comply with the needs of the Brazilian animal conservation program.

Table 9. Summary of quatic animal components of the Embrapa Frozen Biobank.

Group	Species/breeds	Number of animals sampled	Number of samples obtained
Aquatic freshwater fish	10	9,206	34417
Aquatic marine fish	1	128	2,312
Turtles	2	254	1,167
Whole Embrapa collection	43/95	32,978	1,950,602

[30] www.embrapa.br/en/international
[31] an.cenargen.embrapa.br/database_collaboration_page_dev
[32] https://agrin.ars.usda.gov/genomic_handling_dev?language=EN&record_source=BR&only_genomic=Y

*EMPRABA g*ene banking of aquatic species

The EMBRAPA's conservation program for aquatic species has two main branches: an *ex situ in vivo* and an *ex situ in vitro* collection.

The *ex situ in vivo* component consists of live animals maintained at the EMBRAPA Fisheries and Aquaculture Research Center in Palmas, Tocantins State. Since 2014, it focuses on three native species: tambaqui (*Colossoma macropomum*), caranha (*Piaractus brachypomus*), and pirarucu (*Arapaima gigas*). These are native freshwater finfish from the Amazon Basin that are of great commercial interest. They are being produced in several regions of the country, have high productive potential, and are at risk of loss of genetic diversity due to hybridization (in the case of tambaqui and caranha) or local extinction due to overfishing (pirarucu). The individuals in the gene bank were collected from the wild, from rivers of the Araguaia-Tocantins, Amazon and Paraguay basins, as well as from fish farms in the North and Northeast regions of Brazil. Genetic material for the three species is cryopreserved at the EMBRAPA gene bank, a facility for plant, animal and microorganism genetic resources located in Brasília Federal District, in the EMBRAPA Genetic Resources and Biotechnology Research Center (Table 9). This repository is not restricted to species from the *ex situ in vivo* program. It includes also cryopreserved semen from the Pantanal wetland in western Brazil, the largest periodically flooded area of the Americas, a Ramsar Site and a Biosphere Reserve of the United Nations Educational, Scientific and Cultural Organization (UNESCO). Most of those samples were collected from the wild, starting in the 1990s. They are a unique collection of the genetic diversity of finfish from the region and provide genetic material for the development of breeding programs of tambaqui and surubim (*Colossoma macropomum* and *Pseudoplatystoma corruscans*), two species of particular interest in Brazilian aquaculture.

Table 9. Composition of the EMBRAPA finfish gene bank.

Scientific name	Common name	Semen doses	# semen donors	DNA samples	# DNA Donors	Tissue samples	# tissue Donors	Type
Characidae: *Brycon microlepsis*	Piraputanga	322	24	0	0	0	0	Freshwater
Characidae: *Colossoma macropomum*	Tambaqui	3,033	147	6,779	95	17,980	8,760	Freshwater
Characidae: *Piaractus brachypomus*	Caranha/ pirapitinga	364	17	0	0	4	2	Freshwater
Characidae: *Piaractus mesopotamicus*	Pacu	449	25	0	0	0	0	Freshwater
Characidae: *Salminus maxillosus*	Dourado	1,692	51	0	0	0	0	Freshwater
Osteoglossidae: *Arapaima gigas*	Pirarucu	0	0	4,173	101	46	23	Freshwater
Pimelodidae: *Leiarius marmoratus*	Jundiá	0	0	173	18	0	0	Freshwater
Pimelodidae: *Pinirampus pirinampu*	Barbado	0	0	179	17	0	0	Freshwater
Pimelodidae: *Pseudoplatystoma corruscans*	Pintado	1,324	40	0	0	0	0	Freshwater

Pimelodidae: *Pseudoplatystoma reticulatum*	Surubim/ cachara	623	15	0	0	0	0	Freshwater
Rachycentridae: *Rachycentron canadum*	Bijupirá	0	0	2312	128	0	0	Marine
TOTAL	**11**	**7,807**	**319**	**13,616**	**359**	**18,030**	**8,785**	

Challenges, solutions and lessons learned

Several challenges have impeded the further development of the finfish gene banking system.

The biggest challenges have been funding and adequately trained personnel. This has been particularly challenging for the live gene bank component, but the on-going cost of liquid nitrogen for the frozen gene bank has also been problematic.

Moving the broodstock of the live gene bank from contracted to in-house facilities has improved its management. To help resolve the problems of the frozen gene bank, we combined the fish gene bank with the centrally located gene bank at EMBRAPA's Research Center in Brasilia, DF. This facility stores genetic material from animal, microorganisms and plant genetic resources.

Nevertheless, supplementary and more stable funding is needed, including both improved parliamentary support for guaranteed internal support and funds from outside sources such as industry and international sources.

References

Blackburn, H.D., Wilson, C.S. & Krehbiel, B. 2019. Conservation and utilization of livestock genetic diversity in the United States of America through genebanking. *Diversity*, 11: 244. Available at: https://doi.org/10.3390/d11120244.

FAO. 2012. Cryoconservation of animal genetic resources. *FAO Animal Production and Health Guidelines No. 12*. Rome, FAO.

Joyce, P. & Tavaré, S. 1995. The distribution of rare alleles. *Journal of Mathematical Biology*, 33: 602–618. Available at: https://doi.org/10.1007/BF00298645.

Annex 11
Ex-situ in vitro conservation at the ICAR-NBFGR Fish Genetic Resource Repository and Museum

Kuldeep K Lal, ICAR-Central Institute of Brackishwater Aquaculture, Chennai, India
Vindhya Mohindra, ICAR-National Bureau of Fish Genetic resources (NBFGR), Lucknow India
Rajeev K Singh, ICAR-National Bureau of Fish Genetic resources (NBFGR), Lucknow India
Ajithkumar, ICAR-National Bureau of Fish Genetic resources (NBFGR), Lucknow India
J K Jena, Fisheries Science Division, Indian Council of Agricultural Research, New Delhi, India

Introduction

The Indian Council of Agricultural Research (ICAR) manages collections of genetic resources of crops, livestock, fish, and agriculturally important microorganisms and insects. The National Bureau of Fish Genetic Resources (NBFGR) was established within ICAR in 1983, with a specific focus on scientific research and management of aquatic biodiversity, including aquatic genetic resources for food and agriculture (AqGR). ICAR-NBFGR works through its headquarters located in Lucknow and a regional station in Kochi (the Peninsular and Marine Fish Genetic Resource Centre). The development history of ICAR-NBFGR is described in Silas (2010).

ICAR-NBFGR is the lead institution addressing research issues relevant to AqGR management in India and provides technical support to different departments for fulfilling national and international obligations. The bureau's research programs envision "National Actions and Global Relevance" and the NBFGR has in-house capacity on several aspects related to AqGR management, including: assessment of finfish diversity; genetics and genomics; *ex-situ* and *in-situ* conservation; biosecurity; risk assessment of alien species introductions; and aquatic animal disease resistance and antimicrobial resistance in aquaculture.

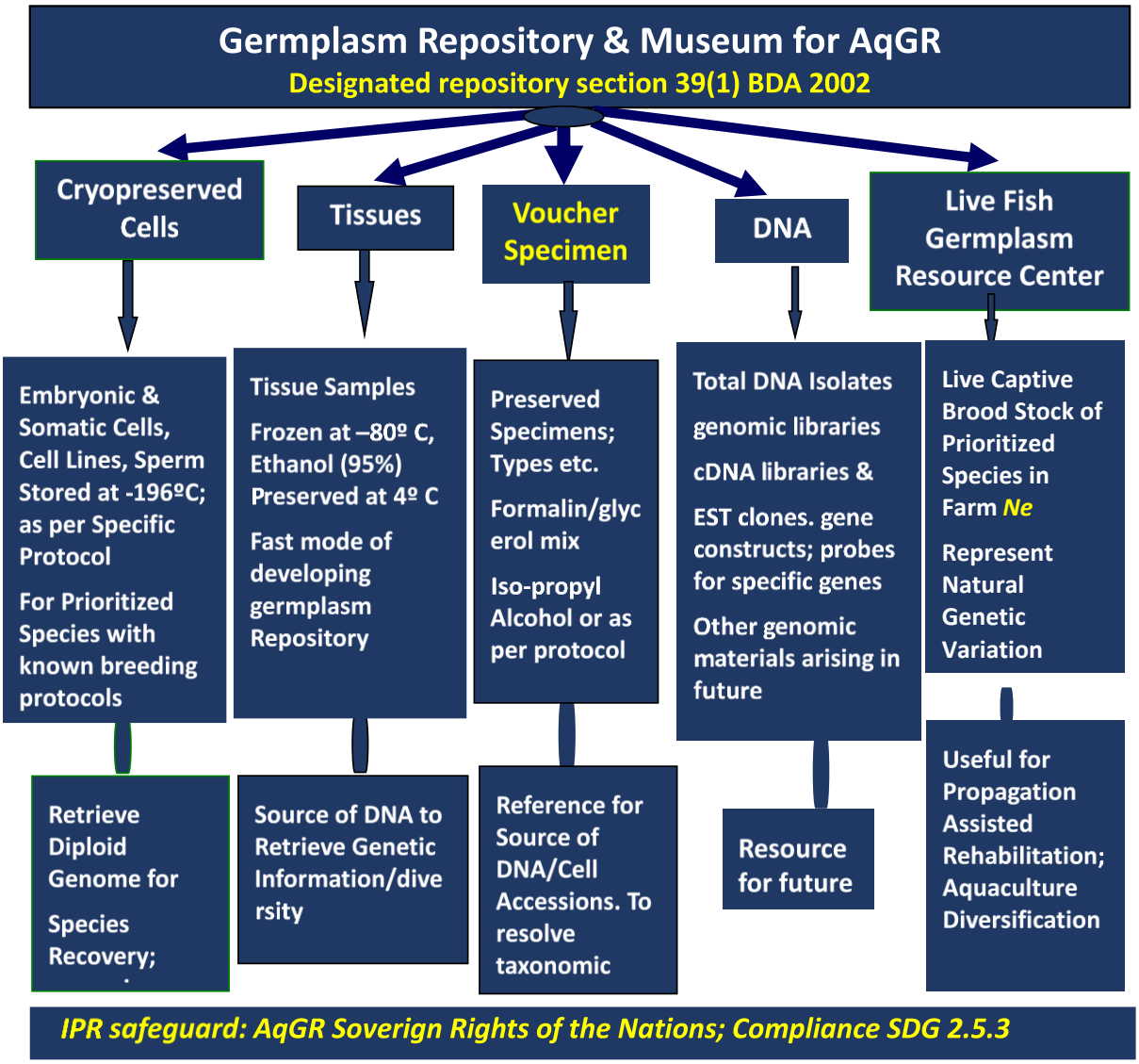
Maintenance of germplasm and capacity building is one of the mandates of NBFGR. NBFGR is a designated repository of fish genetic resources under Section 39 of the Indian Biological Diversity Act of 2002 (http://nbaindia.org/uploaded/pdf/notification/1%20designated%20repositories.pdf).

Conceptual framework of the NBFGR repository

ICAR-NBFGR envisions an integrated, multifunctional facility, the "National Fish Genetic Resource Repository and Museum", with functions of maintenance, enrichment and dissemination of material and knowledge. Figure 12 illustrates the conceptual framework of the NBFGR repository showing the type of AqGR stored at the repositories, the *ex situ in vitro* methodologies utilized and the destination uses of the preserved material.

Repository accessions, in the conceptual framework are categorized as non-retrievable or retrievable (ICAR-NBFGR, 2016).

Figure 12. Conceptual framework of the ICAR-NBFGR repository.



Non-retrievable material: reference voucher specimens and tissue repository

The central role of the NBFGR voucher repository is as future reference for accessions and taxonomic identification. Accessibility to vouchers is critical for correct taxonomic identifications, re-descriptions of species, and genetic studies (including to support legal issues; Buckner *et al.*, 2021). All specimens available as vouchers are also supported by tissue accessions in the NBFGR repository. A digital radiographic facility for the analysis of skeletal characteristics is available as an additional tool for species characterization.

Tissue accessions are a fast way to build a repository of AqGR. The tissues are stored at -80 °C or at 4°C in 95 percent ethanol. In addition, the repository holds extracted DNA stored at -20°C and any processed DNA, such as (bacterial artificial chromosome) BAC libraries or gene constructs for use in future research.

The details of different types of material and their storage procedures are provided in detail in ICAR-NBFGR, 2016 (Chapter 8 on Management of Genomic Resources).

Retrievable material samples for use in restoration

Accession types in this category include sperm, embryonic cells and somatic cells, as primary cultures or continuous cell lines. Cells are cryopreserved for short or long-term *ex situ in vitro* conservation of species and well-performing aquaculture strains.

Cryopreserved sperm can be used for captive reproduction although it represents only the paternal genome. Fish sperm cryopreservation needs the development of species-specific protocols capable of preserving sperm from species in the wild in large quantities. The NBFGR has been engaged for the development of sperm cryopreservation protocols of wild and aquaculture species (Lakra *et. al.*, 2007). So far this has included 30 species (Jena *et. al.*, 2022) and the production of viable progeny for 19 of these species. However, routine application of this technology in commercial aquaculture has not yet been achieved, though NBFGR has begun a program to use the technology in carp hatcheries for genetic exchange. During the field validation, over 11 million larvae have been produced with cryopreserved sperm in 36 hatcheries, in 11 states of India.

Somatic and embryonic cells are also an important component of the repository. They provide *ex situ* conserved genomes for future research on genomics and application in biotechnologies such as surrogate broodstock and nuclear cloning for species recovery.

Somatic and embryonic cells are available as cryopreserved continuous cell lines. A total of 77 authenticated cell lines from 36 finfish species are available in the repository. These cells are developed by NBFGR or are deposited by researchers at the end of projects, for safe storage and future use (Murali *et.al.*, 2020). The cell line repository (https://mail.nbfgr.res.in/nrfc/index.phps) is available to national and international researchers pending approved material transfer agreements (international researchers require approval of the National Biodiversity Authority in compliance with the Biological Diversity Act of 2002). This is amongst the largest collection of conserved fish cell lines in the world. They have been used primarily for fish virus research and have played a vital role in India's National Surveillance Program on Aquatic Animal Diseases. They have also been used as *in vitro* models to find inter-specific risk of emerging pathogens. Cell line development requires extensive research, technical capacity and resources. To date, this is only attempted for priority species of aquaculture and conservation importance.

Somatic cells are being banked on a pilot scale, in the form of cryopreserved primary cultures for conservation of important fish species, including threatened species and elite selected genetic stocks. Primary cultures from 16 species and multiple selected individuals of a few of these species have been cryo-conserved over two years. The aim is a versatile system of diploid cell conservation that can be thawed for research in emerging technologies (Chenais *et. al.*, 2014), such as nuclear cloning, or induced pluripotency etc. The collections have included field collection of somatic cells from distant locations and from cadavers. Some success has been achieved in this, with field validation (publication in prep), and scaling up is being pursued. Stem cells extracted as spermatogonia from adult fish, as well as embryonic cells, will eventually be part of these collections. However, these will likely be limited to the species which can be farmed and captive bred.

The live fish germplasm resource centres

The NBFGR includes a series of live fish germplasm resource centres (LFGRCs) for the *ex situ in vivo* conservation of different life-history stages of aquatic species (Fig. 14), backed up by the cryopreserved material of the NBFGR where needed. LFGRCs are located close to the native distribution of species or genetic stocks, as recommended under the article 9 of the Convention on Biological Diversity (CBD). These are used for conservation breeding as well as evaluating productive value of species or genetic stocks for aquaculture (ICAR-NBFGR 2011 and 2016; Chapter 7 Conservation of germplasm, 34–36 p.). The centres are analogous to pre-breeding evaluation sites of terrestrial agricultural genetic resources but are concerned with both conservation of threatened species and development of aquaculture species. So far, seven LFGRCs have been established and form a network containing, in total, 5066 live accessions from 44 species. These are being used for local restocking, assessing culture potential, recovering threatened species, and supporting community aquaculture.

Information systems

India has also developed the Aquatic Genetic Resource Information System (AqGRISI)[33] that support both the *ex situ* and *in vitro* collections kept at NBFGR and the various LFGRGs. The database contains open-source information for 3157 Indian species in relation to species distribution, biology, taxonomy, type specimens for taxonomy, genetics and genomics, as well as patents. AqGRISI is also linked to different museums, genomic databases and information on type specimens. AqGRISI is also used to generate unique species accession codes and to code the holotypes of new discovered species. AqGRISI was designed to function as a repository and to provide numbers of accessions, however, its repository functions are available only to authorized users.

[33] https://aqgrisi.nbfgr.res.in/

References

Buckner, J.C., Sanders, R.C., Faircloth, B.C., & Chakrabarty, P. 2021. The critical importance of vouchers in genomics. *Elife*, 10: e68264. doi: 10.7554/eLife.68264. PMID: 34061026; PMCID: PMC8186901.

Chenais, N., Depince, A., Le Bail, P.Y., & Labbé, C. 2014. Fin cell cryopreservation and fish reconstruction by nuclear transfer stand as promising technologies for preservation of finfish genetic resources. *Aquaculture International: Journal of the European Aquaculture Society*, 22(1): 63–76. DOI: 10.1007/s10499-013-9671-4.

ICAR-NBFGR. 2011. *Species Prioritisation for ex-situ conservation and Freshwater Aquaculture*. ICAR-National Bureau of Fish Genetic Resources, Lucknow, India, 22 p. www.nbfgr.res.in/images/publication/books-anuals/pdf/Proceeding_SpeciesPrioritization.pdf.

ICAR-NBFGR. 2016. *Guidelines for management of fish genetic resources in India*. ICAR-National Bureau of Fish Genetic Resources, Lucknow, India, 64+ xxiii p. www.nbfgr.res.in/site/writereaddata/siteContent/FGR-Guidelines_NBFGR.pdf.

ICAR-NBFGR. 2021. *Annual Report 2021*, ICAR-National Bureau of Fish Genetic Resources, Lucknow – 226002 (U.P.), India. www.nbfgr.res.in/site/writereaddata/siteContent/20221018094249517 3Annual%20Report%20NBFGR%202021%20English%20for%20web.pdf.

Jena, J.K., Gopalakrishnan, A., Ravishankar, C.N., Lal, K.K., Das, B.K., Das, P.C., Panigrahi, A.K., Shinoj, P., & Madhu, V.R. 2022. Achievements in fisheries and aquaculture in Independent India. In: *Indian Agriculture after Independence*. Indian Council of Agricultural Research, New Delhi, pp. 168–208. ISBN 978-81-7164-256-4. www.researchgate.net/publication/365449731_Achievements_in_Fisheries_and_Aquaculture_in_Independent_India#fullTextFileContent.

Lakra, W.S., Mohindra, V., & Lal, K.K. 2007. Fish genetics and conservation research in India: status and perspectives. *Fish Physiology Biochemistry*, 33: 475–487. doi: 10.1007/s10695-007-9168-z.

Murali, S.K., Basdeo, K., Akhilesh, K.M., Ravindra, K., & Kuldeep, K.L. 2020. National repository of fish cell lines: accelerating fish cell line research in fisheries and aquaculture. *Current Science*, 119(4): 589–590.

Silas, E.G. 2010. Phylogeny and evolutionary aspects of Indian fishes: Challenges for the future. *Indian Journal of Animal Sciences*, 80(4 Suppl.): 8–15.

Annex 12
Russian experience with cryotechnologies for preserving genetic resources of aquatic animals

Valentin Ananiev, Laboratory of Cryobiology, Filial VNIRO (VNIIPRH), Moscow region, Dmitrov area

Introduction

Over the past decades, the Russian Federation has developed cryopreservation protocols for numerous aquatic species and established several gene banks for ensuring the conservation of aquatic biodiversity, including species threatened with extinction and species used for aquaculture purposes.

Russian gene banks and their role in scientific research

The main, largest gene bank for *ex situ in vitro* conservation of finfish sperm is at the Federal Research Institute of Fisheries and Oceanography (VNIRO) Cryobank, at the Affiliate of Freshwater Fisheries in the Dmitrovsky district of the Moscow Region. Research on cryopreservation of fish sperm has been carried out in this institute since the late 1980s. Technologies for the cryopreservation of sperm of sturgeon, carp and salmon species were developed (Ananiev, 1996, 1998, 2002) and a collection of cryopreserved sperm was established. The VNIRO Cryobank collection includes approximately 2000 samples of sperm from more than 32 species (Table 10). Of particular value, are the sperm samples of rare or endangered species and populations listed in the Red Book of Russia (such as beluga, kaluga, Sakhalin sturgeon, Amur sturgeon, spike, white fish) (Anon, 2021;Vasilyeva *et al.*, 2019). Samples of these endangered species and populations were deposited about 25 years ago from males caught in natural reservoirs.

Table 10. Volume and origin of finfish milt held at the VNIRO Cryobank (Kovalev et al., 2020).

Finfish group	Volume of cryopreserved milt (ml)	Number of individuals
Sturgeons[a]	15751.65	697
Salmon[b]	2192.32	540
Cyprinids[c]	10459.99	1029
Whitefish[d]	401.97	322
Mullet [e]	43.25	26

Species conserved for each finfish group listed in the table include*:*

a. Acipenser baeri (2 populations), A. medirostris, A. ruthenus, A. steelatus, A. nudiventris, A. queldenstaedti, Husu huso, Poliodon spathula

b. Oncorynchus mykiss (7 cultured varieties, 1 wild), Samo salar (3 wild populations), O. keta, Salvelinus lepechini, Stenodus leucichthys (extinct in the wild).

c. Cyprinus carpio (16 varieties), Hypophthalmichthys moltrix, Arstichthys nobilis, Ctenopharyngodon Idella, Mytophartngodon piceus, Ictiobus cyprinellus

d. Coregonus albula, C. lavaretus, C. peled

e. Mugil soluy

The largest part of the collection consists of cryopreserved sperm of commercially important finfish species grown for aquaculture. These species are not endangered but the cryopreserved samples represent a source of genetic diversity of critical value to the aquaculture industry.

A second important cryobank is in Astrakhan, on the Volga River. It was created mainly for the cryoconservation of sturgeon and whitefish species by scientists of the Astrakhansky State University and the South Scientific Center of the Russian Academy of Sciences. Cryoconservation methods for these fish were developed here. At present, the cryobank contains over 12 liters of frozen milt, including sperm of Russian (*Acipenser gueldenstaedti/baeri*), beluga (*Huso huso*), stellate (*A stellatus*) , sterlet (*A. ruthenus*) and horn (*A. nudiventris*) sturgeons, paddlefish (*Polydon spathula*), and whitefish (*Coregonus*) species.

Finally, a third cryobank for genetic material of rare and endangered species of animals and plants including finfish and marine invertebrates (sea urchins, sea cucumbers, crabs), is at the Institute of Biophysics of the Russian Academy of Sciences, based in Krasnoyarsk, Siberia (Utechev and Gakhova, 2005; Uteshev *et. al*., 2019, Browne *et al.*, 2011). Its collection of cryopreserved finfish sperm partly duplicates that of the Affiliate of Freshwater Fisheries of the All-Russian Research Institute of Fisheries. Sperm collection and freezing were usually made jointly by employees of both institutes.

Between 1990 and 2000, an attempt was made to create more regional cryobanks in various regions of the Russian Federation (Ananiev, 1998). Cryobiological studies were carried out in these regions but were curtailed due to lack of funding, with the consequence that some collections of finfish sperm were lost (unpublished), compromising the capacity for restoration efforts if they are needed.

Many finfish species and populations are listed in the Red Books of Russia (Anon, 2021). Cryopreserved sperm of Russian sturgeons, listed in the Red Book and whose natural populations are threatened, are given special consideration in terms of *ex situ in vitro* conservation and related scientific research to address cryopreservation challenges (e.g., Krasilnikova and Tikhomirov, 2018).

One of the important areas of research is the cryopreservation of embryos and pre-larvae of finfish, to support alternative restoration strategies and aquaculture. Some progress was recently made in this field (Manokhina and Ananiev, 2019). Particularly, it was found that ultra-high-speed freezing technologies (vitrification) is needed for cryopreserving embryos and pre-larvae, and a unique cryofreezer (*КриоБласт*™ KryoBlast) was invented for this (Katkov *et al.*, 2017).

The larvae of sea urchins and crab germ cells are also preserved in the Siberian cryobank and methods for the cryopreservation of embryos of sea urchins (Gakhova *et al.*, 1988), larvae of oysters and nauplii larvae of barnacles (Gakhova *et al.*, 1990) have been developed.

Policy

The importance of gene banking and cryopreservation is recognized by the Russian federation by both legal and policy levels. The country developed the I Russian Federal Law №7-FZ of January 10th, 2002 which addresses the need for preserving rare and endangered species listed in the country's Red Books, including through cryoconservation. In response to this law, the Affiliate of Freshwater Fisheries of the All-Russian Research Institute of Fisheries developed a policy document which, amongst other things, regulates: the legal status of genetic collections and related insurance funds; the access and use of genetic resources; as well as their registration and deposition in the repositories.

The document has been approved by the Scientific council and management of VNIRO and, as of 2024, is waiting to receive final approval.

References

Ananiev, V.I. 1996. Problems of conservation and stable employment of biological diversity with the use of deep freezing methods of hydrobionts. *Froid et Aquaculture. Refrigeration and Aquaculture*, Bordeaux, France, 1996: 33-42.

Ananiev, V.I. 1998. Research Results and Perspectives of the Program "Low Temperature Gene Bank of Marketable, Rare and Endangered Fish and Aquatic Invertebrate Species". *Action Before Extinction. An International Conference on Conservation of Fish Genetic Diversity*, Vancouver, British Columbia, Canada, February 16-18, 1998, pp. 147-161.

Ananiev, V.I. 2002. The role of low-temperature gene banks in the system of measures of conservation of rare and endangered fish species and in aquaculture. *Conference "Cryopreservation and Safe Keeping of Cells and Tissues*, May 2002, Hradec Kralove (Czech Republic), p. 39.

Ananiev, V.I., & Manokhina, M.S. 2019. Cryolarvae of hydrobionts - the best way to low temperature genetic banks and reproductive material insurance funds. *56th Annual Meeting of the Society for Cryobiology. Abstracts*, July 22-25, 2019. Wyndham San Diego Bayside San Diego, CA, United States. P. 82.

Anon. 2021. Red Book of the Russian Federation, volume "Animals". 2nd edition. M.: FGBU "VNII Ecology", 2021. 1128 p. Available at: https://cicon.ru/

Browne, R.K., Li, H., Robertson, H., Uteshev, V.K., Shishova, N.V., McGinnity, D., Nofs, S., Figiel, C.R., Mansour, N., Lloyd, R.E., Agnew, D., Carleton, C.L., Wu, M., & Gakhova, E.N. 2011. Reptile and amphibian conservation through gene banking and other reproduction technologies. *Russ. J. Herpetol.*, 18(3), 165 – 174.

Dokina, O.B., Pronina, N.D., Kovalev, K.V., Milenko, V.A., & Tsvetkova, L.I. 2019. Improved technology of carp sperm cryopreservation in a large-scale cryobank. *Rybnoe khozyaystvo*, No. 5. P. 97–105.

Ernst, L.K., Vinogradov, M.E., Ananiev, V.I., Gakhova, E.N., Katasonov, V.Ya., Kopeyka, E.F., Manokhina, M.S., & Ananiev, V.I. 1996. Low-temperature cryopreservation of gametes and embryos of hydrobionts and aquaculture biotechnology. State and prospects of scientific and practical developments in the field of mariculture in Russia: Mat. adv–ce. M.: VNIRO, S. 196-198.

Gakhova, E.N., Krasts, I.V., Naidenko, T.Kh., Savelieva, N.A., Bessonov, B., Butsuk, S.V., & Veprintsev, B.N. 1988. The development of sea urchin embryos after cryopreservation. *Ontogenez*, 19(2), 175-180.

Gakhova, E.N., Korn, O.M., & Butsuk, S.V. 1990. Freezing of barnacle larvae *Balanus improvises* to -196ºC. *Biology of the Sea*, No. 4, 62-65.

Gakhova, E.N. 1998. Genetic cryobanks for conservation of biodiversity. The development and current status of this problem in Russia. *Cryo-letters*, suppl. 1, 1998.

Kopeika, E.F. 1998. Cryopreservation of Fish Gametes and Embryos in the Ukraine: Yesterday, Today, and Tomorrow. *Action Before Extinction. An International Conference of Conservation of Fish Genetic Diversity*, Vancouver, British Columbia, Canada (February 16-18, 1998), 1998: 143-145.

Krasilnikova, A.A., & Tikhomirov, A.M. 2018. Obtaining viable Russian sturgeon fry using cryopreserved sperm and evaluating the behavioral reactions of cryoprogeny. *Agricultural Biology*, 53(4): 762–768.

Katkov, I., Bolyukh, V.F., & Sukhikh, G.T. 2017. CryoBlast™ is a new technology for superfast cryopreservation of cells and tissues. 1. Thermodynamic aspects and prospects of applications in reproductive and regenerative medicine. *Cellular technologies in biology and medicine*, 2017, No 4. (in Russian). View at: https://core.ac.uk/reader/155234528

Manokhina, M.S. 1998a. Cryopreservation of the genetic material of aquatic invertebrates. Fisheries. Series: Aquaculture. Inform. plastic bag. M., VNIERH, 1998a, issue. 5:27-36.

Manokhina, M.S. 1998b. Cytoembryological aspects of cryoresistance of gametes and early embryos. Materials of the XV working meeting. (Pushchino, October 13-15, 1998), 1998b: 75-78.

Manokhina, M.S., & Ananiev, V.I. 1996. Low-temperature cryopreservation of gametes and embryos of hydrobionts and aquaculture biotechnology. The state and prospects of scientific and practical developments in the field of mariculture in Russia. Meeting materials. M., VNIRO, 1996: 196-198.

Manokhina, M.S., & Ananiev, V.I. 2003. Low-temperature conservation of embryos of fish and invertebrates with a lecitrophic type of early development (methodological difficulties and prospects for overcoming them). *International Symposium "Cold-water Aquaculture: Start in the XX-I Century"* (St. Petersburg, September 8-13, 2003), 2003: 218.

Manokhina, M.S., Rott, N., & Tsvetkova, L. 1998. Experience in creating and implementing a comprehensive program of B.N. Veprintsev "Cryobank of Fish" (1990-1996). Conservation of genetic resources. Pushchino. S.-14 – 23.

Manokhina, M.S., & Ananiev, V.I. 2018. Biological sciences. Preservation of *Danio rerio* and Carp, embryos and prolarvae in cryobanks, problems and perspectives. *Fundamentalis Scientiam*, 21. (Madrid, Spain) P. 14-19. & The 55th Annual Meeting of the Society for Cryobiology. CRYO2018. July 10-13, 2018 Madrid. Abstracts. P.121.

Uteshev, V.K., Gakhova, E.N., Kramarova, L.I., Shishova, N.V., Kaurova, S.A., & Browne, R.K. 2019. Cryobanking of amphibian genetic resources in Russia: past and future. *Russ. J. Herpetol.*, 26: 319–324.

Uteshev, V.K., & Gakhova, E.N. 2005. Gene cryobanks for conservation of endangered amphibian species, IN: N. Ananjeva and O. Tsinenko (eds.), *Herpetologia Petropolitana*, Proc. of the 12th Ord. Gen. Meeting of the Soc. Eur. Herpetol., 12 – 16 August 2003, St. Petersburg, Russia, pp. 233 – 234.

Vasilyeva, L.M., Elhetawy, A.I.G., Sudakova, N.V., & Astafyeva, S.S. 2019. History, current status and prospects of sturgeon aquaculture in Russia. *Aquac. Res.*, 50, 979–993.

Veprintsev, B. N., & Rott, N.N. 1979. Conserving genetic resources of animal species. *Nature*, 280: 633–634.

Veprintsev, B.N., & Piliev, S.A. 2002. Preserve the gene pool of fish and aquatic invertebrates. Selected works of VNIIPRKh (in 4 volumes), book. 1, vol. I-II. Dmitrov, pp: 366-369.

Annex 13
Norwegian salmonid gene banking spins off international service provider for AqGR

Maureen Ritter, Cryogenetics Services Inc., Black Creek BC, Canada
Joachim Carolsfeld, World Fisheries Trust, Victoria, Canada

Introduction

Norway established a governmental national gene bank in 1986 to assist in the preservation of the biodiversity of its salmon heritage, including restoration of threatened populations. The effort was stimulated, in part, by the widespread infection of native Atlantic salmon (*Salmo salar*) by an introduced parasite, *Gyrodactylus salaris* (World Fisheries Trust, 1998). The core collection consists of frozen milt of 174 Atlantic salmon populations, as well as representatives of brown trout, arctic char, and landlocked salmon. A minimum of 50 individuals of each population were targeted, collected over a period of at least three years, and tested for genetic diversity in comparison to the source populations. Complimentary living gene banks have also been established as a temporary measure to re-establish or supplement populations that are at critically low levels. This combination was used to successfully re-establish 32 of these populations after purposeful extirpated to control the spread of *Gyrodactylus* (Sandodden *et al.*, 2018).

Initially, cryopreservation was performed using 0.5 ml pellets of milt/diluent frozen on dry ice, with subsequent storage in liquid nitrogen (Stoss and Refstie, 1983). Refinement of the process included a joint project between governmental research companies, AquaGen and Geno. Key innovations included streamlined optimizing and standardization of the transport of fresh sperm, the freezing process (especially freezing rates and solutions) thawing rates, and fertilization protocols. An 11 ml "Squarepack" of frozen milt, developed to better suit the hatchery process of salmonids, is considered a key factor in making cryopreservation a practical component of the conservation work and aquaculture applications (Boe *et al.*, 2021). Quality control of the milt before freezing (motility and sperm density) are also key factors in ensuring reproducible results. Whereas the original frozen gene bank was primarily a repository of genetic material adequate for emergency broodstock development, the modernized version with greater through-put capacity is more integrated with the live gene banks and maintenance of recuperated stocks. Expressed advantages of the cryobanks include reducing the cost of maintaining a male broodstock adequate to provide adequate genetic diversity and avoiding culture-based selection of live gene banks (Boe *et al.*, 2021).

A spin-off of the research collaboration was the formation, in 2002, of Cryogenetics,[34] a company that now provides technical support to the global aquatic gene banking market, with offices in Canada, Chile, England, Norway, and the United States of America.

Clients of Cryogenetics include aquaculture companies, government, indigenous groups, and conservation groups. The cryopreservation services include standardized proprietary methods optimized for individual species (see manual – (Cryogenetics, sd)), mobile programmable freezing, central storage facilities, and data management. Their experience has demonstrated that shipping sperm in short term storage conditions to a facility or truck with well-controlled freezing conditions is better than relying upon variable field freezing, and that in-house small repositories are risky due to human error and/or the cost of maintaining

[34] www.cryogenetics.com

adequate levels of liquid nitrogen (see also Chapter 3). The company thus provides storage and maintenance services, though the samples generally remain the property of the clients. Consulting services on conservation and breeding protocols and strategies are also offered, generally in collaboration with governmental and non-governmental stakeholders.

Cryogenetics was formed in response to a need identified first in the Norwegian environment but has developed a global market. Similarly, the Akvaforsk Genetics Centre was developed in the Norway but now serves a global market through Benchmark Genetics with consulting services on genetic improvement and sales of selectively bred Atlantic salmon ova (produced with the help of in-house milt cryopreservation). Other larger aquaculture companies also have in-house selective breeding programs with a cryopreservation element, assisted by Cryogenetics, Benchmark, other service providers, and/or their own staff. The role of private enterprise in *ex situ in vitro* gene banking and use of cryopreserved finfish AqGR, now appears to be support for "in-house" programs for aquaculture production, sale of selectively bred offspring, and conservation programs, rather than the sale of cryopreserved products typical of the terrestrial livestock industry.

Lessons learned:

- Well standardized, tested protocols are key elements to consistent success.
- Centralized storage facilities are far better than relying on more risky and smaller in-house storage.
- Production applications of cryopreservation in aquaculture are showing considerable and growing use in breeding programs, especially with complementary support for genetic program development.
- Conservation applications require long term visions and funding that are challenging to achieve.
- Collaboration and networking are key elements to future success for everyone.
- In contrast to the terrestrial livestock gene banking industry, the current finfish private gene banking market tends to be more focused on provision of integrated technical support and in the case of aquaculture, the sale of resulting selectively bred offspring rather than on the sale of cryopreserved products.

Reference

Bøe, K., Bjøru, B., Bårdsen, B.T., Wist, A.N., Wolla, S., & Sivertsen, A. 2021. Opportunities and challenges related to sperm cryopreservation in Atlantic salmon genebanks. *Conservation Science and Practice*, 3:e552.

Cryogenetics. Nd. Reproduction handbook for use at hatcheries. Salmonids. 33pp. Available at: https://s33002.pcdn.co/wp-content/uploads/Reproduction-Handbook-Samonids.pdf

Sandodden, R., Brazier, M., Sandvik, M., Moen, A., Wist, A.N., & Adolfsen, P. 2018. Eradication of *Gyrodactylus salaris* infested Atlantic salmon (*Salmo salar*) in the Rauma River, Norway, using rotenone. *Management of Biological Invasions*, 9(1).

Stoss, J., & Riefste, T. 1983. Short-term storage and cryopreservation of milt from Atlantic salmon and sea trout. *Aquaculture*, 30(1–4): 229-236.

Walso, O. 1998. The Norwegian gene bank program for Atlantic salmon (*Salmo salar*). IN: Harvey, B. et al. (eds). *Action Before Extinction: An International Conference on Conservation of Fish Genetic Diversity*. World Fisheries Trust.

Annex 14
The dream and the reality: two decades of running an independent fish gene bank

Brian Harvey, Nanaimo, Victoria, Canada

Introduction

The first attempt to operate a finfish gene bank in North America was made by the International Fisheries Genebank (IFGB), a not-for-profit organization founded by Brian Harvey in British Columbia in 1992 (Harvey 1993b), based on prior research work on freezing of fish gametes (Harvey 1982, 1983a and b, 1993a; Harvey and Kelly 1984 a and b, 1988; Harvey *et al.*, 1982, 1983; Harvey and Chamberlain, 1982; Harvey and Ashwood-Smith, 1983). The IFGB had support from a number of institutional donors and based its gene banking on an innovative use of "dry shipper" containers (liquid nitrogen vapour containers) for simple and rapid cryopreservation of fresh semen in the field, eliminating the need to ship semen to a distant facility for freezing. The IFGB provided to its clients numbered, high-volume straws, cryoprotectant and a thawing solution. The version tailored to salmonids was enthusiastically received by the First Nations fisheries programs and a number of salmon farms but at that time it was not attractive to government management agencies which often see gene banking as a failure in genetic resource management. Environmental organizations tended to view gene banking only as "technical fix", and were also not supportive. To build upon initial successes and broaden the range of activities, in 1994 the gene bank was integrated in the programme of work of the World Fisheries Trust (WFT), a Canadian non-profit organization with the broader mandate to work with countries and local communities to protect aquatic biodiversity and sustainably manage aquatic genetic resources for food and agriculture (AqGR).

The actual storage of the growing collection remained where it had been from the beginning, in secure nitrogen tanks equipped with alarms in a large commercial facility, in British Columbia, for the collection and storage of farmed animal semen. The WFT went on to develop its own database program (Spermsaver) as well as training courses that were provided to the First Nations, salmon farms and government hatcheries.

Although the WFT's gene banking package was used in British Columbia by a number of salmon farms for routine broodstock management and for the collection of wild genetic material where permitting agreements existed, the primary aim of the founders of the gene bank was the conservation of wild germplasm as a tool to prevent the loss of genetic diversity in wild stocks. Carrier-Sekani, a northern BC First Nation, was the first to successfully incorporate thawed semen into its population rebuilding program in 2019 (Lirette, 2019). This successfully deployed gene-banked semen was obtained 20 years previously and cryopreserved by the WFT-trained Indigenous fisheries technicians. The use of the cryopreserved sperm was needed as a major landslide blocked the salmon's migratory route in the mainstream Fraser River, and the wild stock at the spawning grounds was critically low. A hatchery using this sperm was able to supplement the low stock.

Since gene banking did not initially receive significant attention and support by government agencies, securing funds for gene banking initiatives was challenging and most of the collections managed by WFT dwindled to the point that about 80 percent of the material has now been discarded. For a while, the national Canadian fisheries agency used its own gene banking method, based on WFT's, but resurging interest is now mostly carried out in collaboration with Cryogenetics, a private firm (see Appendix 14).

The WFT methodology was transferred to Brazil, Colombia, and Venezuela in the 1990s, with support from the Canadian International Development Agency (CIDA), the International Development Research Centre (IDRC), and local partners (Harvey, 1996, 1999; Harvey and Mace, 1994). It was easily applicable to warmwater species as demonstrated by the work with zebrafish (Harvey *et al.*, 1982) and tilapia (Harvey and Kelley, 1984a, 1988). In Brazil, it was empirically adapted with little change, to commercially important characid fish (Carolsfeld *et al.*, 2003) and used in research and in hatcheries of electrical power companies to populate reservoirs. The protocols have subsequently been adapted and used for many South American species (Appendix 10 and 11, and Chapter 5).

Lessons learned

- The cryopreservation of finfish sperm, whether at the field collection site or in a centralized freezing facility, is not technically difficult. Cryoprotectant formulas are easily adjustable and uncomplicated. Putting the entire procedure in the hands of trained field technicians can work very well, and in fact the main challenge is making sure that thawing and fertilization are done properly (freezing and thawing damages some of the sperm cells, so their fertilization window closes earlier than in the wild). The better the training, the less chance of a problem.
- Samples storage and management, including shipment to and from repository, are straightforward and best done securely with a large facility, which however requires regular on-going funding.
- Raising general public's awareness on the importance of gene banking for conservation is fundamental in keeping it funded. There is a need for a multi-year public awareness campaign to make the general public aware of why it should care about the risks and the benefits of AqGR gene banking. Proponents of gene banks will still need to look long and hard at their own motives because the arguments against gene banking are strong.

References

Carolsfeld, J., Godinho, H.P., Zaniboni Filho, E., & Harvey, B.J. 2003. Cryopreservation of sperm in Brazilian migratory fish conservation. J. Fish Biology. 63(2): 472-489.

Harvey, B. 1999. Fish Genetic Resource Conservation in Canada and Brazil: Action and Reaction**.** In: "Toward Policies for Conservation and Sustainable Use of Aquatic Genetic Resources, "R.Pullin, D. Bartley and J. Kooiman, Eds. ICLARM Conference Proceedings 59. 277 pp.

Harvey, B. 1996. Banking Fish Genetic Resources: The Art of the Possible. In: Biodiversity: Science and Development, F. Di Castri and T. Younes, Eds. CAB International, Wallingford, UK.

Harvey, B. and T. Mace. 1994. Field experiences with cryopreservation in the preservation of fish genetic resources. FAO Expert Consultation on the Utilization and Conservation of Aquatic Genetic Resources, Rome, November 1992.

Harvey, B. 1993a. Cryopreservation of fish spermatozoa. In: Genetic conservation of salmonid fishes. Ed: J. Cloud and G. Thorgaard. Plenum Press, New York. pp 175-181.

Harvey, B. 1993b. The International Fisheries Genebank: A new mechanism for conserving fish genetic resources. Presented at the 15th Annual General Meeting of the American Fisheries Society, North Pacific International Chapter, Richmond, B.C., March 1993 (in press).

Harvey, B. 1982. Cryobiology and the storage of teleost gametes. Int. Symp. on Reproductive Physiology of Fishes. Wageningen. pp 123–127. Pudoc, Wageningen.

Harvey, B., and R.N. Kelley. 1988. Practical methods for chilled and frozen storage of tilapia spermatozoa. In: R.S.V. Pullin, T. Bhukaswan, K. Tonguthai and J.L. Maclean (eds.). The Second International Symposium on Tilapia in Aquaculture. ICLARM Conference Proceedings 15: 187-189.

Harvey, B., and R.N. Kelley. 1984. Chilled storage of *Sarotherodon mossambicus* spermatozoa. Aquaculture 36: 85-95.

Harvey, B., and R.N. Kelley. 1984. Short-term storage of *Sarotherodon mossambicus* ova. Aquaculture 37: 391-395.

Harvey, B. 1983. Cryopreservation of *Sarotherodon mossambicus* spermatozoa. Aquaculture 32: 313-320.

Harvey, B. 1983. Cooling of embryonic cells, isolated blastoderms and intact embryos of the zebrafish to -196 C. Cryobiology 20: 440-447.

Harvey, B., R.N. Kelley and M.J. Ashwood-Smith. 1983. Permeability of intact and dechorionated zebrafish embryos to glycerol and dimethylsulfoxide. Cryobiology 20: 432-439.

Harvey, B., and M.J.Ashwood-Smith. 1982. Cryoprotectant penetration and supercooling in the eggs of salmonid fishes. Cryobiology 19: 29-40.

Harvey, B., and J.B. Chamberlain. 1982. Water permeability in the developing embryo of the zebrafish *Brachydanio rerio.* Can. J. Zool. 60: 268-270.

Harvey, B., R.N. Kelley and M.J. Ashwood-Smith. 1982. Cryopreservation of zebrafish spermatozoa using methanol. Can. J. Zool. 60: 1867-1870.

Lirette, D. 2019. To save endangered salmon, scientists use 20-year-old frozen sperm. CBC News · Posted: Sep 13, 2019.

Marliave, J., Harvey, B., and C. Sanford. 1994. Storage and use of cryopreserved oyster trochophores as food for larvae. Presented at the 1994 Annual Meeting, American Association of Zoological Parks and Aquaria, Atlanta, September 1994.

Glossary

Accession	A distinct, uniquely identifiable sample of a genetic resource, which is maintained in storage for conservation and use
Activator	A solution that enhances and/or prolongs the motility of thawed sperm. Generally based on ionic mechanisms, enhanced energy (ATP activity) and/or enhanced flagella activity. Used only in some protocols and the functioning is not yet well understood
Aquatic genetic resources for food and agriculture	Genetic material of aquatic organisms, including molecular sequences, genes, chromosomes, tissues, gametes, embryos and other early life history stages, individuals, farmed types, wild stocks, and communities of aquatic organisms of current actual or future potential value for food and agriculture
Broodstock	A group of sexually mature individuals used in aquaculture for breeding purposes
Cell culture	Culturing of living cells in an artificial culture medium
Cell line	The culture of cells that originated from a primary cell culture (see def.), generally used to expand cell populations and prolong life span through a permanently established cell culture
Chilled storage	Process whereby biological material is maintained viable by holding at a temperature between ambient and freezing, typically on ice in a cooler for short-term storage or transport
Cooling	In cryopreservation literature refers to the freezing process
Cryopreservation	Process whereby organelles, cells, tissues, extracellular matrix, organs, or any other biological constructs are preserved by cooling to cryogenic temperatures.
Cryoprotectant	Chemicals used to protect cells from damage during freezing and thawing, they are often classified by whether they penetrate the cell (referred to as “permeating”) or remain outside the cell (“non-permeating”)
Cryoprotectant agent	Chemicals that reduce freezing damage. Penetrating CPAs, like DMSO, methanol or glycerol, facilitate the dehydration of cells to reduce formation of ice crystals while also stabilizing internal solutes; non-penetrating CPAs either create an external osmotic environment that also facilitates dehydration (like sugars) or stabilize membranes (like egg yolk or milk powder)
Dewar (for cryogenic storage)	A specialized type of vacuum flask used for storing liquid nitrogen or nitrogen vapour at cold temperatures (down to -196ºC)
Diluent	A more general term for solutions such as an extender, that increase the volume of a suspension such as semen. At times used interchangeably with “extender” but also differentiated by some authors as an extender prior to the addition of a CPA
Ex situ conservation	Conservation of genetic resources outside of their natural habitats
Ex situ in vivo conservation	“Live gene bank,” maintaining whole live organisms outside of their natural habitat
Ex situ in vitro conservation	Cells or tissues maintained, alive or in suspended animation, in laboratory conditions and outside the original intact organism. Includes cryopreservation. Contrasted with *in vivo*

Extender	A solution of salts, sometimes including organic compounds such as sugars (external CPAs), that inhibits the triggering of motility, maintaining sperm viability prior to and during the freezing process, and increasing the volume of the semen to make it easier to handle. Depending on their properties, extenders can extend the functional life and fertilizing capability of the sperm. Penetrating cryoprotectants are added to the extender either initially or just prior to freezing
Farmed type	A descriptor applied to farmed aquatic organisms at a level below species, including strain, variety, hybrid, triploid, monosex group, other genetically altered forms or wild-sourced
Framework	Used in this document to describe the organization of networks and protocols
Gamete	A mature haploid (containing only one set of chromosomes) male or female germ cell (see def.) which can unite with another of the opposite sex in sexual reproduction to form a zygote (a diploid cell resulting from the fusion of two haploid gametes)
Gene bank	A repository for the collection, preservation, and management of genetic resources
Germplasm	Living genetic resources used for breeding, conservation, or research
In vitro fertilization	Artificial fertilization of an ovum or ova outside the body of a female, including in standard hatchery practices, but also in tissue culture
Oocyte	An egg cell, which gives rise to a mature ovum by meiosis
Pathway	Used in this document to indicate the describe the sequence of steps of a protocol of manipulation
Primary cell culture	The *in vitro* culture of cells directly isolated from original tissue of interest.
Primordial germ cells	Germ cells that have not yet differentiated into their eventual tissues
Protocol	Series of actions established for achieving a purpose, as in a protocol to freeze a cell type
Germ cell	Any biological cell that gives rise to the gametes (see def.) of an organism that reproduces sexually
Repository	A fully integrated gene bank that has good stability and reliable results (e.g. most of governmental gene banks)
Repository network	Ensemble of interactions between biobanks and community members, which can include universities, hatcheries, government agencies, international agencies, farms, etc.
Somatic cell	Non-reproductive cell
Sperm	In animals, the male gamete. Also called a spermatozoon (plural: spermatozoa)
Thermocouple	A thermoelectric device for measuring temperature
Tissue culture	*In vitro* culture of cells, tissues, organs, and their components under defined physical and chemical conditions
Voucher	A permanently preserved specimen that is labelled and catalogued in a collection that is accessible to other researchers