Progress, challenges and perspectives on fish gamete cryopreservation

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AQUAGAMETE

IMPRESS
Improved production strategies for endangered freshwater species

cost
EUROPEAN COOPERATION IN SCIENCE AND TECHNOLOGY

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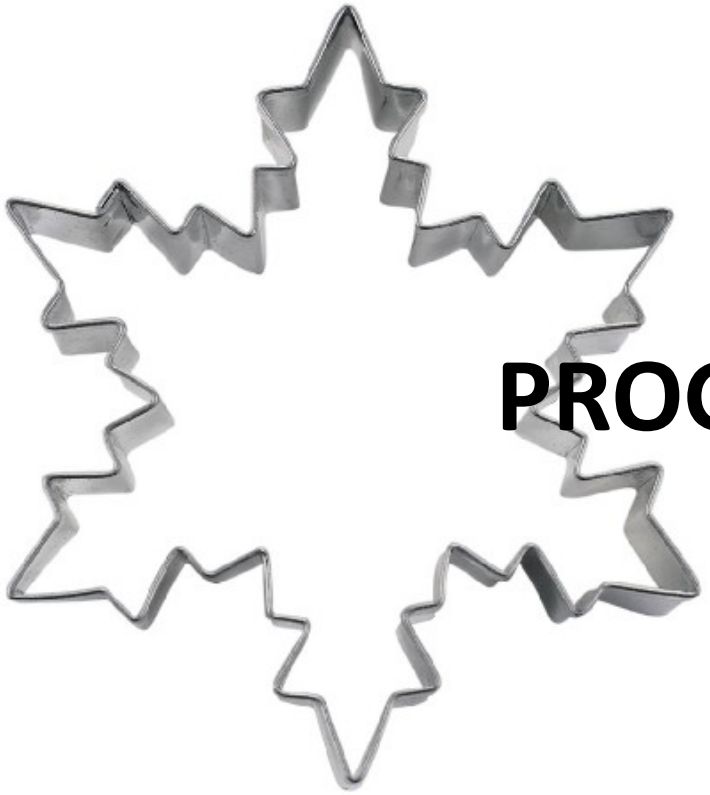
GAB

CCMAR



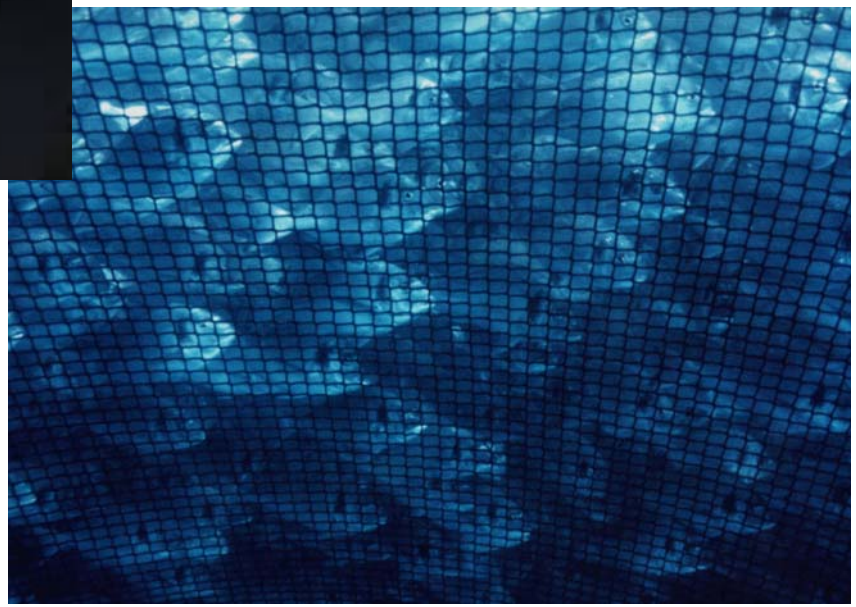
PROGRESS

Freezing protocols have been developed for many different fish species, in special, freshwater ones.

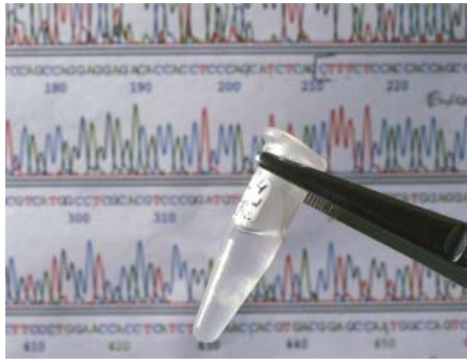
Salmonids and cyprinids have centred a good part of the attention.

Cryopreservation of fish gametes has evolved during the last decades due to the increasing number of potential applications.

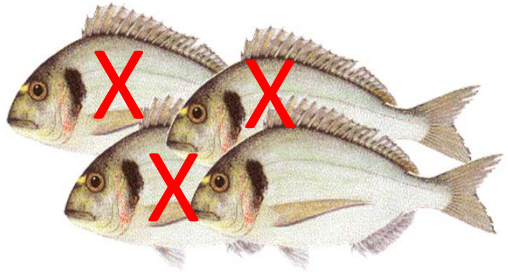
Aquaculture purposes, improvement of broodstock management at hatcheries (modifying the offspring production season),

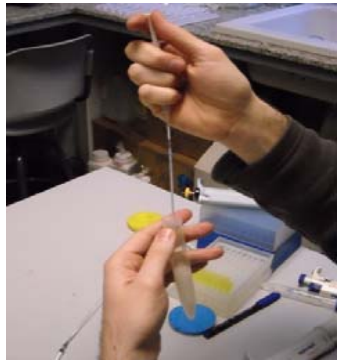


…preserving the genetically selected strains…

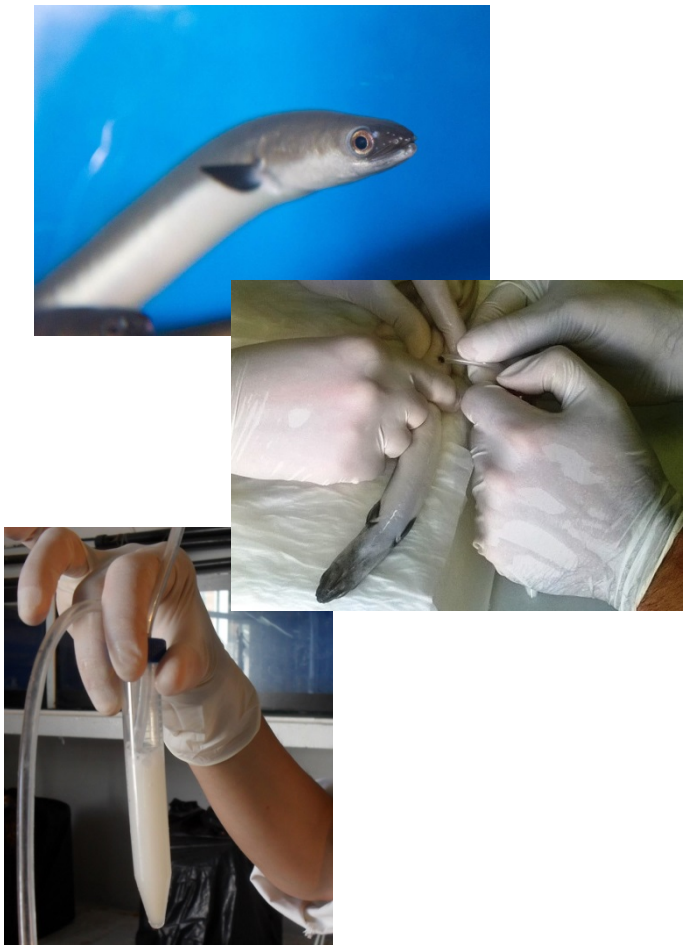


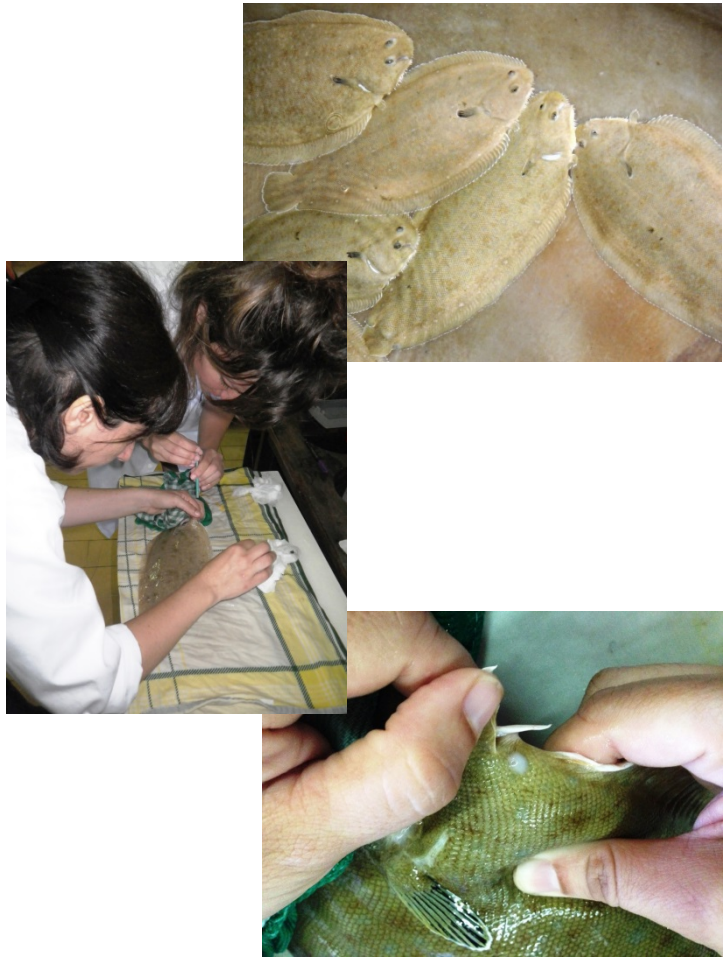
CRIOGEN,
SELECTSPARUS &
SELECTBREAM Projects
(2003-2010)





…helping with species having reproductive problems…





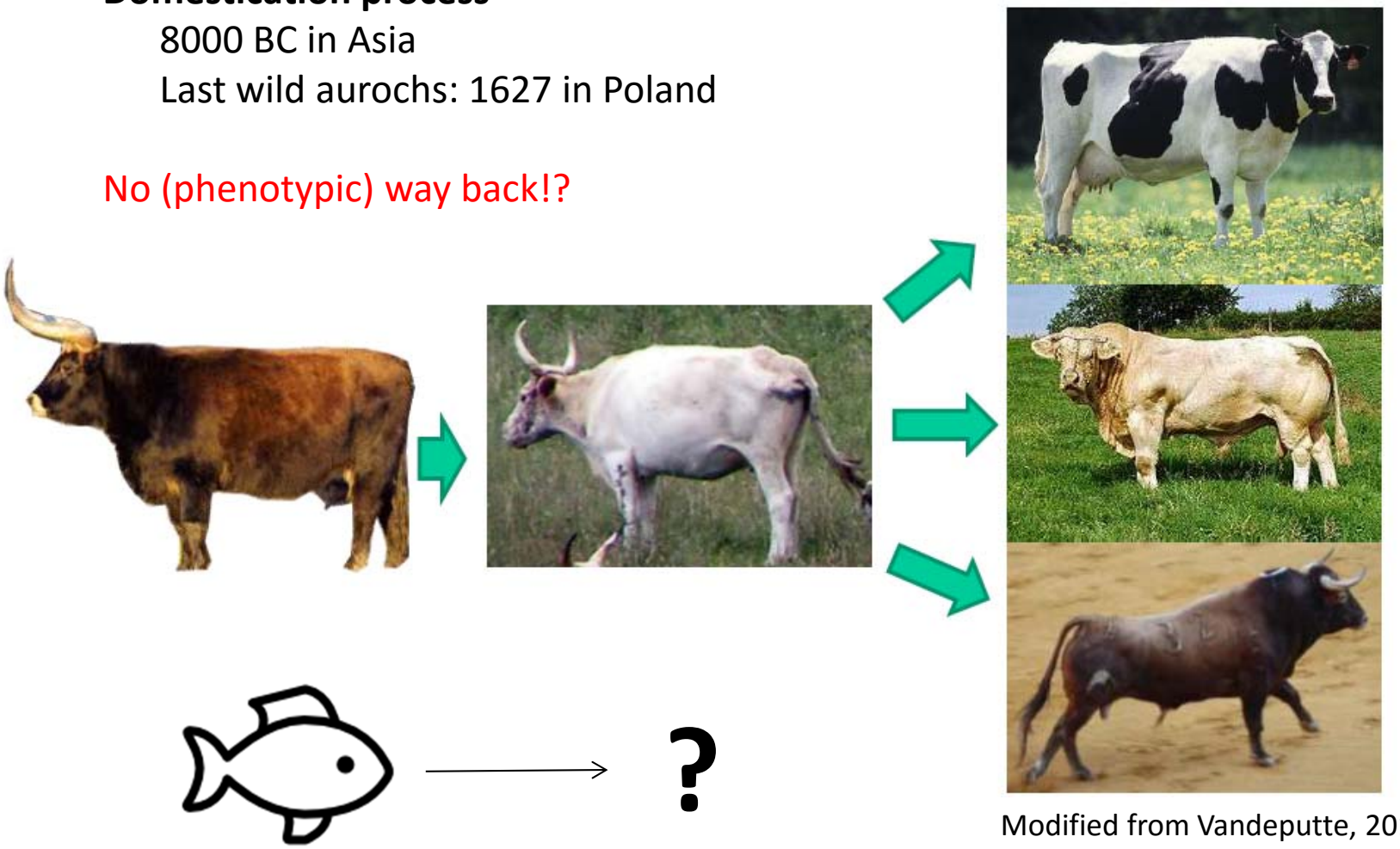
…or even preserving the original wild genotypes for the recovery of genes in the future.

Domestication process

8000 BC in Asia

Last wild aurochs: 1627 in Poland

No (phenotypic) way back!?



Modified from Vandeputte, 2011

In fish, natural populations still exist

Modified from Vandeputte, 2011

Storage of genetic resources

-Increasing number of fish in the lists of endangered species. Thousands of them in the *International Union for Conservation of Nature and Natural Resources* (IUCN) Red List.

-Applications in restocking and conservation programs (biodiversity cryobanking).

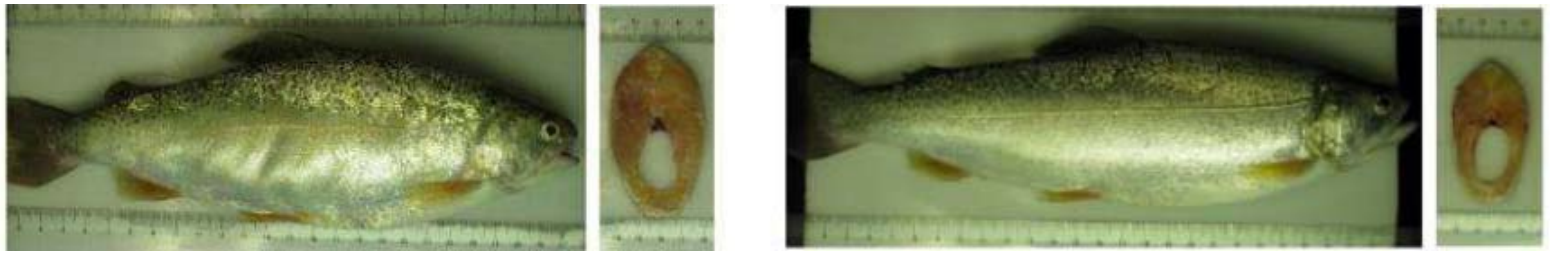
-New species attracts the interest of cryobiologists and aquaculturists (mainly in South America and Asia).



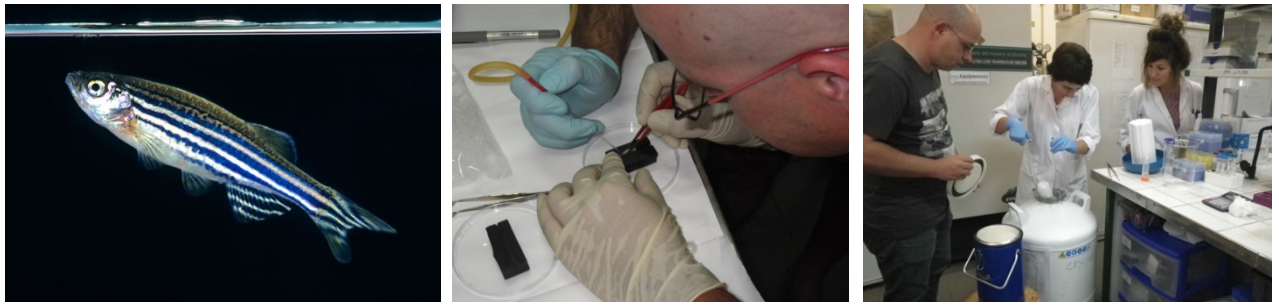



Transgenics and other special cases

-First trials in fish in the early 90s
-Main target trait: growth rate (extra copies of the GH gene)
"Six-pack" trout (Medeiros et al., 2009)



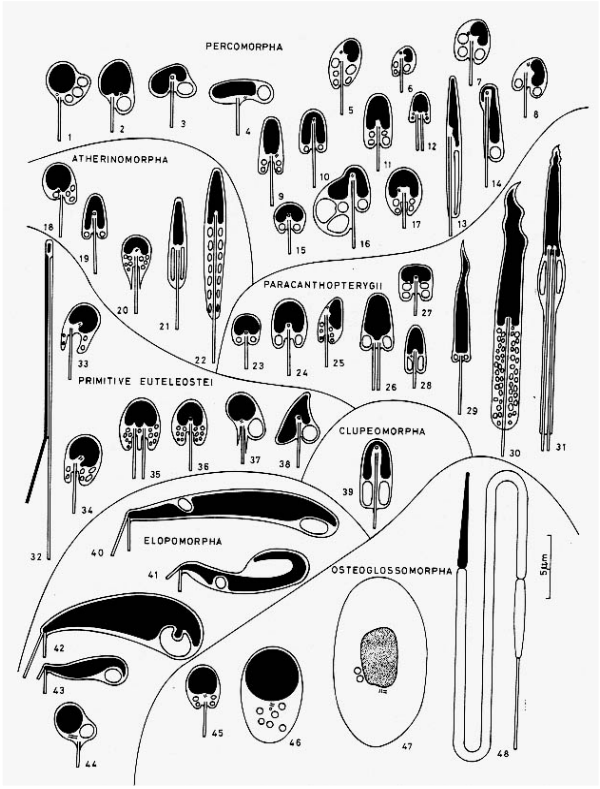
-Increasing use of aquatic biotechnology models such as zebrafish requires the use of thousands of transgenic lines, knockout and mutant strains that need adequate storage and management.
-Many strains have reproductive limitations.



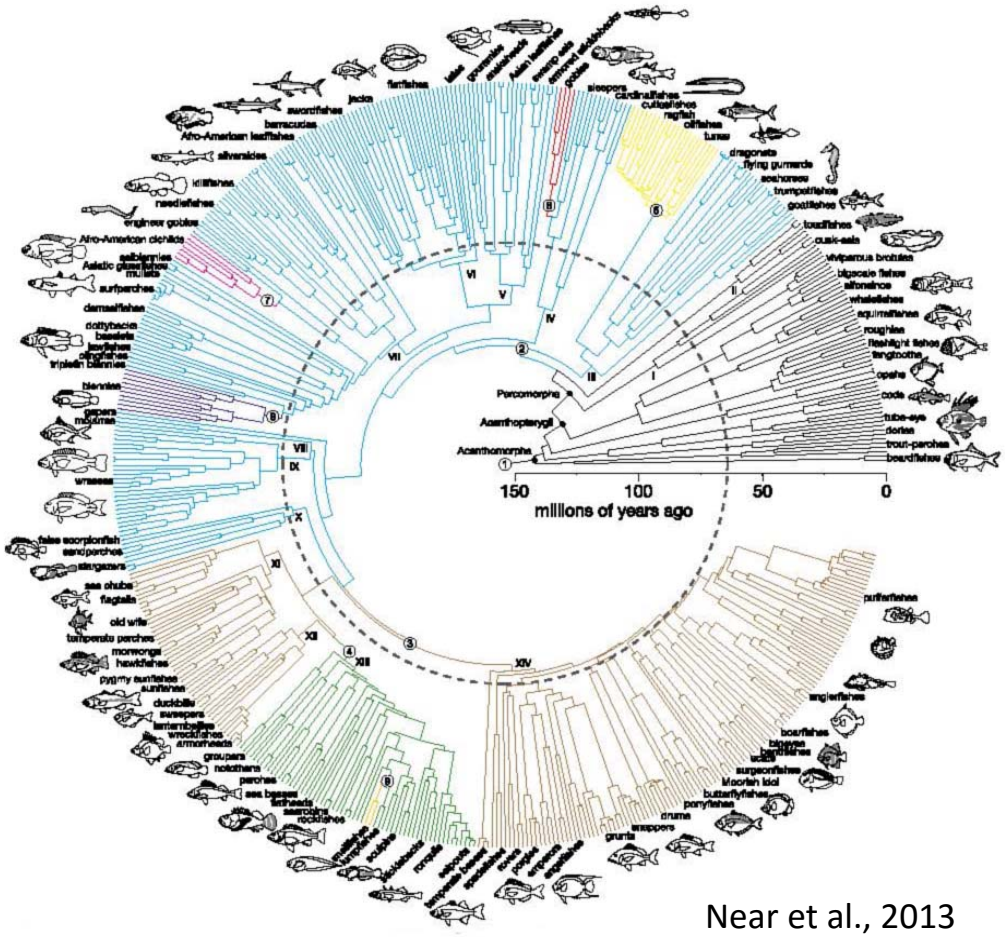
CHALLENGES

Diversity

The increasing numbers of studies describing methods to cryopreserve sperm in many species, evidences the extreme diversity of fish.



Mattei, 1991



Near et al., 2013

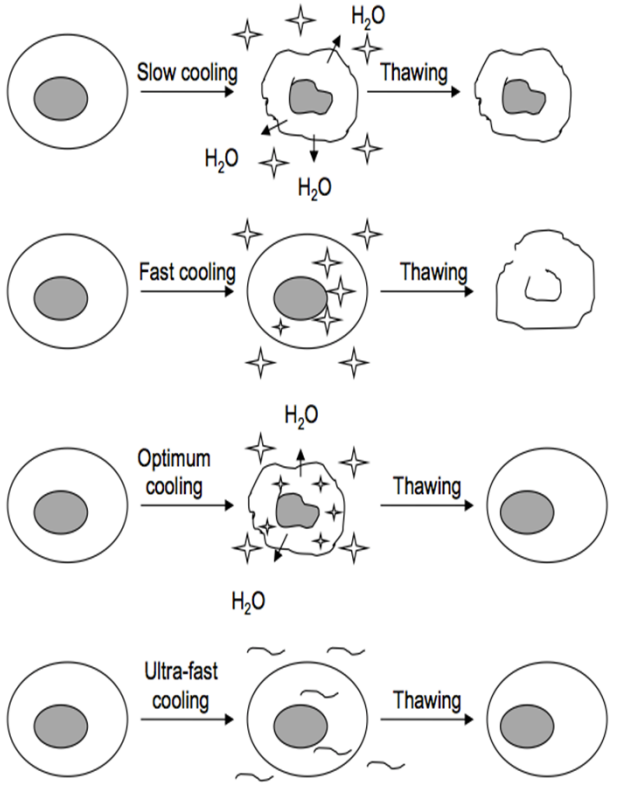
Gamete morphology and biology

Biophysical and chemical processes

During cooling, freezing, and thawing processes happens osmotic changes, dehydration and rehydration, cell volume variations, ice crystals formation, cryoprotectants toxicity, etc.

Cells (types, species) are more or less sensitive to these changes.

Cryopreservation protocols must be adapted to find specific compromises.



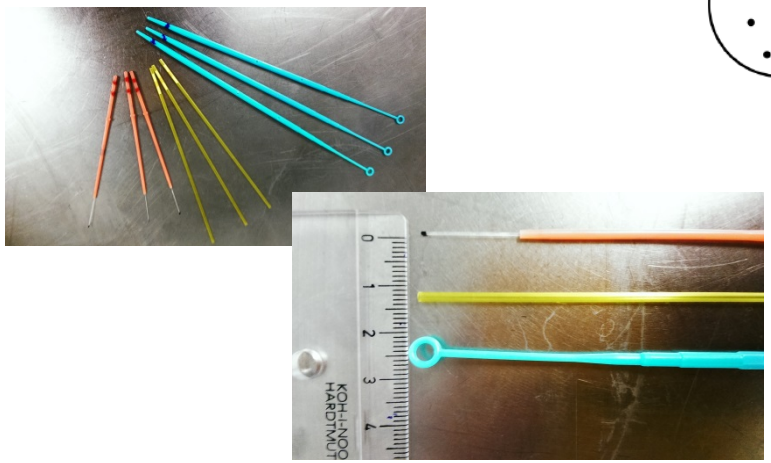
Horváth, pers. com.

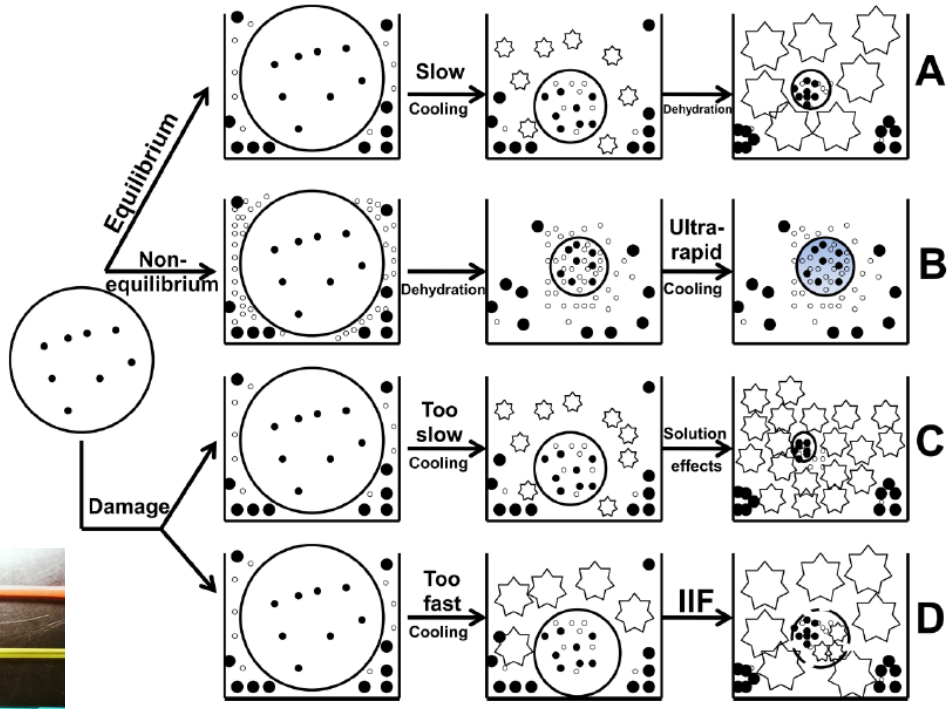
Vitrification

Trying to prevent the negative effects of crystallization, mixed cryoprotectants at very high concentration (30-50%) and using very high freezing rates (10^6-10^{10} °C/s), getting the solidification of external and internal media into an amorphous/glassy state without formation of harmful ice crystals (Fahy *et al.*, 1984).

Concentrated cryoprotective solution are toxic for cells.

Very high freezing rates can be difficult to achieve with large samples. Limited to low volumes (2-4 µl; cryoloops or cryotops).





Cuevas-Uribe, 2011

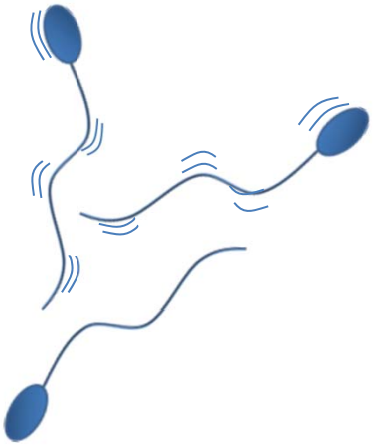
Published results in fish sperm vitrification

- Green swordtail (*Xiphophorus hellerii*) – 12% membrane integrity, **7% motility** (Cuevas-Uribe et al., 2011)
- Channel catfish (*Ictalurus punctatus*) – 50% membrane integrity, **25% fertilization** (Cuevas-Uribe et al., 2011)
- Rainbow trout (*Onchorynchus mykiss*) – 11,1% DNA fragmentation, 98,4% plasma membrane integrity, 36,2% mitochondrial membrane integrity, **31,0% fertilization** (Figueroa et al., 2013)
- Atlantic salmon (*Salmo salar*) – 9,2% DNA fragmentation, 98,6% plasma membrane integrity, 47,2% mitochondrial membrane integrity, **44,1% motility, 46,2% fertilization** (Figueroa et al., 2015)
- Tambaqui (*Colossoma macropomum*) – 9,9% plasma membrane integrity, 40,4% mitochondrial functionality, 96,6% DNA integrity, **49,9% viability**, **0% motility** (Varela et al., 2015)

It does not seem to be able to replace conventional freezing used in most fish species.

A real practical application is feasible in small model fishes as zebrafish due to the low volumes of sperm that they produces.

Not only sperm



Fish genome cryobanking has been faced using different cells types: spermatozoa, oocytes, spermatogonia and primordial germ cells, as well as somatic cells, blastomeres and embryos.

Spermatozoa have been the objective of most of the studies, making of sperm cryopreservation the technique better established and more commercialized.

Easy to collect in most of the fish species

Small cell size and high chilling resistance, making them easy to preserve in many fish species

Reconstruction of individuals can be done by normal fertilization (or androgenesis)

BUT

Allows the preservation of only male germplasm

Oocyte cryopreservation

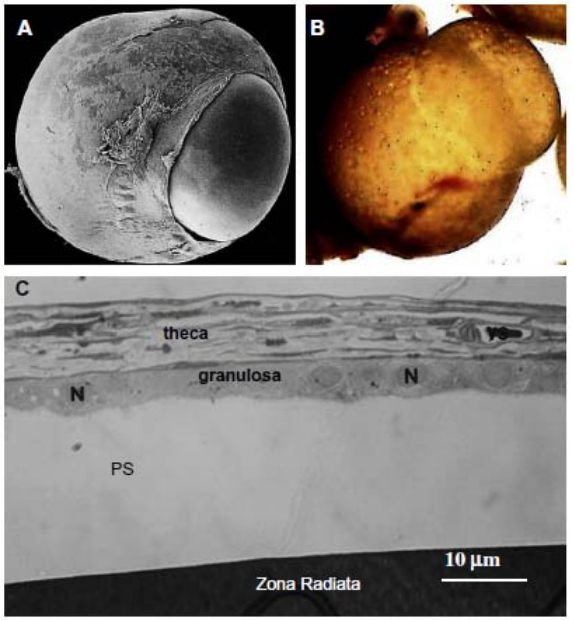
Big cell volume, presence of chorion, low permeability to cryoprotectants, chilling sensitivity

Studies has been carried out in zebrafish, as well as other marine and freshwater species.

Cryoprotectant toxicity, chilling sensitivity, membrane permeability and cryopreservation (cooling rates, vitrification) of oocytes at different stages of development or ovarian fragments (Zhang et al., 2007; Godoy et al., 2013; Streit Jr. et al., 2014).

Moreover, using cryopreserved oocytes needs the development of protocols for *in vitro* maturation of ovarian follicles after cryopreservation (Seki et al., 2008, 2011; Tsai et al., 2010).

Still far of aquaculture applications.



Lubzens et al., 2010

Spermatogonia and primordial germ cell cryopreservation

The preservation of these cells guarantee the full individual genome.

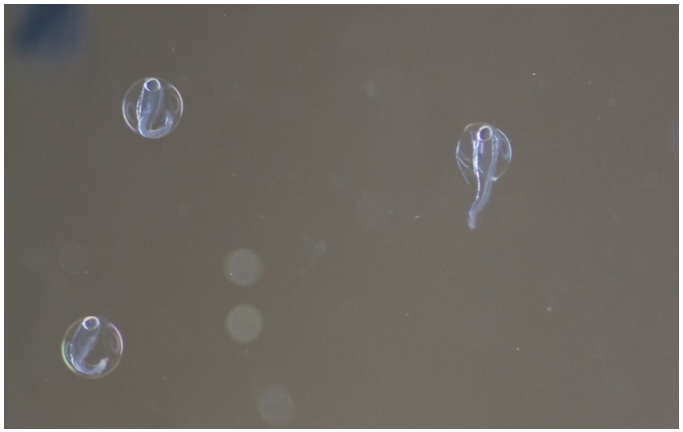
They have been cryopreserved successfully in several fish species (Yoshizaki et al., 2011; Robles et al., *in press*).

They require the development of specific biotechnological tools, such as transplantation.

Fish embryo cryopreservation

It could be perfect for the establishment and management of genetic selection programs in fish farms.
However, they have:

- low membrane permeability
- low surface-to-volume ratio
- large size of yolk
- high chilling sensitivity



Very limited preliminary positive results (Chen and Tian, 2005; Robles et al., 2005).

Somatic cells cryopreservation

They are diploid cells, so full individual genome could be preserved.

They can be easily collected . Fins are well regenerated.

They require cell culture and nuclear transfer for individual restoration (Siripattarapravat et al., 2011; Chenais et al., 2014).

Still far of aquaculture applications.

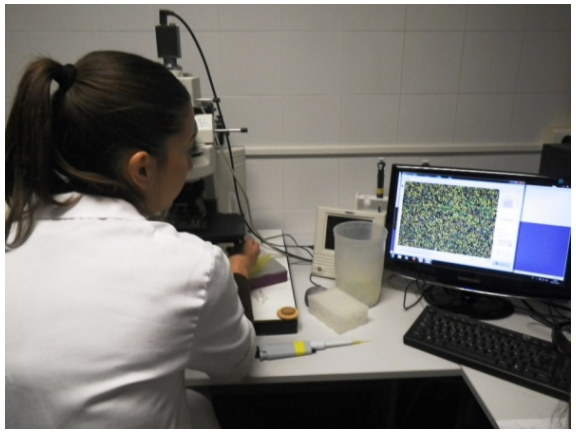
Evaluation of gametes quality

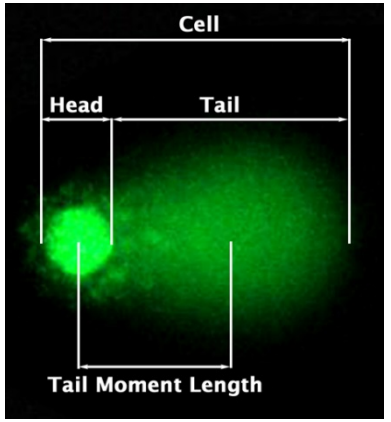
Needed both for selection of sperm samples and for the establishment of sperm cryopreservation programs and companies.

The techniques for sperm quality evaluation has been improved, giving a deeper information about the effects of the processes happening during the freezing-thawing that reduces the sperm quality.

- Cell motility (CASA) and morphometry (ASMA)
- Antioxidant status
- Chromatin integrity
- Proteomic information about cryodamages
- Fertilization rate
- Development of the progeny.

The improvement of these techniques allows the development of better cryopreservation methods.





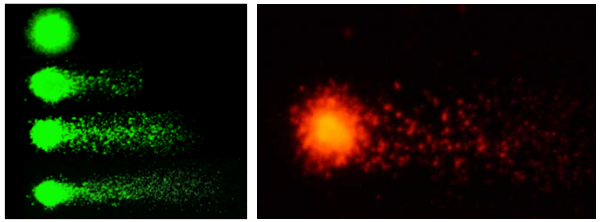
Genetic damages

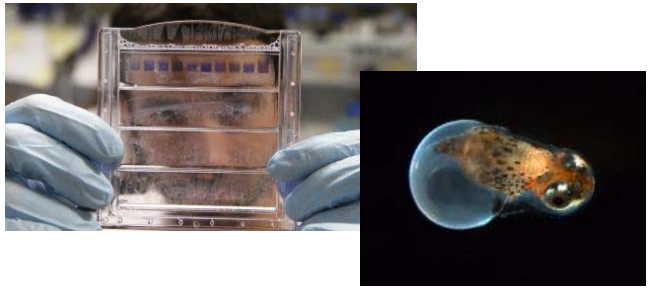
-The genetic information can be damaged during the freezing and thawing processes.
-Damages can be due to:

Mechanical events (ice formation, nuclear membrane disruption)
Oxidative events (free radicals formation)
Bases oxidization
DNA strain breaks (single and double)

-Specially weak regions have been identified.
-Specific mRNAs associated with gamete quality and fertilization events (i.e.: gilthead seabream sperm; Cartón-García et al., 2013; Guerra et al., 2013).
-The damages can mean losses of genetic information and further abnormalities in the development of embryos and juveniles.
-Different techniques have been used to assess them.

SCGE (Single cell gel electrophoresis or Comet assay)
Neutral SCGE.
Alkaline SCGE.
SCGE combined with specific endonucleases for the detection of oxidized DNA bases.
TUNEL
SCSA (Sperm Chromatin Structure Assay)
Alkaline gel electrophoresis
Q-PCR
RT-PCR (Centromeres and Telomeres length)





(Reviewed by Cabrita et al., pers. com.)

Lack of standardization

- Definitions
  - Extenders
  - Cryoprotectant concentrations
  - Dilution ratios
- Methods
  - Sperm concentration determination
  - Sperm cryopreservation methods
    - Equilibration
    - Handling of straws
    - Polystyrene box or controlled-rate freezer
    - Vials
    - Thawing
  - Calculation of fertilization and hatch rates
  - Osmolality measurements
  - CASA measurements
- Publication
  - Lack of guidelines or good laboratory practices
  - Lack of standard procedures
  - Lack of definitions
- Problems with the industry
  - Standards exist but are they applicable to aquaculture?
  - Lack of quality control

(Reviewed by Á. Horváth; 1st AQUAGAMETE Training School)

Cryopreservation and industry

In mammals (both cattle and humans) cryopreservation means good business, but not in fish species (yet).

However, during the last years the development of genetic improvement programmes has happened and there is a growing offer of cryobanking services.

Cryopreservation services are available for:

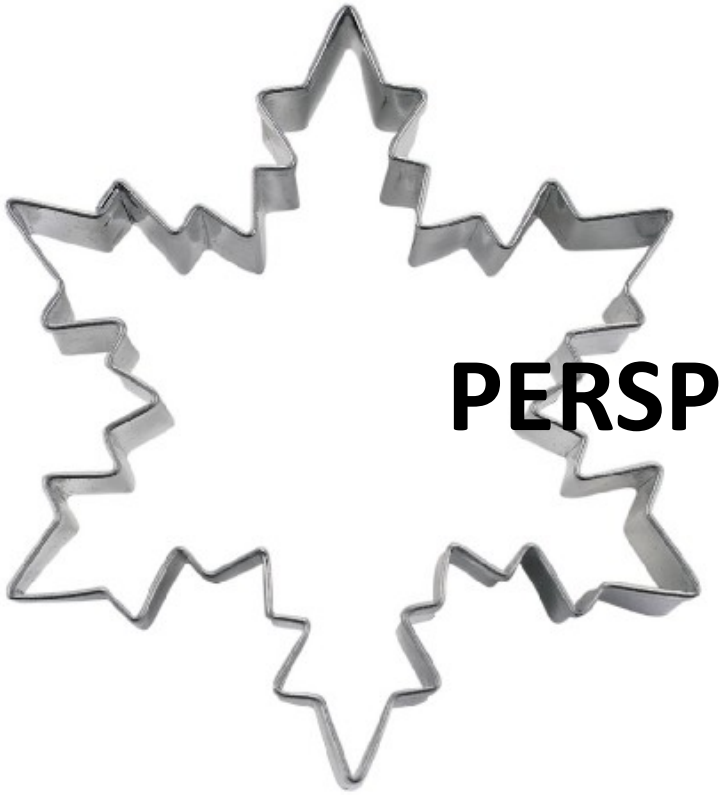
several salmonids

Atlantic salmon; Rainbow trout
Coho salmon; Chinook salmon
Arctic char; Sockeye salmon
Brown trout; Brook trout

others: sablefish, zebrafish and lumpsucker.

Specific media commercialized for gamete preparation (for salmonids, turbot, sea bream or tilapia).

(Reviewed by Martínez-Páramo et al., in prep.)



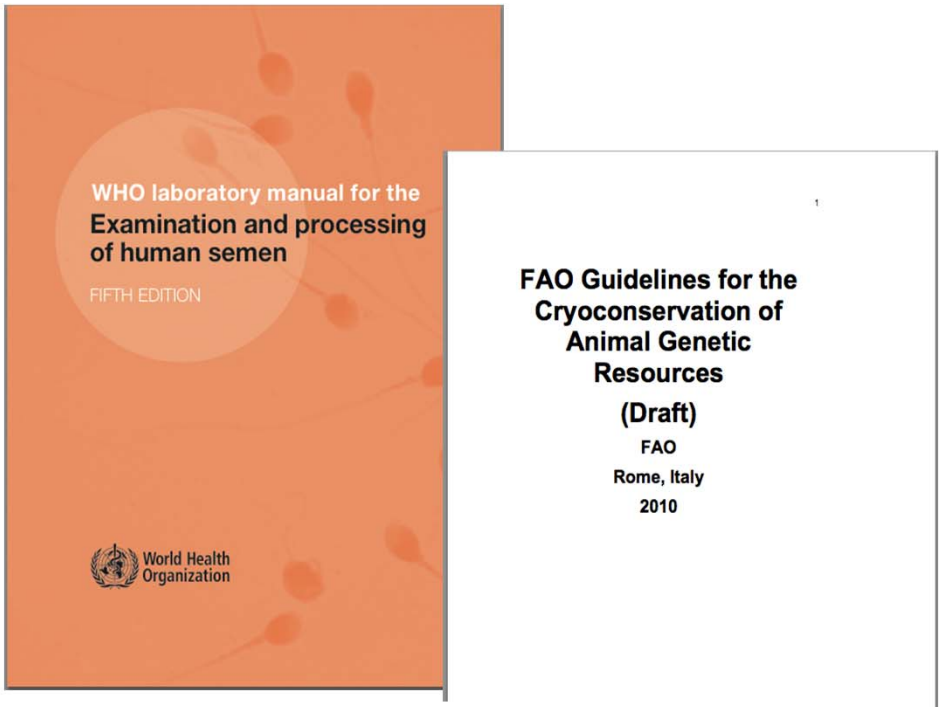
PERSPECTIVES

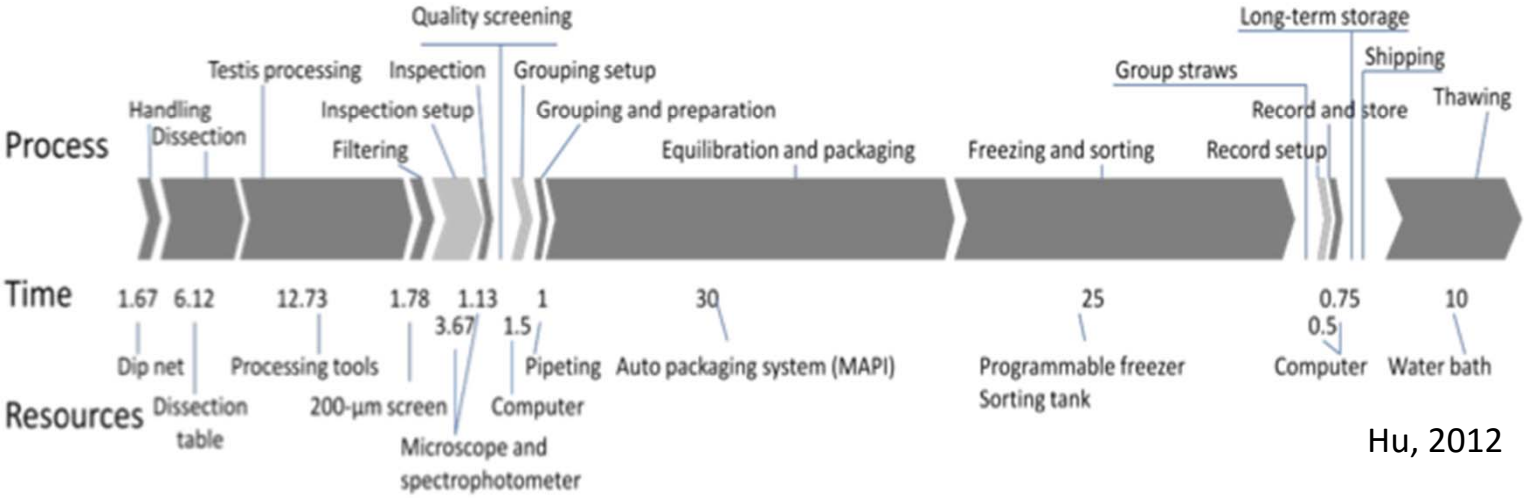
Standardization

Standardization of definitions

Standardization of protocols

Standardization of reporting





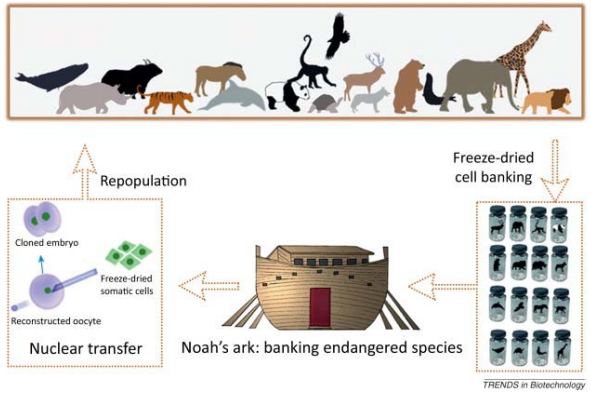
Hu, 2012

(Reviewed by Á. Horváth; 1st AQUAGAMETE Training School)

Main perspectives

Research
-Scaling up the methods
-Testing new biotechnologies
-Development of biotechnology centers

Fish industry
-Development of services centers allowing:
- Standardization
- Quality assessment
- Cryobanking (linked to genetic programmes)

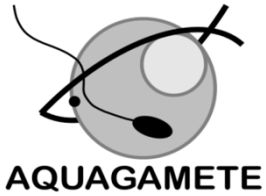


Loi et al., 2013

Cryobanking (Endangered species / Special samples)
-National or supranational management of cryobanks to ensure the safety of unique genetic resources.
-Specific objectives in relation with conservation programmes
-Cryobanks of aquatic species are established in Europe, USA, Brazil, Australia and New Zealand

(Reviewed by C. Labbé, pers. com.; Reviewed by Martínez-Páramo et al., in prep)

Acknowledgements



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