AQUATIC ANIMAL REPORTS 2(2) (2024) : 36-29
DOI: 10.5281/zenodo.11473601
aqar@scopesscience.com

AQUATIC ANIMAL REPORTS

Journal homepage: https://scopesscience.com/index.php/aqar

Received: 29 April 2024; Received in revised form: 07 May 2024
Accepted: 13 May 2023; Available online: 25 August 2024 REVIEW

Citation: Güngör, E. & Ekici, A. (2024). Methods used for the long-term preservation of primordial and early-stage germ cells in fish. *Aquatic Animal Reports*, *2*(2), 36-59. https://doi.org/10.5281/zenodo.11473601

METHODS USED FOR THE LONG-TERM PRESERVATION OF PRIMORDIAL AND EARLY-STAGE GERM CELLS IN FISH

Ege GÜNGÖR[1*], Aygül EKİCİ[2]

[1] Kalenderhane Mahallesi, Onaltı Mart Şehitleri Caddesi, No: 2 P.K 34134 Vezneciler Fatih İstanbul 1, Aquaculture Programme, Institute of Graduate Studies in Science and Engineering, Istanbul University, Istanbul, Turkey. (Corresponding author) Ege Güngör1, Turkey.egegun@gmail.com*
[2] Kalenderhane Mahallesi, Onaltı Mart Şehitleri Caddesi, No: 2 P.K 34134 Vezneciler Fatih İstanbul 2, Department of Aquaculture, Faculty of Aquatic Sciences, Istanbul University, Istanbul, Turkey.

Aygül Ekici2: aekici@istanbul.edu.tr, ORCID ID: https://orcid.org/0000-0003-1285-0304

**Corresponding author: Ege GÜNGÖR, egegun@gmail.com, +90-555 639 37 36*

Abstract

In sexually reproducing organisms, the continuous transmission of genetic data across generations is based on the biology of germ cells. High plasticity and capacity to form both gametes make Primordial Germ Cells (PGC) and Early-stage Germ Cells (eGC) (Oogonia or Spermatogonia) suitable candidates for long-term storage and retrieval of genetic data. This review examines cryopreservation techniques for these cells because of their key roles in gametogenesis. A wide range of current studies was examined in the review, from the embryonic origins of germ cells to advanced techniques used for isolation, enrichment, and cryopreservation in fish species. The development of germ-cell cryopreservation techniques has expanded our understanding of reproductive biology, opening novel possibilities for aquaculture and the preservation of genetic diversity. Enzymatic separation and enrichment methods that increase the viability and purity of isolated germ cells are important factors in the success rates of germ cell transplantation. The review also highlighted the potential of cryopreservation techniques, which offer the potential to preserve the genetic material of

endangered species and improve aquaculture practices by preserving valuable genetic traits. With a comprehensive review of the existing modalities and their applications, this review highlights the contribution of germ cell studies to the fields of developmental biology, conservation, and aquaculture, emphasizing their importance for the sustainable management of biological resources and the conservation of biodiversity.

Keywords: Primordial germ cell, early-stage germ cell, enrichment, cryopreservation.

1. Introduction

All organisms that reproduce sexually have two different cell lines developing in the early embryonic stage (Kunwar and Lehmann, 2006). The living body develops from the somatic line and is mortal because somatic cells cannot transmit their genetic data to their offspring. The germ cell line, on the other hand, results in the production of gametes with its ability to renew itself and undergo meiosis. Primordial Germ Cells (PGCs), which first develop in the embryo in the development of germ cells, are considered the source of germ cells playing vital roles in gametogenesis (Starz-Gaiano and Lehmann 2001). On the other hand, Early Stage Germ Cells (eGC), which is a broader definition, is employed to define Type A and Type B Oogonia (OG) or Spermatogonia (SG), which have the potential to turn into eggs and spermatozoa (Pšenička et al., 2015; Barroca et al., 2009).

PGCs carry genetic data from parents to their offspring (McLaren, 2003), and they provide the genetic link between all generations with this characteristic, which makes the germ cell line considered immortal. On the other hand, PGCs also differ morphologically from somatic cells because they have larger diameters (10-20 μm) and larger nuclei (6-10 μm) (Braat et al., 1999). These morphological characteristics make it easier to separate PGCs from somatic cells.

PGCs form spermatogonial or oogonial stem cells that are capable of self-renewal after the migration to the germline, producing female and male germ cells with gametogenesis, which can continue throughout the life of males. For this reason, PGC and transplantation of spermatogonial stem cells can be employed in reproductive medicine, including infertility treatments. These cells are also powerful tools for transgenic studies. The first PGC transplantation technique used to produce donor-derived offspring successfully was developed in chickens (*Gallus domesticus*) (Tajima et al., 1993). The donor PGCs that were taken from the blood of early chicken embryos were transferred intravenously to genetically different

carriers in this method. Later, a spermatogonial transplantation technique was developed for mice (*Mus musculus*), in which a testicular cell suspension that contained spermatogonial stem cells was transplanted into the testicular tubules of compatible carriers (Brinster and Zimmermann, 1994; Brinster and Avarbock, 1994).

When the first studies were conducted in this field, germ cell transplantation techniques could not be used for lower vertebrates such as fish, where very little was known about the functions of germ cells. However, techniques for PGC transplantation have been developed for fish since 2003 (Takeuchi et al., 2003), and spermatogonial transplantation was performed in fish after these studies (Okutsu. et al., 2006). These techniques can be employed for transplantation between allogenic strains (Takeuchi et al., 2003; Okutsu et al., 2006) and xenogenic species (Takeuchi et al., 2004). The advances brought by these techniques, which are still valid in our present day, include fish production by using transgenic germ cells, obtaining gametes of economically important fish with large body mass from closely related smaller-size species, and preserving the genetic resources of endangered species by combining them with cryopreservation of germ cells, paving the way for innovations in bioengineering.

2. Enzymatic dissociation and enrichment methods of eGC and PGC.

In eGC and PGC transplantation studies, one of the most important steps is the enzymatic separation and enrichment of these cells from the gonad tissue, which enables the use of enzymes to digest the extracellular matrix and connective tissue surrounding the germ cells in the gonad tissues, and obtain living single cells from the resulting cell suspension by using various methods and enrich the target cells.

The isolation of gonadal cells by enzymatic digestion has been used commonly in mammalian and teleost species (Bellve et al., 1977; Kaul et al., 2012). For this purpose, collagenase (*Labeo rohita*) (Panda et al., 2011), trypsin-EDTA (*O. niloticus, A. baerii, O. mykiss, Stfario, T. thymallus*, *Ictalurus furcatus*) (Farlora et al., 2014; Pšeniˇcka et al., 2016; Shikina et al., 2008; Kise et al., 2012; Kobayashi et al., 2004), dispase (*Seriola quinqueradiata*) (Morita et al., 2021), and DNase I combinations (*O. niloticus*, *A. ruthenus*, *C. carpio*) (Hong et al., 2004) are used widely. The enzymes used most commonly to decompose testicular tissue are trypsin and collagenase in fish (Table 1). Enzymatic separation requires considering factors such as fish species, sex, enzyme types, concentrations (Shikina et al., 2008), and incubation times

(Pšeniˇcka et al., 2015). Inadequate enzyme digestion may cause reduced single-cell yield, and excessive digestion can cause cell damage. The detachment of the epitopes from the surface of the cell membrane has been observed during trypsin digestion, which may disrupt germ cell functions (Ichida et al., 2019a; Zou et al., 2011; Garcia and Hofmann, 2012). It was also argued that sub-lethal damage that may occur during enzymatic digestion may affect the survival rate of cells during cryopreservation (Boonanuntanasarn et al., 2023, Hagedorn et al., 2018).

Table 1. Enzymatic dissociation procedure from testicular tissue in some fish species. T: Trypsin; A: Collagenase.

Fish Species	Enzyme	Enzyme percentage	Incubation duration	Viability	References
Tilapia (*Oreochromis niloticus*)	T	0.5%	3 hours	----	(Farlora et al., 2014)
Siberian sturgeon (*Acipenser baerii*)	T	0.3%	2 hours	90%	(Pšeniˇcka et al., 2016)
Rohu (*Labeo rohita*)	C.	500 units/mL	2 hours	----------	(Panda et al., 2011)
Rainbow trout (*Oncorhynchus mykiss*)	T	0.3%	2 hours	--------	(Shikina et al., 2008)
Rainbow trout (*Oncorhynchus mykiss*)	T	0.5%	2 hours	----------	(Kise et al., 2012)
Rainbow trout (*Oncorhynchus mykiss*)	T	0.5%	1.5 hours	93.2%	(Kobayashi et al., 2004)
Japanese amberjack (*Seriola quinqueradiata*)	C + dispase II	0.4% C + 0.03% dispase II	2 hours	----------	(Morita et al., 2021)
Tilapia (*Oreochromis niloticus*)	T+C	2% C + 0.25% T	C 4 hours + 30min T	----------	(Lacerda et al., 2010)
Sterlet (*Acipenser ruthenus*)	T+C	1) 0.1% T and 0.1% C, 2) 0.3% T, 3) 0.1% T, 4) 0.3% C, 5) 0.1% C	2 hours	86%	(Pšeniˇcka et al., 2015)
Common carp (*Cyprinus carpio*)	T+C	0.15% T + 0.1% C	1.5 hours	----------	(Franěk and Pšenička, 2020)
Brown trout (*Salmo trutta m. fario*)/European grayling (*Thymallus thymallus*)	T	0.2%	1.5 hours	85%	(Lujić et al., 2017)
Blue catfish (*Ictalurus furcatus*)	T	0.25% T - EDTA / 0.05% T - EDTA	1 hour	0.25% T 83.5%/ 0.05% T 98.2%	(Shang, 2013)

In donor cell populations, the purity of eGCs is one of the important factors that affect the success of germ cell transplantation. The cells that make up the gonads include supporting cells (Sertoli and Leydig cells), germ cells of various stages, fibroblasts, and red blood cells. Among these, only PGCs and eGCs are capable of colonizing host gonads, self-renewal, and developing into functional gametes (Yoshizaki et al., 2010; Yano et al., 2008). For this reason, a higher success rate is achieved when enriched cells are used instead of undifferentiated cells when equal numbers of cells are transplanted. Many modalities were tried in the past for the enrichment of eGCs (e.g., Density Gradient Centrifugation (DGC), Differential Plating (DP), Centrifugal Elutriation (CE), Fluorescence-Activated Cell Sorting (FACS), and Magnetic-activated Cell Sorting (MAC). These methods have advantages and disadvantages compared to each other.

DGC is a simple process and is based on the principle of separating cells according to their densities and does not require materials other than silica-based media such as percoll/ficoll and equipment other than a centrifuger. Although it has the disadvantage of obtaining low purity, it is still used often for the enrichment of fish eGCs with a purity rate of 60-83.6% because of its practicality in zebrafish (*Danio rerio*) (Wong et al., 2011), medeka (*Oryzias latipes*) (Ryu and Gong, 2020), eel (*Misgurnus anguillicaudatus*) (60% purification) (Yoshikawa et al., 2009), Japanese Flounder (*Paralichthys olivaceus*) (83.6% purification) (Ren et al., 2021), and in Siberian sturgeon (*Acipenser baerii*) (79.4% purification) (Pšeničcka et al., 2015).

DP purification method is performed by using different adhesion characteristics of cells in which cells are incubated briefly on surfaces covered with culture medium so that more adherent cells can be separated from non-adherent cells by pipetting or gentle shaking. In general, eGCs can be separated from somatic cells such as fibroblasts because they adhere weakly to substrates, which show relatively tight binding (Shikina et al., 2013; Sato et al., 2013). This method also offers a simple procedure, but low purity and risks of spontaneous differentiation during in vitro culture cause this method to be considered disadvantageous. Studies conducted on rainbow trout (*Oncorhynchus mykiss)* reported that over 90% purity could be achieved with serial applications of DP (Shikina et al., 2008, 2013).

Cells are purified in the CE method by using centrifugation according to their physical characteristics. Unlike typical centrifuges, the centrifuge required in this method has a special separation chamber where the cells are subjected to a centrifugal force and countercurrent flow.

This method allows the separation of different cell groups because the size, shape, and density of cells affect sedimentation rates (Liu et al., 2021). In a previous study conducted by Bellaiche et al., cell fractions that contained Type A Spermatogonia with greater than 90% purity were obtained with CE from immature testes of rainbow trout (*Oncorhynchus mykiss*) following the pre-enrichment with DGC (Bellaiche et al., 2014). CE requires specialized equipment for high enrichment and precise determination of conditions specific to the cells to be separated. For this reason, this method has only been employed in a few fish germ cell enrichment and transplantation studies to date (Liu et al., 2021).

The FACS Method allows separating cells based on light scattering parameters and for the purification process, cells are exposed to laser light in a flow cytometry device and their fluorescence characteristics are measured in this method. Then, a positive or negative electrical charge is applied to individual cells, which are separated by delivering them to different channels through an electrostatic deflection system (Hu et al., 2016; Adan et al., 2017). In particular, cells that are labeled with fluorescent proteins or stains (Fluorescein isothiocyanate, Green fluorescent protein, red fluorescent protein, PKH26) can be easily enriched by FACS with their strong fluorescence intensity. The process of fluorescent marking of germ cells in fish is generally performed by Vasa (Okutsu et al., 2008; Yoshizaki et al., 2003; Wong et al., 2011) and Nanos1 (nos1 3'UTR) (Saito et al., 2008; Higaki et al., 2010) RNA-binding media that are specific to these cells with fluorescent expression of cell surface proteins. Fluorescent marking of germ cells is performed with microinjection of mRNAs encoding fluorescent protein (Saito et al., 2010), fluorophore-conjugated dextran (Saito et al., 2015; Pšeničcka et al., 2020) by using fluorophore-conjugated antibodies or transgenic fish expressing germ cell-specific fluorescent protein (Takeuchi et al., 2003). In studies conducted on fish, PGCs and eGCs could be enriched to purity levels of 93.2% (Kobayashi et al., 2004) and 93.2% (Kise et al., 2012), respectively in transgenic rainbow trout (*Oncorhynchus mykiss*) with germ cells expressing Green Fluorescent Protein (GFP) (pvasa-GFP). GFP-nanos1 mRNAs were injected into embryos at the 1 to 4-cell stage to mark PGCs in non-transgenic fish, and 100% purity was achieved after FACS (Goto-Kazeto et al., 2010). Fluorophore-Conjugated Antibodies produced to attach to fish eGC surface proteins (vasa/nanos1 + GFP) were detected in brown trout (*Salmo trutta*) (Ichida et al., 2021), Pacific bluefin tuna (*Thunnus orientalis*) (Ichida et al., 2019b), and rainbow trout (Hayashi et al., 2019) and provided eGC purification with rates of 70.7-80.9% after FACS.

Although high purity is achieved in isolation using the FACS method, it also has a disadvantage because of the need for high knowledge and specialized expensive equipment. Also, the processing capacity is low (107 cells/hour) because the cells are separated one by one in this method; and for this reason, it is inefficient for studies in which a large number of samples are used (Ichida et al., 2019a; Plouffe et al., 2015).

Cells are separated in the MACS Method according to their magnetic characteristics, not their light scattering characteristics. The cytometry device does not have a laser and detector duo in this method, but a magnetic column. Cells are marked using antibodies conjugated with magnetic nanoparticles against cell surface antigens before separation and are passed through the column and separated into positive and negative according to their magnetism (Pan et al., 2012). When highly specific antibodies are used, MACS enables the acquisition of largely pure cell populations. Enrichment of eGC by MACS in fish was reported in a study conducted on rainbow trout (*Oncorhynchus mykiss*) using No 172 antibody, which showed that 81.7% of testicular cells and 54.8% of ovarian cells purified by MACS were vasa-positive (Ichida et al., 2019a). When compared to mammalian studies in which greater than 95% purity was achieved with MACS, the purity of fish eGC was lower, suggesting that antibody No 172 is not very specific for eGCs (Ichida et al., 2019a). Unlike FACS, MACS cannot separate cells with gates customized based on light scattering characteristics and for this reason, more specific antibodies are required to achieve a higher purity of eGC. However, FACS requires fewer and cheaper consumables and skills (Zhu and Murthy, 2013). Also, MACS can be applied on large scales because a period of 15 minutes is required to separate magnetically labeled cells, regardless of cell number (Ichida et al., 2019a).

3. Germ Cell Cryopreservation

Techniques for PGC and eGC isolation and cryopreservation need to be developed to use germ cell transfer techniques efficiently in fish. While freezing germ cells prevents disease outbreaks in aquaculture, loss of valuable fish species because of natural disasters or technological disruptions, long-term cryopreservation of genetic resources decreases the need to keep large amounts of brood stock. Despite attempts to preserve maternal genetic information through embryo cryopreservation and egg cryopreservation, current techniques have not been successful (Kumari and Maurye, 2021). Therefore, the only practical methods currently available for preserving maternal genetic information, apart from maintaining brood stock, are PGC and eGC cryopreservation (Wylie et al., 2023).

The main target of freezing techniques in fish is to create a method to preserve the integrity of tissues or cells at sub-zero temperatures for long periods. Each freezing process consists of the following steps (1) the preparation of the cooling medium with the most appropriate preservatives and additives, (2) performing the freezing process by using fast or slow freezing methods, (3) cryogenic storage of the biological material, and (4) recovering the frozen material by heating, each of which must be optimized to minimize the freezing damage and ensure the recovery of a high percentage of healthy living cells (Pegg, 2015).

Two main methods are employed to freeze the germ cells, the first of which is ultra-rapid freezing and vitrification by using concentrated cryoprotectants. Here, the sample is quickly frozen, creating a glass-like condition to prevent the formation of ice crystals. The second method is slow freezing, in which a lower concentration of cryoprotectant is employed and the sample is frozen at a rate according to the protocol (usually 1°C/minute). The slow freezing method is based on the slow removal of water from the cell cytoplasm to prevent intracellular ice formation by increasing viscosity. The cell suspension is then cryopreserved and stored at -196°C. No changes that may harm the cells are expected to occur at this temperature (Mazur, 1984).

The use of fish germplasm conservation studies in aquaculture is still in the developmental stage. Only sperm-freezing processes were developed to cover the needs of aquaculture (Cabrita et al., 2010). In our present day, breeding programs are used in some fish species to protect genetic resources and efforts are being made to ensure the continuity of such species with transgenic or mutant lines (Carmichael et al., 2009; Robles et al., 2009). As well as sperm freezing, great efforts were also made to freeze fish oocytes. Preservation of oocytes in fish is limited because of their different biological characteristics when compared to sperm. Fish oocytes have low cryoprotectant permeability and high water contents in their cytoplasm because of the presence of chorion and large yolk volume, which makes it difficult to protect oocytes from freezing damage caused by ice crystals. Studies that investigated the freezing of vitellogenic oocytes (Godoy et al., 2013) and early-stage oocytes (Guan et al., 2008, 2010; Tsai et al., 2010) were conducted in zebrafish. However, ovarian follicles were damaged severely after freezing and failed during their growth under in vitro conditions (Tsai et al., 2010; Anil et al., 2018).

As another technique, embryo cryopreservation focuses on increasing the permeability of cryoprotectants or embryo manipulation. One of the major obstacles in this field is how to ensure proper thawing and prevent ice formation. To the best of our knowledge, these problems were solved successfully by simultaneous injection of cryoprotectant and nanoparticles into the zebrafish embryos, which were then thawed by using a laser pulse to ensure uniform heating by stimulating the nanoparticles fully (Khosla et al., 2017). However, procedures for performing this procedure on a larger scale and in different fish embryos have not yet been developed.

3.1. Primordial Germ Cell Cryopreservation

PGC freezing is an important step for studies in the field of germ cell preservation because preserving these high pluripotency cells is advantageous in many terms. These cells, which are diploid with their ability to transform into gametes of both sexes, enable the recovery of genetic data of both sexes after they are transferred to the host organism following the freezing and thawing processes (Inoue et al., 2012). Even one single PGC is adequate to restore the reproductive capacity of an individual (Saito et al., 2008). Cryopreservation of PGCs can be achieved by marking them with a Green fluorescent protein that is labeled Nanos 1 mRNA Injection (GFP-nos1-30 UTR), which is used to track primordial germ cells (Saito et al., 2006; Kawakami et al., 2010), or it depends of their distinction from other cells by using transgenic lines. Because these cells must be separated from other cells before cryopreservation and transplantation (Kobayashi et al., 2007; Riesco et al., 2012). PGCs are localized at the blastula stage (Higaki et al., 2010), at the migration stage (Riesco et al., 2012), and at the genital ridge and can be cryopreserved in the post-migration phase (Kobayashi et al., 2007). However, it is necessary to be aware that PGC migration ability will gradually decrease when donors are used at later embryonic stages (Saito et al., 2010).

In a previous study of eel embryos at the somitogenesis stage marked with the GFP-nos1-30 UTR, the embryos were treated with cryoprotectant and vitrified, and the thawed embryos were dissociated in citric acid. It was reported that when one single PGC that was isolated from these fish was transplanted into a carrier at the blastula phase, the cells maintained their viability and migrated towards the genital ridge actively (Inoue et al., 2012). In another study that was conducted on rainbow trout, genital ridge cryopreservation was performed with slow freezing (-1 C°/minute) to obtain donor-derived offspring. The obtained PGCs were transplanted into rainbow trout fry and donor-derived offspring were obtained (Kobayashi et al., 2007). Many

studies were conducted on zebrafish and rainbow trout and on the process of producing live fish from vitrified PGCs to optimize cryopreservation processes and maximize post-thaw survival. In a previous study in which the entire embryo of zebrafish, enzymatically dissociated genital ridge cells, and single PGCs were compared with different cryopreservation methods, a 98% survival rate was reported after thawing (Riesco et al., 2012). PGC cryopreservation and transplantation were also successfully performed in other fish species such as carp (*Cyprinus carpio*) and Japanese eel (*Anguilla japonica*) (Kawakami et al., 2012a, b).

3.2. Early Germ Cell (Spermatogonia and Oogonia) Cryopreservation

Cryopreservation of eGCs obtained from immature and adult individuals was investigated by using different cryopreservation techniques in many fish species, especially salmonid and cyprinid fish species (Table 2).

In these studies, species-specific optimized protocols were created to increase the viability of cells after thawing. To begin with, we must say that oogonia cryopreservation is the only way known to preserve genetic data of maternal origin with current techniques. Genetic data can also be recovered by transferring frozen oogonia to a carrier. The only alternative method to this is to grow the rootstocks of the target species continuously under culture conditions. Of course, this also brings risks such as disease, genetic drift, and failure to meet culture conditions because of technical reasons. However, all techniques for eGC cryopreservation are much more advantageous in terms of time, equipment, and economy when compared to the preservation in culture conditions and PGC cryopreservation.

Unlike PGC cryopreservation, target cells can be obtained in higher numbers because the gonad is larger in immature (8-12 months in salmonid species) and adult periods when eGC is isolated. If the isolation timing is planned accurately, the target cell can be detected densely in the tissue. Analyzes of histological sections of immature salmonid gonads were also reported in previous studies and it was shown that the gonad contains a large proportion of Type A Spermatogonia (Lujić et al., 2018; Kise et al., 2012; Shikina et al., 2008).

Table 2. List of selected studies on the cryopreservation of eGCs in fish.*

Species	Cryopreservation method	Cryopreserved material	Successful cryoprotectant	Post-thaw viability	Reference
Striped catfish (*Pangasianodon hypophthalmus*)	SRF	T	1.3 M DMSO	65.64% ± 2.36%,	(Boonanuntanasarn et al., 2023)
Rainbow trout (*Oncorhynchus mykkis)*	SRF	T	1.3 M DMSO, 0.1 M trehalose	35.1±5.3%	(Lee et al., 2013)
		T	1.3 M DMSO 0.1 M trehalose	No data	(Lee et al., 2016a)
		O	1 M DMSO, 0.1 M trehalose	72.9 ±6.2%	(Lee et al., 2016b)
Manchurian trout (*Brachymystax* lenok)	SRF	T	1.3 M ME, 0.2 M trehalose	81.0 ±1.3%	(Lee and Yoshizaki, 2016)
Common carp (*Cyprinus carpio)*	SRF	T	2 M DMSO, 0.3 M trehalose	40.7±9.2%	(Franěk et al., 2019a)
		O	1.5 M DMSO, 0.3 M glucose	66.0 ±8.6%	(Franěk et al., 2019c)
Goldfish (*Carassius auratus)*	SRF	TS	2 M DMSO, 50 mM glucose	~72%	(Marinović et al., 2016)
Nile tilapia (*Orechromis niloticus*)	SRF	TS	1.3 M DMSO, 10% FBS	No data	(Lacerda et al., 2010)
Siberian sturgeon (*Acipenser baerii)*	SRF	T	1.5 M EG, 50 mM glucose	64.3±6.1%	(Pšenička et al., 2016)
		O	1.5 M EG, 50 mM glucose	52.0 ±7.3%	
Starry gobby (*Asterropteryx semipunctata*)	SRF	T, TS	1.3 M DMSO, 0.1 M trehalose	~60%	(Hagedorn et al., 2018)
Tench (*Tinca tinca*)	SRF	TS	1.5 M GLY, 50 mM glucose	57.7 ±16.8%	(Linhartová et al., 2014)
		T	3 M DMSO, 50 mM glucose	~55%	(Marinović et al., 2016)
Black rockfish (*Sebastes schlegelii*)	SRF	T	DMSO %47 (vol/vol) egg yolk powder, D (+)-trehalose dihydrate	~90%	(Li et al., 2023)
Tiger puffer (*Takifugu rubripes)*	SRF	T	1.3 M DMSO, 0.1 M trehalose	61.2 ±2.7%	(Yoshikawa et al., 2018)
Honmoroko (*Gnathopogon caerulescens*)	V	OS	5 m DMSO	110%	(Higaki et al., 2018)
		TS	5 M PG	50%	
Medaka (*Oryzias latipes*)	V	T	5 M EG, 21% Ficoll, 0.35 M sucrose	~43%	(Seki et al., 2017)
Zebrafish (*Danio rerio*)	NIV	T	ES: 1.5 M ME and PG VS: 3 M DMSO and PG	50−72 ±1−13%	(Marinović et al., 2018)
Brown trout (*Salmo trutta*)	NIV	O	ES: 1.5 M ME, 1.5 DMSO VS: 3 M PG, 3 M DMSO	40.34%	(Lujić et al., 2017)
Sterlet (*Acipenser ruthenus*)	NIV	O	1.5 M PG and 1.5 M Me2SO	55.7% ± 11.5%	(Lujić et al., 2023)
Russian sturgeon (*Acipenser gueldenstaedtii*)	NIV	O	1.5 M MeOH and 1.5 M Me2SO	49.4% ± 17.1%	(Lujić et al., 2023)

*SRF slow-rate freezing NIV needle-immersed vitrification, V vitrification, O ovarian tissue, T testicular tissue, TS testicular cell suspension, OS ovarian cell suspension, ME methanol, DMSO dimethyl sulfoxide, PG propylene glycol, GLY glycerol, EG ethylene glycol, ES equilibration solution, VS vitrification solution.

In general, eGCs can be cryopreserved by slow freezing technique in programmable -80°C freezers by maintaining a cooling rate of approximately -1°C/minute (Franěk et al., 2019a, b; Lee and Yoshizaki, 2016; Pšenička et al., 2016). Aside from this, it was reported that they can be cryopreserved effectively with ultra-fast vitrification (Seki et al., 2017). The donor was killed for dissection of the gonad in previous studies conducted with these techniques, but in relatively large-sized species such as shortnose sturgeon (*Acipenser brevirostrum*), non-lethal gonad isolation methods can also be used with minor surgical intervention (Matsche et al., 2013). This removed piece of gonad can be cryopreserved as tissue or as an enzymatically dissociated cell suspension, as reported in a study on Siberian sturgeon (*Acipenser baerii)* (Pšenička et al., 2016). This study also found that cryopreservation of the tissues was better in terms of cell survival rates than cryopreservation of separated cells. Also, in a trial in which the tissue was cryopreserved as a whole, the cells that died in the cryopreservation step were completely decomposed in the enzymatic separation step. In this way, dead cells that may affect the success of the transplantation process are almost eliminated in transplantation studies conducted with this method (Pšenička et al., 2016).

Another recently developed cryopreservation strategy is the Needle-Immersed Vitrification (NIV) method in which pieces of gonad tissue placed on the tip of an insulin needle are exposed briefly to equalization and vitrification solution and immersed directly in liquid nitrogen (-196°C). As well as being fast, this method is also advantageous because it reduces the equipment requirement (Lujić et al., 2017; Marinović et al., 2018). The simplest method that has been developed for eGC cryopreservation is to freeze the whole fish by placing it in a -80°C freezer or on dry ice, where the cooling rate is reported to be close to or the same as the generally accepted optimum (-1°C/min). A study conducted on rainbow trout reported no statistical differences in the number of spermatogonia that were recovered after thawing over an observation period of 1-1113 days when whole fish were stored continuously at -80°C or frozen to -80°C and then transferred to liquid nitrogen (Yoshizaki and Lee, 2018). For this reason, this method has a significant advantage because the fish can be frozen immediately without any preparations. However, it must be taken into consideration that freezing the entire fish will not be advantageous in conditions that require working with a large number of samples or requiring rapid thawing in which possible parallel experiments on the tissue before cryopreservation cannot be performed.

Regardless of the technique used, cryoprotectants are employed against the formation of ice crystals in most cryopreservation processes. Cryoprotectants such as low-concentration (2 M%) ethylene glycol, dimethyl sulfoxide, and methanol were generally determined to be optimal in the slow freezing technique. Over time, studies also tried supplements that supported cell survival (e.g., glycerol and protein), and 40-90% live germ cells were obtained after thawing. Similar cryoprotectants are used in high concentrations in the vitrification technique (20%). Also, unlike the slow freezing technique, vitrification involves drawing enzymatically separated cells into pipettes (Seki et al., 2017; Higaki et al., 2018), using gonad pieces or whole gonads (in zebrafish, medaka, or young trout) by using the NIV Method (Lujić et al., 2017; Marinović et al., 2018), or by dipping the gonads placed on a metal grid (e.g., copper grid) into liquid nitrogen (Seki et al., 2017). After vitrification of fish germ cells (around 50%), thawing viability is lower in most cases than slow-rate freezing. After thawing, although the viability rate is low in studies using the vitrification method, this technique is very suitable for field sampling because of its advantages such as being able to cryopreserve samples very quickly and requiring little equipment. However, it must be noted that vitrification is not an ideal method for all species. For example, studies conducted on carp (*Cyprinus carpio*) testicular cells reported that these cells had a fourfold lower survival rate after vitrification when compared to the slow-freezing method (Franěk et al., 2019a).

4. Whole tissue cryopreservation

Although several techniques were used to preserve genetic resources in fish, this field is still in the development stage. Traditionally, the strategy of preserving the species with cryopreservation of gametes is the most common approach. In this method, the purpose is to preserve genetic data by fertilizing fresh eggs with cryopreserved sperm of the species or genetic line desired to be preserved throughout different generations (Di Iorio et al., 2019; Liu et al., 2015; Diwan et al., 2020). However, the high yolk content and low membrane permeability of eggs reduce survival rates greatly after cryopreservation (Zhang et al., 1993; Guan et al., 2008; Guan et al., 2010), making the recovery of embryos derived from cryopreserved gametes difficult. Embryos of the species intended to be preserved can be reproduced by fertilizing eggs whose DNA has been inactivated with frozen sperm in Androgenesis, which is another method in which cryopreserved sperm is used (Thorgaard et al., 1990; Babiak et al., 2002). In this method, embryo production success is very low and the genetic data of the female is not preserved.

On the other hand, PGCs and eGCs are the precursors of the gametes and can sustain gametogenesis (spermatogenesis or oogenesis) after the transplantation (Okutsu et al., 2006). The potential of these cells to develop into functional gametes and their ability to transmit genetic data to subsequent generations make them candidates for species conservation studies through cryopreservation and germline chimera production (Yoshizaki et al., 2003; Caires et al., 2010). However, it becomes difficult to use these cells for conservation studies because PGCs can only be isolated from the genital ridges or embryos of fish larvae and these processes require extensive knowledge and equipment. Long-term preservation of eGCs with cryopreservation of the entire tissue, which has been developed in recent years as an alternative to the previous methods, provides results suitable in terms of the preservation of species and genetic lines because it eliminates the disadvantages mentioned above. Cryopreservation of the entire testicular tissue containing eGC was studied in many species including rainbow trout (*Oncorhynchus mykiss*) (Lee et al., 2013), carp (*Cyprinus carpio*) (Franěk et al., 2019a), Manchurian trout (*Brachymystax lenok*) (Lee and Yoshizaki, 2016) and zebrafish *(Danio rerio) (Marinović et al., 2019)* (Table 3). It was also shown in various studies that spermatogonia isolated from these tissues can be transplanted to produce donor-derived offspring (Lee et al., 2013; Lee et al., 2016; Marinović et al., 2019). In previous studies that compared whole testicular tissue freezing with enzymatically dissociated cell cryopreservation, it was shown that freezing whole testicular tissue increased the survival rates after thawing or reduced the number of dead cells (Pšeničcka et al., 2016). In another study in which the same comparison was made, the highest viability was reported to be 67% when judia *(Rhamdia quelen)* eGCs were cryopreserved as whole tissue. When samples that were first separated enzymatically were cryopreserved, this rate was 27% (Rosa et al., 2023). In a previous study that was conducted on starry goby *(Asterropteryx semipunctata*) testicular cells, it was found that whole tissue cryopreservation had an advantage in terms of post-thawing viability when compared to cryopreservation of enzymatically dissociated cells and it was found to be more successful in preserving the viability of large cells in particular (Hagedorn et al., 2018).

Another approach that can be considered within the scope of cryopreservation of the entire gonad tissue is freezing the entire fish. In a previous study that was conducted to cryopreserve eGC of rainbow trout *(Oncorhynchus mykiss)*, 1019 ± 251 eGC/fish was obtained after 1113 days from fish frozen whole at -80°C (Lee et al., 2015).

Table 3. Studies involving the cryopreservation of entire gonad tissue in some fish species.

Species	T or O	Cryoprotectant	Cryopreservation Method	Successful cryoprotectant	Cryopreservation duration	Viability	References
Murray River Rainbowfish (*Melanotaenia fluviatilis*)	T	DMSO, EG, methanol, glycerol	SRF	1.3 M DMSO	24 h	Large cell" (> 9 µm) viability (72.6% ± 10.5%)	(Rivers et al., 2020)
Siberian sturgeon (*Acipenser baerii*)	T and O	DMSO, EG, glycerol	SRF	1.5 M EG	30 days	90%	(Pšeničcka et al., 2016)
Rainbow trout (*Oncorhynchus mykiss*)	O	DMSO, EG, glycerol	SRF	1.0 M DMSO	1185 days	72.9% ± 6.2%	(Lee et al., 2016)
Zebrafish (*Danio rerio*)	O	DMSO, EG, PG, glycerol	NIV	1.3 M DMSO	-	50%	(Marinović et al., 2018)
Brown trout (*Salmo trutta*)	O	DMSO, methanol, PG	NIV	3M Me2SO + 3M PG	2 days	40%	(Lujic et al., 2017)
Striped catfish (*Pangasianodon hypophthalmus*)	T	DMSO, EG, PG	SRF	1.3M PG	7 days	65.64%	(Boonanuntanasarn et al., 2023)
American Shad (*Alosa sapidissima*)	T	DMSO	SRF	10% DMSO + 40% FBS + 50% DMEM	120~350 days	65% to 93.8%	(Xu et al., 2022)
Starry goby (*Asterropteryx semipunctat*)	T	DMSO	SRF	0.1 M trehalose, 9.2% (v/v) DMSO (1.3 M), and 10% (v/v) Fetal Bovine Serum (FBS) in L15 medium.	24 h	The number of intact spermatogonial cells was found to be unaffected by the cryopreservation process.	(Bouwmeester et al., 2022)
Common carp (*Cyprinus carpio*)	T	Me2SO	SRF	2 M Me2SO	At least 1 day	>40%	(Franˇek et al., 2019a)
Manchurian trout (*Brachymystax lenok*)	T	methanol, EG, PG, Me2SO, glycerol	SRF	1.3 M Methanol, 0.2 M Trehalose, 10% Egg yolk	2 days	81.0% ± 1.3%	(Lee and Yoshizaki, 2016)
Rainbow trout (*Oncorhynchus mykiss*)	T	DMSO, EG, PG, glycerol	SRF	1.3 M DMSO, 0.1 M trehalose, 10% egg yolk	Up to 939 days	35.1 ± 5.3% at 1 d, 33.5 ± 7.1% at 728 d	(Lee et al., 2013)
Zebrafish (*Danio rerio*)	T	Me2SO, MeOH, PG	SRF, NIV	1.3 M Me2SO, 0.1 M trehalose, 1.5% BSA.	-	~50%	(Marinović et al., 2019)

5. Conclusions

When the species-specific optimized cryoprotectant, cryopreservation methods, and the viability rates of the obtained cells were evaluated in previous studies on PGC and eGC cryopreservation, a high comparability was detected between the results. Based on this, it is possible to predict that the techniques mentioned in this review will be customized for more species in the future and their usability in the field of conservation of endangered species and aquaculture will also increase. As the articles examined in this review pointed out, the issue in the field of eGC and PGC cryopreservation is no longer about whether it is possible or not, but about the species-specific development of practical application methods that are suitable for use in vivo. These cells are suitable candidates for long-term storage and recovery of genetic data because of their high plasticity and capacity to form both gametes. However, there are currently only two methods to recover the species cryopreserved by the above-mentioned methods (surrogate production and transplantation for the production of donor-derived gametes) (Goto and Saito, 2019) and/or in vitro gamete differentiation through cell culture (Xie et al., 2020). It is not correct to argue that there is a currently completed protection strategy because these two methods require high knowledge, time, and expensive equipment. Other steps must also be developed for a whole protection strategy, such as determining suitable carrier species, sterilizing these species, and determining the optimal transplantation method. Despite all these developmental steps, cryopreservation of eGC and PGC is currently the only method to recover genetic resources properly because of their potential to transform into germ cells of both sexes. With the techniques mentioned in this review, cells that carry both paternal and maternal genetic resources can be cryopreserved with a high viability rate after thawing (Franěk and Pšenička, 2020).

Ethical approval
Not applicable

Informed consent
Not available.

Data availability statement
"The authors declare that data can be provided by corresponding author upon reasonable request."

Conflicts of interest
There is no conflict of interests for publishing this study.

Funding organizations
No funding available for this study.

Contribution of authors
Ege Güngör1: Writing original draft.
Aygül Ekici2: Writing original draft.

All authors have read and agreed to the published version of the manuscript.

References

Adan, A., Alizada, G., Kiraz, Y., Baran, Y., & Nalbant, A. (2017). Flow Cytometry: Basic Principles and Applications. Critical Reviews in Biotechnology, 37, 163–176.

Anil, S., Rawson, D., & Zhang, T. (2018). Development of molecular markers for zebrafish (*Danio rerio*) ovarian follicle growth assessment following in-vitro culture in cryopreservation studies. Cryobiology, 83, 75–83. https://doi.org/10.1016/j.cryobiol.2018.05.004

Babiak, I., Dobosz, S., Goryczko, K., Kuzminski, H., Brzuzan, P., Ciesielski, S. (2002). Androgenesis in rainbow trout using cryopreserved spermatozoa: the effect of processing and biological factors. Theriogenology, 57(4), 1229–1249.

Barroca, V., Lassalle, B., Coureuil, M., Louis, J. P., Le Page, F., Testart, J., Allemand, I., Riou, L., & Fouchet, P. (2009). Mouse differentiating spermatogonia can generate germinal stem cells in vivo. Nature Cell Biology, 11(2), 190-196. https://doi.org/10.1038/ncb1826

Bellaiche, J., Lareyre, J. J., Cauty, C., Yano, A., Allemand, I., & Le Gac, F. (2014). Spermatogonial Stem Cell Quest: nanos2, Marker of a Subpopulation of Undifferentiated Spermatogonia in Trout Testis. Biology of Reproduction, 90, 1–14.

Bellve, A. R., Cavicchia, J. C., Millette, C. F., O'Brien, D. A., Bhatnagar, Y. M., & Dym, M. (1977). Spermatogenic cells of the prepuberal mouse: Isolation and morphological characterization. Journal of Cell Biology, 74, 68–85.

Boonanuntanasarn, S., Sreebun, S., Booncherd, K., Khaosa-Art, P., Sooksawat, T., Ichida, K., Pirarat, N., & Yazawa, R. (2023). Cryopreservation of testicular cell in striped catfish (*Pangasianodon hypophthalmus*) and its effects on apoptosis, germ-cell specific gene expression and germ cell transplantability. Cryobiology, 100, 1-9. https://doi.org/10.1016/j.cryobiol.2021.05.007

Bouwmeester, J., Daly, J., Henley, E. M., et al., (2022). Conservation of coral reef fishes: a field-hardy method to cryopreserve spermatogonial cells. Coral Reefs, 41, 855–861. https://doi.org/10.1007/s00338-022-02268-1

Braat, A. K., Speksnijder, J. E., & Zivkovic, D. (1999). Germ line development in fishes. International Journal of Developmental Biology, 43, 745–760.

Brinster, R. L., & Avarbock, M. R. (1994). Germline transmission of donor haplotype following spermatogonial transplantation. Proceedings of the National Academy of Sciences of the USA, 91, 11303–11307.

Brinster, R. L., & Zimmermann, J. W. (1994). Spermatogenesis following male germ-cell transplantation. Proceedings of the National Academy of Sciences of the USA, 91, 11298–11302.

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. https://doi.org/10.1111/j.1439-0426.2010.01556.x

Caires, K., Broady, J., & McLean, D. (2010). Maintaining the male germline: regulation of spermatogonial stem cells. Journal of Endocrinology, 205, 133–145.

Carmichael, C., Westerfield, M., & Varga, Z. M. (2009). Cryopreservation and in vitro fertilization at the zebrafish international resource center. In Methods in Molecular Biology (Clifton, N.J.), 546, 45–65. https://doi.org/10.1007/978-1-60327-977-2_4

Di Iorio, M., Esposito, S., Rusco, G., Roncarati, A., Miranda, M., Gibertoni, P. P., & Iaffaldano, N. (2019). Semen cryopreservation for the Mediterranean brown trout of the Biferno River (Molise-Italy): Comparative study on the effects of basic extenders and cryoprotectants. Scientific Reports, 9(1), 1–9.

Diwan, A. D., Harke, S. N., & Panche, A. N. (2020). Cryobanking of fish and shellfish egg, embryos and larvae: An overview. Frontiers in Marine Science, 7, 251.

Farlora, R., Hattori-Ihara, S., Takeuchi, Y., et al., (2014). Intraperitoneal Germ Cell Transplantation in the Nile Tilapia Oreochromis niloticus. Marine Biotechnology, 16, 309–320. https://doi.org/10.1007/s10126-013-9551-y

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(e0205481). https://doi.org/10.1371/journal.pone.0205481

Franěk, R., Tichopád, T., Fučíková, M., Steinbach, C., & Pšenička, M. (2019b). Production and use of triploid zebrafish for surrogate reproduction. Theriogenology, 140, 33–43. https://doi.org/10.1016/j.theriogenology.2019.08.016

Franěk, R., Tichopád, T., Steinbach, C., Xie, X., Lujić, J., Marinović, Z., Horváth, Á., Kašpar, V., Pšenička, M., Lujić, J., Horváth, Á., & Pšenička, M. (2019c). Preservation of female genetic resources of common carp through oogonial stem cell manipulation. Cryobiology, 87, 78–85. https://doi.org/10.1016/j.cryobiol.2019.01.016

Franěk, R., & Pšenička, M. (2020). Cryopreservation of Germ Stem Cells in Fish. In Betsy, J., & Kumar, S. (Eds.), Cryopreservation of Fish Gametes. Springer, Singapore. https://doi.org/10.1007/978-981-15-4025-7_12.

Garcia, T., & Hofmann, M.C. (2012). Isolation of Undifferentiated and Early Differentiating Type A Spermatogonia from Pou5f1-GFP Reporter Mice. In Germline Development. Springer, New York, NY, USA, pp. 31–44.

Godoy, L. C., Streit, D. P., Zampolla, T., Bos-Mikich, A., & Zhang, T. (2013). A study on the vitrification of stage III zebrafish (*Danio rerio*) ovarian follicles. Cryobiology, 67, 347–354. https://doi.org/10.1016/j.cryobiol.2013.10.002

Goto, R., & Saito, T. (2019). A state-of-the-art review of surrogate propagation in fish. Theriogenology, 133, 216-227. https://doi.org/10.1016/j.theriogenology.2019.03.032.

Goto-Kazeto, R., Saito, T., Takagi, M., Arai, K., & Yamaha, E. (2010). Isolation of Teleost Primordial Germ Cells Using Flow Cytometry. International Journal of Developmental Biology, 54, 1487–1492.

Guan, M., Rawson, D. M., & Zhang, T. (2008). Cryopreservation of zebrafish (*Danio rerio*) oocytes using improved controlled slow cooling protocols. Cryobiology, 56, 204–208. https://doi.org/10.1016/j.cryobiol.2008.03.001

Guan, M., Rawson, D. M., & Zhang, T. (2010). Cryopreservation of zebrafish (*Danio rerio*) oocytes by vitrification. Cryo-Letters, 31, 230–238.

Hagedorn, M. M., Daly, J. P., Carter, V. L., Cole, K. S., Jaafar, Z., Lager, C. V. A., Parenti, L. R. (2018). Cryopreservation of fish spermatogonial cells: The future of natural history collections. Scientific Reports, 8, 1–11. https://doi.org/10.1038/s41598-018-24269-3

Hayashi, M., Ichida, K., Sadaie, S., Miwa, M., Fujihara, R., Nagasaka, Y., Yoshizaki, G. (2019). Establishment of novel monoclonal antibodies for identification of type A spermatogonia in teleosts. Biology of Reproduction, 101, 478–491.

Higaki, S., Todo, T., Teshima, R., Tooyama, I., Fujioka, Y., Sakai, N., Takada, T. (2018). Cryopreservation of male and female gonial cells by vitrification in the critically endangered cyprinid honmoroko *Gnathopogon caerulescens*. Fish Physiology and Biochemistry, 44, 503–513. https://doi.org/10.1007/s10695-017-0449-x

Higaki, S., Eto, Y., Kawakami, Y., Yamaha, E., Kagawa, N., Kuwayama, M., Nagano, M., Katagiri, S., Takahashi, Y. (2010). Production of fertile zebrafish (*Danio rerio*) possessing germ cells (gametes) originated from primordial germ cells recovered from vitrified embryos. Reproduction, 139, 733–740. doi:10.1530/REP-09-0549

Hong, Y., Liu, T., Zhao, H., Xu, H., Wang, W., Liu, R., Chen, T., Deng, J., Gui, J. (2004). Establishment of a normal medakafish spermatogonial cell line capable of sperm production in vitro. Proceedings of the National Academy of Sciences of the USA, 101, 8011–8016. doi: 10.1073/pnas.0308668101.

Hu, P., Zhang, W., Xin, H., Deng, G. (2016). Single cell isolation and analysis. Frontiers in Cell and Developmental Biology, 4, 116.

Ichida, K., Hayashi, M., Miwa, M., Kitada, R., Takahashi, M., Fujihara, R., Boonanuntanasarn, S., Yoshizaki, G. (2019a). Enrichment of transplantable germ cells in salmonids using a novel monoclonal antibody by magnetic-activated cell sorting. Molecular Reproduction and Development, 86, 1810–1821.

Ichida, K., Kawamura, W., Miwa, M., Iwasaki, Y., Kubokawa, T., Hayashi, M., Yazawa, R., Yoshizaki, G. (2019b). Specific visualization of live type A spermatogonia of Pacific bluefin tuna using fluorescent dye-conjugated antibodies. Biology of Reproduction, 100, 1637–1647.

Ichida, K., Matsushita, Y., Amano, Y., Miwa, M., Nagasawa, K., Hayashi, M., Mizutani, H., Takahashi, M., Boonanuntanasarn, S., Yoshizaki, G. (2021). Visualization and tracking of live type A spermatogonia using a fluorescence-conjugated antibody in Salmo species. Aquaculture, 533, 736096.

Inoue, D., Fujimoto, T., Kawakami, Y., Yasui, G. S., Yamaha, E., Arai, K. (2012). Vitrification of primordial germ cells using whole embryos for gene-banking in loach, *Misgurnus anguillicaudatus*. Journal of Applied Ichthyology, 28, 919–924. https://doi.org/10.1111/jai.12058

Kaul, G., Kumar, S., & Kumari, S. (2012). Enrichment of CD9+ spermatogonial stem cells from goat (*Capra aegagrus hircus*) testis using magnetic microbeads. Stem Cell Discovery, 2, 92–99.

Kawakami, Y., Goto-Kazeto, R., Saito, T., Fujimoto, T., Higaki, S., Takahashi, Y., Arai, K., Yamaha, E. (2010). Generation of germ-line chimera zebrafish using primordial germ cells isolated from cultured blastomeres and cryopreserved embryoids. International Journal of Developmental Biology, 54, 1491–1499. https://doi.org/10.1387/ijdb.093059yk

Kawakami, Y., Ishihara, M., Saito, T., Fujimoto, T., Adachi, S., Arai, K., & Yamaha, E. (2012a). Cryopreservation of green fluorescent protein (GFP)-labeled primordial germ cells with GFP fused to the 3′ untranslated region of the nanos gene by vitrification of Japanese eel (Anguilla japonica) somite stage embryos. Journal of Animal Science, 90, 4256–4265. https://doi.org/10.2527/jas.2011-4884

Kawakami, Y., Saito, T., Fujimoto, T., Goto-Kazeto, R., Takahashi, E., Adachi, S., Arai, K., & Yamaha, E. (2012b). Technical note: Viability and motility of vitrified/thawed primordial germ cell isolated from common carp (*Cyprinus carpio*) somite embryos. Journal of Animal Science, 90, 495–500. https://doi.org/10.2527/jas.2011-4329

Khosla, K., Wang, Y., Hagedorn, M., Qin, Z., & Bischof, J. (2017). Gold nanorod induced warming of embryos from the cryogenic state enhances viability. ACS Nano, 11, 7869–7878. https://doi.org/10.1021/acsnano.7b02216

Kise, K., Yoshikawa, H., Sato, M., Tashiro, M., Yazawa, R., Nagasaka, Y., ... & Yoshizaki, G. (2012). Flow-Cytometric Isolation and Enrichment of Teleost Type A Spermatogonia Based on Light-Scattering Properties. Biology of Reproduction, 86(4). https://doi.org/10.1095/biolreprod.111.093161

Kobayashi, T., Takeuchi, Y., Takeuchi, T., & Yoshizaki, G. (2007). Generation of viable fish from cryopreserved primordial germ cells. Molecular Reproduction and Development, 74, 207–213. https://doi.org/10.1002/mrd

Kobayashi, T., Yoshizaki, G., Takeuchi, Y., & Takeuchi, T. (2004). Isolation of highly pure and viable primordial germ cells from rainbow trout by gfp-dependent flow cytometry. Mol Reprod Dev, 67(1), 91-100. doi:10.1002/mrd.20003

Kumari, K., Maurye, P. (2021). Cryopreservation in Aquaculture. In: Pandey, P.K., Parhi, J. (eds) Advances in Fisheries Biotechnology. Springer, Singapore. https://doi.org/10.1007/978-981-16-3215-0_13

Kunwar, P. S., & Lehmann, R. (2006). Germ-cell attraction. Developmental Biology, 421, 226–227.

Lacerda, S. M. S. N., Batlouni, S. R., Costa, G. M. J., Segatelli, T. M., Quirino, B. R., Queiroz, B. M., Kalapothakis, E., & França, L. R. (2010). A new and fast technique to generate offspring after germ cells transplantation in adult fish: The Nile tilapia (*Oreochromis niloticus*) model. PLoS ONE, 5, e0010740.

Lee, S., Iwasaki, Y., Shikina, S., & Yoshizaki, G. (2013). Generation of functional eggs and sperm from cryopreserved whole testes. Proceedings of the National Academy of Sciences, 110, 1640–1645. https://doi.org/10.1073/pnas.1218468110

Lee, S., Iwasaki, Y., & Yoshizaki, G. (2016a). Long-term (5 years) cryopreserved spermatogonia have high capacity to generate functional gametes via interspecies transplantation in salmonids. Cryobiology, 73, 5–9. https://doi.org/10.1016/j.cryobiol.2016.08.001

Lee, S., Katayama, N., & Yoshizaki, G. (2016b). Generation of juvenile rainbow trout derived from cryopreserved whole ovaries by intraperitoneal transplantation of ovarian germ cells. Biochemical and Biophysical Research Communications, 478, 1478–1483. https://doi.org/10.1016/j.bbrc.2016.08.156

Lee, S., & Yoshizaki, G. (2016). Successful cryopreservation of spermatogonia in critically endangered Manchurian trout (*Brachymystax lenok*). Cryobiology, 72, 165–168. https://doi.org/10.1016/j.cryobiol.2016.01.004

Lee, S., Seki, S., Katayama, N., et al., (2015). Production of viable trout offspring derived from frozen whole fish. Scientific Reports, 5, 16045. https://doi.org/10.1038/srep16045Lee, S., Katayama, N., & Yoshizaki, G. (2016b). Generation of juvenile rainbow trout derived from cryopreserved whole ovaries by intraperitoneal transplantation of ovarian germ cells. *Biochemical and Biophysical Research Communications, 478*,1478–1483. https://doi.org/10.1016/j.bbrc.2016.08.156

Linhartová, Z., Rodina, M., Guralp, H., Gazo, I., Saito, T., & Pšenička, M. (2014). Isolation and cryopreservation of early stages of germ cells of tench (*Tinca tinca*). Czech Journal of Animal Science, 59, 381–390.

Liu, Q. H., Ma, D. Y., Xu, S. H., Xiao, Z. Z., Xiao, Y. S., Song, Z. C., & Li, J. (2015). Summer flounder (*Paralichthys dentatus*) sperm cryopreservation and application in interspecific hybridization with olive flounder (*P. olivaceus*). Theriogenology, 83(4), 703–710.

Liu, Y., Nan, B., Niu, J., Kapler, G. M., & Gao, S. (2021). An optimized and versatile counter-flow centrifugal elutriation workflow to obtain synchronized eukaryotic cells. Frontiers in Cell and Developmental Biology, 9, 905.

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. https://doi.org/10.1016/j.cryobiol.2017.04.005

Lujić, J., Marinović, Z., Bajec, S. S., Djurdjevič, I., Urbányi, B., & Horváth, Á. (2018). Interspecific germ cell transplantation: A new light in the conservation of valuable Balkan trout genetic resources? Fish Physiology and Biochemistry, 44(6), 1487–1498. https://doi.org/10.1007/s10695-018-0510-4

Lujić, J., Franěk, R., Marinović, Z., Kašpar, V., Xie, X., Horváth, Á., Pšenička, M., & Urbányi, B. (2023). Vitrification of the ovarian tissue in sturgeons. Theriogenology, 196, 18–24. https://doi.org/10.1016/j.theriogenology.2022.11.009

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. Scientific Reports, 9(1), 13861.

Marinović, Z., Lujić, J., Kása, E., Bernáth, G., Urbányi, B., & Horváth, Á. (2016). Cryosurvival of isolated testicular cells and testicular tissue of tench *Tinca tinca* and goldfish *Carassius auratus* following slow-rate freezing. General and Comparative Endocrinology. https://doi.org/10.1016/j.ygcen.2016.07.005

Marinović, Z., Lujić, J., Kása, E., Csenki, Z., Urbányi, B., & Horváth, Á. (2018). Cryopreservation of Zebrafish Spermatogonia by Whole Testes Needle Immersed Ultra-Rapid Cooling. Journal of Visualized Experiments, (133), 56118. https://doi.org/10.3791/56118

Matsche, M., Rosemary, K., Brundage, H., & O'Herron, J. (2013). Reproductive demographics, intersex, and altered hormone levels in shortnose sturgeon, *Acipenser brevirostrum*, from Delaware River, USA. Journal of Applied Ichthyology, 29, 299–309. https://doi.org/10.1111/jai.12133

Mazur, P. (1984). Freezing of living cells: Mechanisms and implications. American Journal of Physiology, 247(3 Pt 1), C125–C142. https://doi.org/10.1152/ajpcell.1984.247.3.C125

McLaren, A. (2003). Primordial germ cells in the mouse. Developmental Biology, 262(1), 1–15. https://doi.org/10.1016/S0012-1606(03)00214-8

Morita, T., Miwa, M., Kumakura, N., Morishima, K., Miki, T., Takeuchi, Y., & Yoshizaki, G. (2021). Production of functional sperm from cryopreserved testicular germ cells following intraperitoneal transplantation into allogeneic surrogate in yellowtail (*Seriola quinqueradiata*). Cryobiology, 100, 32-39. https://doi.org/10.1016/j.cryobiol.2021.04.001

Okutsu, T., Suzuki, K., Takeuchi, Y., Takeuchi, T., & Yoshizaki, G. (2006). Testicular germ cells can colonize sexually undifferentiated embryonic gonad and produce functional eggs in fish. Proceedings of the National Academy of Sciences of the USA, 103(8), 2725–2729.

Okutsu, T., Takeuchi, Y., & Yoshizaki, G. (2008). Spermatogonial Transplantation in Fish: Production of Trout Offspring from Salmon Parents. Fisheries for Global Welfare and Environment, 209–219.

Pan, Y., Du, X., Zhao, F., & Xu, B. (2012). Magnetic Nanoparticles for the Manipulation of Proteins and Cells. Chemical Society Reviews, 41, 2912–2942.

Panda, R. P., Barman, H. K., & Mohapatra, C. (2011). Isolation of enriched carp spermatogonial stem cells from *Labeo rohita* testis for in vitro propagation. Theriogenology, 76(2), 241–251. https://doi.org/10.1016/j.theriogenology.2011.01.031

Pegg, DE. (2015) Principles of cryopreservation. Methods Mol Biol. 1257:3-19. doi: 10.1007/978-1-4939-2193-5_1. PMID: 25428001.

Plouffe, B. D., Murthy, S. K., & Lewis, L. H. (2015). Fundamentals and Application of Magnetic Particles in Cell Isolation and Enrichment: A Review. Reports on Progress in Physics, 78(1), 016601.

Pšenička, M., Saito, T., Linhartová, Z., & Gazo, I. (2015). Isolation and transplantation of sturgeon early-stage germ cells. Theriogenology, 83(6), 1085–1092. https://doi.org/10.1016/j.theriogenology.2014.12.010

Pšenička, M., Saito, T., Rodina, M., & Dzyuba, B. (2016). Cryopreservation of early stage Siberian sturgeon Acipenser baerii germ cells, comparison of whole tissue and dissociated cells. Cryobiology, 72, 199–122. https://doi.org/10.1016/j.cryobiol.2016.02.005

Ren, Y., Sun, Z., Wang, Y., Yu, Q., Wang, G., He, Z., Liu, Y., Jiang, X., Kang, X., & Hou, J. (2021). Production of Donor-Derived Offsprings by Allogeneic Transplantation of Oogonia in the Adult Japanese Flounder (*Paralichthys olivaceus*). Aquaculture, 543, 736977.

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(1), 122–130. https://doi.org/10.1016/j.theriogenology.2011.07.024

Rivers, N., Daly, J., Jones, R., et al., (2020). Cryopreservation of testicular tissue from Murray River Rainbowfish, *Melanotaenia fluviatilis*. Scientific Reports, 10, 19355. https://doi.org/10.1038/s41598-020-76378-7

Robles, V., Cabrita, E., & Paz Herráez, M. (2009). Germplasm cryobanking in zebrafish and other aquarium model species. Zebrafish, 6(3), 281–293. https://doi.org/10.1089/zeb.2009.0592

Rosa, I. F., Martinez, E. R. M., Digmayer, M., Doretto, L. B., Nóbrega, R. H. (2023). Successful Cryopreservation of Spermatogonia Stem Cells of Neotropical Catfish (Rhamdia quelen) and Enriched Germ Cell Transplantation into Common Carp (*Cyprinus carpio*) Testes. Fishes, 8(10), 478. https://doi.org/10.3390/fishes8100478

Ryu, J. H., & Gong, S. P. (2020). Enhanced Enrichment of Medaka Ovarian Germline Stem Cells by a Combination of Density Gradient Centrifugation and Differential Plating. Biomolecules, 10, 1477.

Saito, T., Fujimoto, T., Maegawa, S., Inoue, K., Tanaka, M., Arai, K., & Yamaha, E. (2006). Visualization of primordial germ cells in vivo using GFP-nos1 3'UTR mRNA. International Journal of Developmental Biology, 50(8), 691-9. https://doi.org/10.1387/ijdb.062143ts

Saito, T., Goto-Kazeto, R., Arai, K., & Yamaha, E. (2008). Xenogenesis in teleost fish through generation of germ-line chimeras by single primordial germ cell transplantation. Biology of Reproduction, 78, 159–166. https://doi.org/10.1095/biolreprod.107.060038

Saito, T., Goto-Kazeto, R., Fujimoto, T., Kawakami, Y., Arai, K., & Yamaha, E. (2010). Inter-species transplantation and migration of primordial germ cells in cyprinid fish. International Journal of Developmental Biology, 54, 1479–1484. https://doi.org/10.1387/ijdb.103111ts

Saito, T., & Psenicka, M. (2015). Novel Technique for Visualizing Primordial Germ Cells in Sturgeons (*Acipenser ruthenus, A. gueldenstaedtii, A. baerii*, and *Huso huso*). Biology of Reproduction, 93, 1–7.

Sato, T., Katagiri, K., Kubota, Y., & Ogawa, T. (2013). In Vitro Sperm Production from Mouse Spermatogonial Stem Cell Lines Using an Organ Culture Method. Nature Protocols, 8, 2098–2104.

Seki, S., Kusano, K., Lee, S., Iwasaki, Y., Yagisawa, M., Ishida, M., Hiratsuka, T., Sasado, T., Naruse, K., & Yoshizaki, G. (2017). Production of the medaka derived from vitrified whole testes by germ cell transplantation. Scientific Reports, 7, 43185. https://doi.org/10.1038/srep43185

Shang, M. (2013). Isolation, identification, culture, cryopreservation, genetic transformation and transplantation of catfish germ line stem cells. PhD Thesis, Auburn University.

Shikina, S., Ihara, S., & Yoshizaki, G. (2008). Culture conditions for maintaining the survival and mitotic activity of rainbow trout transplantable type A spermatogonia. Molecular Reproduction and Development, 75(3), 529-37. https://doi.org/10.1002/mrd.20771

Shikina, S., Nagasawa, K., Hayashi, M., Furuya, M., Iwasaki, Y., & Yoshizaki, G. (2013). Short-Term in Vitro Culturing Improves Transplantability of Type A Spermatogonia in Rainbow Trout (*Oncorhynchus mykiss*). Molecular Reproduction and Development, 80, 763–773.

Starz-Gaiano, M., & Lehmann, R. (2001). Moving towards the next generation. Mechanisms of Development, 105, 5–18. https://doi.org/10.1016/S0925-4773(01)00392-6

Tajima, A., Naito, M., Yasuda, Y., & Kuwana, T. (1993). Production of germ line chimera by transfer of primordial germ cells in the domestic chicken (Gallus domesticus). Theriogenology, 40(3), 509–519.

Takeuchi, Y., Yoshizaki, G., & Takeuchi, T. (2003). Generation of live fry from intraperitoneally transplanted primordial germ cells in rainbow trout. Biology of Reproduction, 69(4), 1142–1149.

Takeuchi, Y., Yoshizaki, G., & Takeuchi, T. (2004). Surrogate broodstock produces salmonids. Nature, 430, 629–630. https://doi.org/10.1038/430629a

Thorgaard, G.H., Scheerer, P.D., Hershberger, W.K., & Myers, J.M. (1990). Androgenetic rainbow trout produced using sperm from tetraploid males show improved survival. Aquaculture, 85(1–4), 215–221.

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(2), 290–303. https://doi.org/10.1016/j.theriogenology.2010.02.013

Wong, T.-T., Saito, T., Crodian, J., & Collodi, P. (2011). Zebrafish germline chimeras produced by transplantation of ovarian germ cells into sterile host larvae. Biology of Reproduction, 84(6), 1190–1197. https://doi.org/10.1095/biolreprod.110.088427

Wylie, M. J., Kitson, J., Russell, K., Yoshizaki, G., Yazawa, R., Steeves, T. E., & Wellenreuther, M. (2023). Fish germ cell cryobanking and transplanting for conservation. Molecular Ecology Resources, 00, 1–8. https://doi.org/10.1111/1755-0998.13868

Xie, X., Nóbrega, R., & Pšenička, M. (2020). Spermatogonial Stem Cells in Fish: Characterization, Isolation, Enrichment, and Recent Advances of In Vitro Culture Systems. Biomolecules, 10(4), 644. https://doi.org/10.3390/biom10040644

Xu, H.-Y., Hong, X.-Y., Zhong, C.-Y., Wu, X.-L., & Zhu, X.-P. (2022). Restoring Genetic Resource through In Vitro Culturing Testicular Cells from the Cryo-Preserved Tissue of the American Shad (*Alosa sapidissima*). Biology, 11, 790. https://doi.org/10.3390/biology11050790

Yano, A., Suzuki, K., & Yoshizaki, G. (2008). Flow-Cytometric Isolation of Testicular Germ Cells from Rainbow Trout (*Oncorhynchus mykiss*) Carrying the Green Fluorescent Protein Gene Driven by Trout vasa Regulatory Regions. Biology of Reproduction, 78(1), 151–158.

Yoshikawa, H., Xu, D., Ino, Y., Yoshino, T., Hayashida, T., Wang, J., Yazawa, R., & Yoshizaki, G. (2018). Hybrid sterility in fish caused by mitotic arrest of primordial germ cells. Genetics, 209(3), 507–521. https://doi.org/10.1534/genetics.118.300777

Yoshikawa, H., Morishima, K., Fujimoto, T., Saito, T., Kobayashi, T., Yamaha, E., & Arai, K. (2009). Chromosome Doubling in Early Spermatogonia Produces Diploid Spermatozoa in a Natural Clonal Fish. Biology of Reproduction, 80(6), 973–979.

Yoshizaki, G., & Lee, S. (2018). Production of live fish derived from frozen germ cells via germ cell transplantation. Stem Cell Research, 29, 103–110. https://doi.org/10.1016/j.scr.2018.03.015

Yoshizaki, G., Takeuchi, Y., Kobayashi, T., & Takeuchi, T. (2003). Primordial germ cell: A novel tool for fish bioengineering. Fish Physiology and Biochemistry, 28(1-4), 453–457. https://doi.org/10.1023/B:FISH.0000030628.91607.2d

Yoshizaki, G., Ichikawa, M., Hayashi, M., Iwasaki, Y., Miwa, M., Shikina, S., & Okutsu, T. (2010). Sexual Plasticity of Ovarian Germ Cells in Rainbow Trout. Development, 137(8), 1227–1230.

Zhang, T., Rawson, D.M., & Morris, J. (1993). Cryopreservation of pre-hatch embryos of zebrafish (*Brachydanio rerio*). Aquatic Living Resources, 6(2), 145–153.

Zhu, B., & Murthy, S.K. (2013). Stem Cell Separation Technologies. Current Opinion in Chemical Engineering, 2, 3–7.

Zou, K., Hou, L., Sun, K., Xie, W., & Wu, J. (2011). Improved efficiency of female germline stem cell purification using fragilis-based magnetic bead sorting. Stem Cells and Development, 20(12), 2197–2204.