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Development of germline stem cell manipulation technologies for improved conservation of the European eel

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List of abbreviations

AI – artificial insemination
ANOVA – analysis of variance
BSA – bovine serum albumin
CF – correction factor
DAB – 3,3′-diaminobenzidine
EG – ethylene glycol
ES – equilibration solution
EtOH – ethanol
FBS – fetal bovine serum
Gly – glycerol
GSC – germline stem cell
GSI – gonadosomatic index
hCG – human chorionic gonadotropin
HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
hpf – hours post-fertilization
IVF – *in vitro* fertilization
KD – gene knock-down
L-15 – Leibovitz L-15 culture medium
lN_2 – liquid nitrogen
mDF – modified Davidson's fixative
Me_2SO – dimethyl sulfoxide
MeOH – methanol
MO – morpholino oligonucleotide
N – number of biological replicates (individuals) used in trials
NBF – neutral buffered formalin
NIV – needle immersed vitrification
OSC – oogonial stem cell
PBS – phosphate buffered saline
PG – propylene glycol
PGC – primordial germ cell
RT – room temperature
SSC – spermatogonial stem cell
TB – trypan blue
VS – vitrification solution
WS – warming solution

1 INTRODUCTION

The European eel (*Anguilla anguilla*) is currently listed as critically endangered by the International Union for Conservation of Nature (IUCN; Pike et al., 2020). Over the past few decades, the population of this species has experienced a dramatic decline, estimated at over 90%. This alarming decrease is largely attributed to a combination of factors including overfishing, habitat loss, pollution, and barriers to migration such as dams and waterway modifications which hinder their natural lifecycle of spawning and returning to the sea. Additionally, the European eel is susceptible to a parasitic nematode, *Anguillicola crassus*, which damages their swim bladder, critically impairing their swimming abilities and survival rates (Molnár et al., 1991).

In response to the critical endangerment of the European eel, numerous conservation efforts have been initiated across various levels. These include strict regulations on fishing, habitat restoration projects, and the installation of eel ladders and passageways that facilitate their migration across water barriers. European Union regulations now mandate member states to develop and implement Eel Management Plans, which are crucial for the species' recovery (Hanel et al., 2019). These plans aim to ensure that at least 40% of adult eels can escape to the ocean to breed, a significant step given their complex life cycle that spans across continents and requires precise environmental conditions to trigger breeding behaviors.

Preserving the European eel is significant not only for maintaining biodiversity but also for the ecological balance of freshwater and marine ecosystems where they play a critical role as both predator and prey. Their presence influences the structure and function of aquatic ecosystems. Additionally, European eels have considerable cultural and economic importance in many European countries, where they are a traditional food source and contribute to local fisheries economies. Protecting this species is thus an integral part of sustaining natural heritage and economic stability in these regions, aligning environmental conservation with socioeconomic benefits. Therefore, novel conservation and management plans and efforts are desperately needed for the conservation of this valuable species.

2 AIMS

The main objective of this dissertation was to develop and adapt the surrogate broodstock technology for the conservation of the European eel. This was done through developing several critical steps for the manipulation of both male and female germline stem cells (GSCs) – spermatogonial stem cells (SSCs) and oogonial stem cells (OSCs). These critical steps were identified as sub-aims of the dissertation, and included:

- Identification of SSCs and OSCs within the gonadal tissue through histological methods;
- Development of the optimal isolation techniques for both SSCs and OSCs;
- Development of optimal short-term (hypothermic storage) and long-term (cryopreservation through slow-rate freezing and vitrification) preservation methods for both male and female gonadal tissues;
- Testing of the functionality of frozen/thawed SSCs through transplantation into common carp *Cyprinus carpio* recipients.

3 LITERATURE OVERVIEW

3.1 Description of the species

3.1.1 Taxonomy and morphology

The European eel *Anguilla anguilla* is a representative of freshwater eels. It is a teleost fish with an elongated body that goes through considerable development and changes from early larval stages to the adult stage characteristic for most of the Anguilliform species. The exact classification of this species is the following:

Kingdom	Animalia
Phylum	Chordata
Subphylum	Vertebrata
Superclass	Gnathostomata
Class	Actinopterygii
Infraclass	Teleostei
Order	Anguilliformes
Suborder	Anguilloidei
Family	Anguillidae
Genus	*Anguilla*
Species	*Anguilla anguilla*

Anguilliform fish, or fish belonging to the Order Anguilliformes, consist of 8 suborders: Protoanguilloidei, Synaphobranchoidei, Muraenoidei, Chlopsoidei, Congroidei, Moringuoidei, Saccopharyngoidei and Anguilloidei and the common name for all of them is eel. There are approximately 800 species in this order. The genus *Anguilla* is one of 111 genera in this order, comprises of 15 species of fresh-water eels distributed worldwide (Figure 1; Inoue et al., 2010). Similar was more recently observed by Tsukamoto et al. (2020) where the authors revised the common names for species and subspecies of genus *Anguilla* according to the International Code of Zoological Nomenclature and determined the existence of 19 species and subspecies of this genera.

The European eel *Anguilla anguilla* (Linnaeus, 1758) can also be found under the synonym name *Muraena anguilla* (Linnaeus, 1758). The name "eel" has a mixed origin, partially descends from the Old English æl, and Common Germanic *ēlaz, but it is also be related to the common Germanic West Frisian iel, Dutch aal, German Aal, and Icelandic áll. Besides its most common name, there are some other names, such as Common eel, River eel,

Glass eel, Silver eel, Weed eel, etc. (Froese and Puley, 2024). Those names are usually related to a certain developmental stage or the habitat of the European eel.

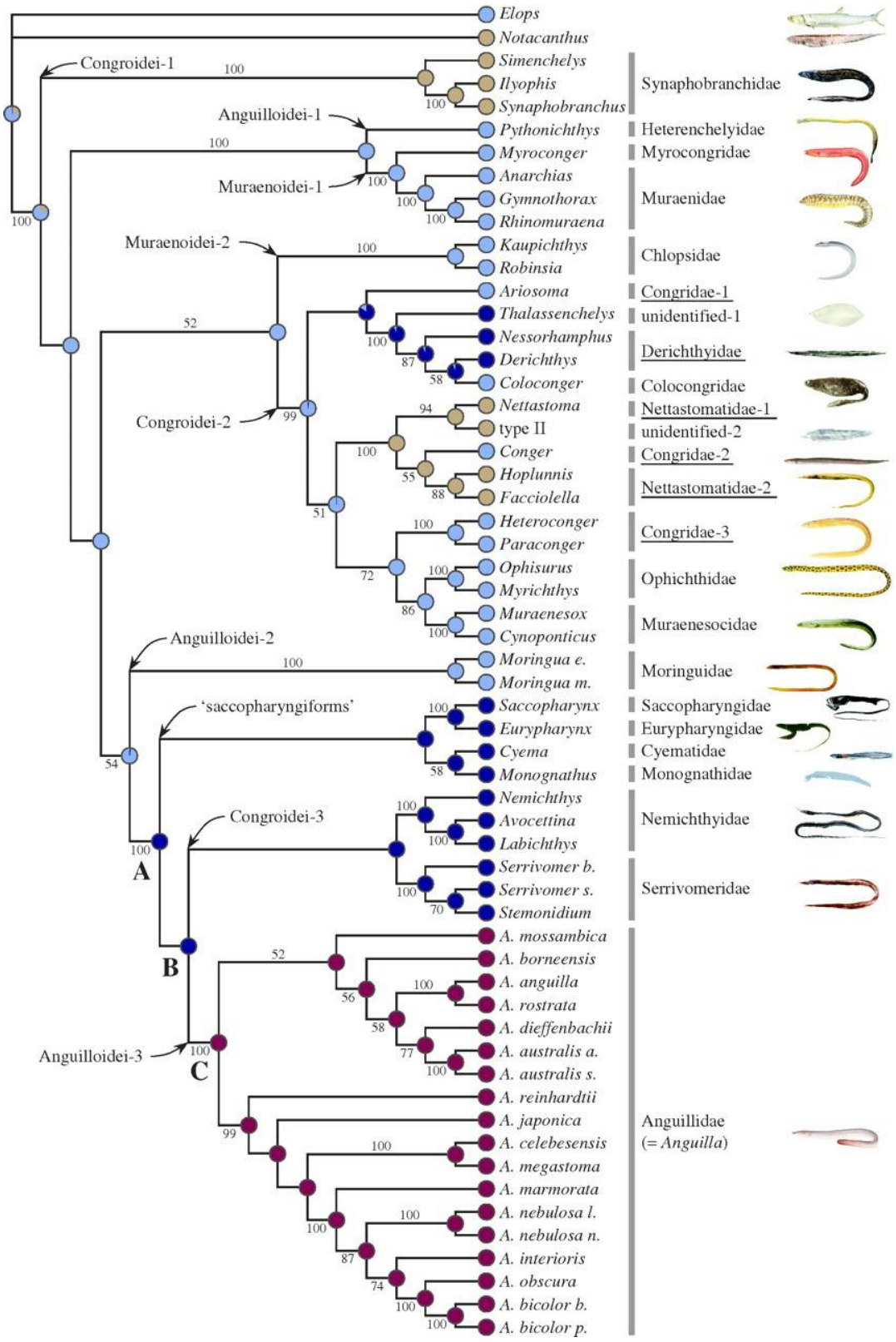

Figure 1. Classification of 56 anguilliforms representing all 15 anguilliform families based on whole mitogenome sequences. Cited from Inoue et al. (2010) with permission from the publisher.

The European eel is a fish with an elongated, cylindrical body. It has small vertical gill openings and a slightly compressed posterior end (Bauchot, 1986, cited from U.S. Fish and Wildlife Service, 2019). The upper jaw is slightly shorter than the lower one. It has one pair of pectoral fins with 15-21 rays, and no pelvic fins. The dorsal fin starts far behind the pectoral fins and is combined with the anal fin, which rounds off inferiorly in the caudal region of the body. This unique fin has more than 500 soft rays in total, including 243-275 dorsal rays, 175-249 anal rays, and 7-12 caudal rays (Berg, 1964, cited from U.S. Fish and Wildlife Service, 2019). The vertebrae count is 110-120, and there are no spines in the dorsal and anal fins.

Although the European eel exhibits sexual dimorphism, it can be challenging to distinguish between males and females based on external characteristics alone. This is because their physical appearance undergoes significant changes during their life cycle. During the juvenile (yellow) and early adult (silver) stages, it is almost impossible to differentiate between the sexes. It is only when they reach sexual maturity, and prepare for migration and spawning season, that the differences become more apparent. At this stage, females have a thicker body with a more rounded snout, while males remain smaller with a more pointed snout and significantly larger eyes. The average length of a female is between 80 and 100 cm, weighing from 400 to 1000 grams, while males range from 30 to 40 cm in length and 80 to 180 grams in weight. In captivity, the European eel can live for about 55 years, while in nature their lifespan ranges between 7 and 85 years depending on their living conditions.

3.1.2 Ecology and habitat preference

The European eel is able to survive in a variety of aquatic habitats with varying ecological conditions including salinity, temperature, and oxygen levels. While the natural habitat of the species only includes waters connected to the sea (Kottelat and Freyhof, 2007), humans have introduced the eel to many other closed habitats. This particular fish species can be found in various bodies of water such as oceans, seas, rivers, streams, ponds, lakes, and brackish water. It typically prefers to live in benthic habitats, and due to its nocturnal nature, it spends most of the day hiding under rocks or burrowed in the mud, only becoming active at dusk. This fish has low food requirements, and its diet mainly consists of marine and freshwater invertebrates such as mollusks and crustaceans, small fish, or even small dead aquatic animals (Maitland, 2003; Sinha et al., 1975). There is evidence to suggest that the European eel is capable of crawling over mud or grass in search of freshwater or terrestrial invertebrates. They

can stay out of water for more than 12 hours, as reported by Deelder (1984). During most of the cold months, these eels hibernate without food (Froese and Puley, 2024).

The European eel is a species of fish that predominantly lives in the dark, usually on the bottom of water bodies where they are protected from direct light. It prefers areas with rocky landslides, bridge pillars, grids, and dark places where it can easily hide. It also enjoys rivers with clay and muddy bottoms. The eel can find good habitat in areas with submerged trees, tangled roots, aquatic plants, or hollow steep banks. These areas provide good shelter for the eel and can also serve as an ambush spot for waiting for the prey.

Although European eels have a clear ecological niche, their preferences vary depending on factors such as their body size, age, sex, climate, and the global environment. Smaller eels (measuring less than 30 cm in total body length) tend to prefer shallow water and avoid high water velocity (higher than 0.1 m/s). They are adept at maneuvering through submersed aquatic macrophytes, which are areas with dense vegetation where larger eels cannot reach. On the other hand, larger eels usually struggle to navigate through the vegetation and are found in deeper waters or along the edges of vegetation masses. Adult female eels prefer to swim to the upper reaches of freshwater streams, while males usually remain in brackish water at the mouth. However, both sexes can be found up to 1000 m above sea level in their search for food. During extremely dry or cold winter seasons as well as hot summers, eels can burrow into the mud, sometimes up to 40 cm deep, and stay there for a couple of days to survive unfavorable conditions.

When eels are ready to mate, they leave fresh water and travel to the sea. On their way through freshwaters, they will overcome any obstacle, including high waterfalls or other barriers, just to enter the sea. Eels usually rest during the day, hidden in mud, water plants, or cavities, while they tend to be more active at night, mostly hunting for food. They are not picky eaters and will consume almost anything, including small fish and their eggs, river crabs and snails, frogs, worms, and insects. They become more careful and aggressive against prey when food is scarce, becoming true predators. Their food intake decreases during mating seasons and when migrating downstream. When they reach the sea, they require less food, and their intestines tend to degenerate. They are highly adaptable and can survive in less-than-ideal conditions compared to some other fish species. They thrive in a diverse environment with plenty of prey and a healthy river ecosystem. Their ability to easily adapt to various environments is a key factor in their survival and overcoming obstacles in the last few decades. However, this adaptability may have a significant impact on their future survival.

The natural distribution of this species entails a vast area that stretches along the Atlantic coast of Scandinavia to Morocco, including all rivers that belong to the North Atlantic basin. They are also present in the Baltic and Mediterranean Seas, as well as along the coast of Europe. Their habitat extends also from the Black Sea to the White Sea (Figure 2; Froese and Puley, 2024; Pike et al., 2020).

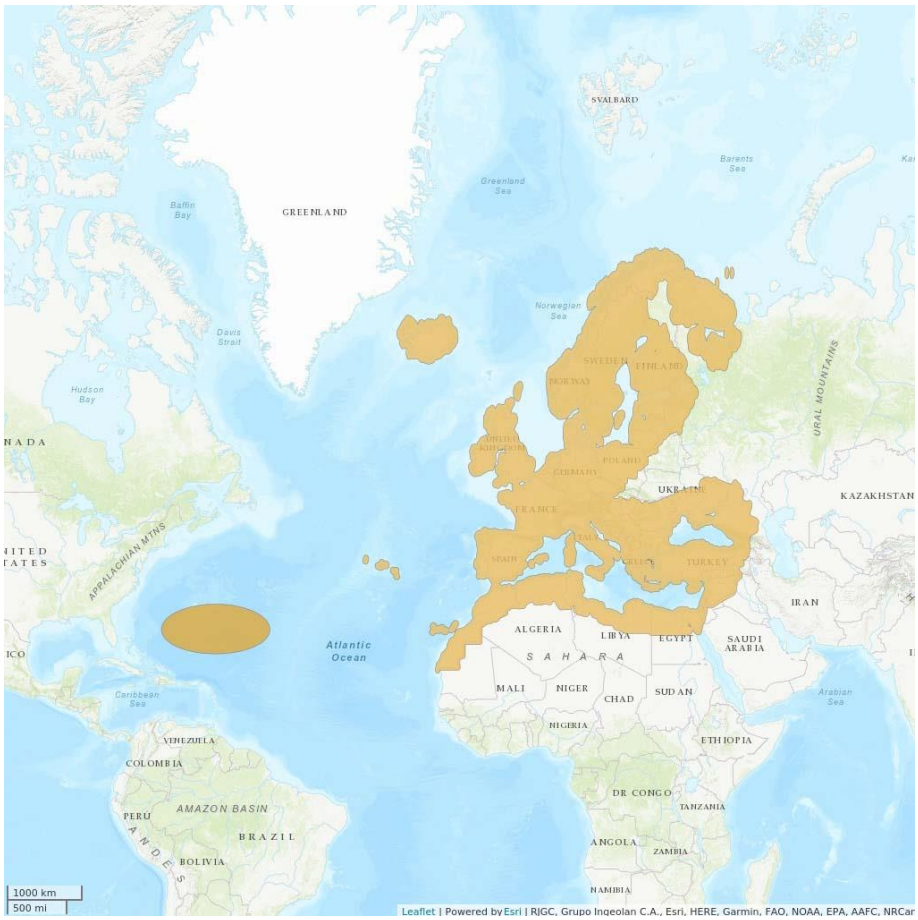

Figure 2. A map displaying the distribution of European eel (orange). Cited from Pike et al. (2020).

3.2 Reproduction and development

3.2.1 Reproduction

European eels have been known as a catadromous species (Cresci, 2020; van Ginneken and Maes, 2005). They spawn and are born in salty water, but spend the majority of their lives in fresh water. It is known that eels engage in a mating ritual in the Sargasso Sea from February to April each year, but no one has yet been able to fully research it. While it is impossible to spawn European eels in captivity, protocols have been developed for some related species, such as *Anguilla japonica*.

Eels mature differently based on their sex, body size, and environment. Males usually mature between 4 to 14 years, while females take 10 to 14 years to mature (Cresci, 2020). Size also plays a role in this process; males typically mature after reaching a total body length of 50 cm, while females are usually around 1.5 m long. Once they are ready for breeding, individuals of both sexes become extremely voracious and eat until they have enough fat deposits for the upcoming migration. At this point, they start their journey to the Sargasso Sea, their mating site. After a long and exhausting trip, they descend to a depth of 100-300 m to spawn in warmer water with higher salinity and pressure. The fertilized eggs are released and rise to the surface. As a small larva, known as "leptocephalus", they begin their journey back to Europe, trying to reach their parents' previous residence (van Ginneken and Maes, 2005). These larvae hatch along the Gulf Stream and reach the European shore after 2 to 3 years, in a very small size of 6 to 7 cm long. Males usually stay in brackish water or the first kilometer of the river, while females, who have stronger and bigger bodies, will try to reach further distances into freshwater but no more than 1000 m above the sea level.

3.2.1.1 Early gonadal development

The early gonadal development of the European eel is very similar to many other fish species. The first differentiated germline cells are the primordial germ cells (PGCs). These cells originate outside of the gonad and have to migrate towards it guided by chemoattractants (Saito et al., 2011). Once PGCs reach the gonadal ridges (the primordial gonad), they incorporate into it, proliferate, and subsequently start to differentiate into male germline cells called spermatogonial stem cells (SSCs) or into female germline cells called oogonial stem cells (OSCs). In the European eel, gonads are considered to be sexually undifferentiated (since they contain predominantly PGCs) until the elver size of around 20 cm (Colombo and Grandi, 1996; Grandi and Colombo, 1997).

In larger elvers of approximately 20 to 22 cm, PGCs start to slowly differentiate into both SSCs and OSCs (Colombo and Grandi, 1996). SSCs are approximately the same size as PGCs, however, they are darker, usually organized in rows and are surrounded by large polymorphic cells that will differentiate into Sertoli cells. OSCs and very early-stage oocytes are organized in cysts enveloped by a layer of flattened dark cells (future granulosa cells). Oocytes on the other hand are of lighter color, have a round nucleus and visible chromosomal threads. The most striking observation in the gonads of this stage is that all early-stage germline cells are present at the same time – PBCs, SSCs and OSCs and early oocytes. This indicates

that the European eels have juvenile hermaphroditic gonads and that they are “undifferentiated” gonochoristic teleosts.

At sizes of 22 to 25 cm, three types of gonads can be identified (Colombo and Grandi, 1996). The first type is similar to the previously described gonads of smaller elvers. The second type also contains both male and female germline cells, but they are organized in separate areas; OSCs and early oocytes form larger areas of oogonial/oocyte cysts while rows of SSCs and PGCs organize male areas. These two types are called the Syrski organs. The third type is predominantly female and contains only female germline cells including larger primary oocytes. As the elvers grow, the gonads continue to develop and differentiate. Syrski organs usually start to show features of degenerating and dying oocytes and further differentiate into male gonads, while the type 3 immature gonads continue to develop and grow into female gonads. As the elvers grow past 30 cm in length, they now have fully differentiated male and female gonads, i.e., there is a clear distinction between the early testes and early ovaries. Early testes retain the lamellar structure and for the most part contain only SSCs, while the early ovaries have a lobular structure and contain OSCs and different types of early-stage oocytes (Colombo and Grandi, 1996; Grandi and Colombo, 1997).

3.2.1.2 Ovarian structure

Oogenesis begins with the proliferation of oogonial stem cells (OSCs). After intense initial proliferation, some of the oogonia commit to differentiation, and start changing in size and developing into primary oocytes. Initially, OSCs usually have one nucleolus and chromosomes are distributed throughout the nucleus. During the primary oocyte stage (specifically during zygotene), chromosomes start to aggregate on the opposite side of the nucleolus (Elkouby and Mullins, 2017). During pachytene, nucleolus moves back to its central position, and the chromosomes are spread throughout the nucleus. Subsequently, the nucleus starts to increase in size and becomes the germinal vesicle, while nucleoli become smaller and move to the periphery. This is the diplotene stage. During this stage, follicle cells surround the oocytes and become visible on histological slides. Early prophase oocytes are mainly located at the medial side of the ovary, while later prophase oocytes form one or two rows running from the medial side to the lamellae on the lateral side (Beullens et al., 1997).

During further development, oocytes continue to grow mostly through yolk accumulation (Beullens et al., 1997). There are three kinds of yolk: yolk vesicles, yolk globules, and oil droplets, and their appearance varies by species. Once the yolk becomes conspicuous,

the inner and outer theca membranes are formed by squamous cells. They line up into two layers outside the follicle cell layer. Thus, the zona radiate is formed, and radial striation is evident. Once vitellogenesis is completed, the germinal vesicle moves and starts grouping with yolk globules and oil droplets. This increases egg diameter, and the egg can absorb high water, so the weight increases as well. The first meiotic division occurs once the germinal vesicle moves to the animal pole, triggering the release of the first polar body. Then, the second meiotic division occurs, and the egg is released. The eggs are arrested in metaphase.

According to Lokman and Young (1998), anguillid oocytes can be classified into 10 stages based on their morphological appearance:

I. Oogonia – the ovarian stem cells;
II. Previtellogenic oocytes – early-stage oocytes with a basophilic cytoplasm and the chromatin organized in a nucleolar or perinuclear structure;
III. Cortical alveolar stage – characterized by a less basophilic cytoplasm and the presence of oil droplets;
IV. Early-vitellogenic oocytes – they contain peripheral yolk granules;
V. Mid-vitellogenic oocytes – contain both peripheral and central yolk granules;
VI. Late-vitellogenic oocytes – peripheral yolk granules begin to fuse;
VII. Migratory nucleus-stage oocyte – the process of oocyte maturation has started and the nucleus is moving towards the periphery;
VIII. Ovulated egg;
IX. Post-ovulatory follicle;
X. Atretic follicle – process of follicle resorption.

Oocyte growth is a complex process that is regulated by various hormones. Among these hormones, gonadotropin (GtH), thyroxine, triiodothyronine, growth hormone, insulin, and insulin-like factors (IGFs) play a crucial role (Aroua et al., 2006). These hormones act directly, indirectly, or synergistically to support oocyte growth. GtH I is known to support vitellogenesis, while GtH II plays a role in the ovulation of mature oocytes (Querat et al., 1991). Both hormones can stimulate steroid secretion in cultured follicles (Jéhannet et al., 2023). However, the presence of GtH II in plasma is only detectable very close to ovulation (Querat et al., 1991). Conversely, GtH I levels increase during vitellogenesis and regulate the production of estradiol-17β. Additionally, GtH I stimulates the production of vitellogenin (VTG) and chorion proteins. The release of GtH is controlled by stimulation and inhibition of neuroendocrine regulation. The hypothalamus produces gonadotropin-releasing hormone (GnRH), which stimulates the regulation of GtH. GnRH cells have a connection with GtH cells

in the hypophysis. The release of GnRH is stimulated by environmental spawning conditions and pheromones.

3.2.1.3 Testicular structure

In male eels, the testis is a paired organ composed of numerous lobules separated by a thin layer of fibrous connective tissue. Each lobule contains SSCs that undergo mitotic divisions to either clonally proliferate, or commit to differentiation and form differentiated spermatogonia type A (Schulz et al., 2010). The process of spermatogenesis can be divided into three stages:

1. Proliferative phase – SSCs commit to differentiation and proliferate to form differentiated type A spermatogonia to late type B spermatogonia;
2. Meiotic phase – late type B spermatogonia enter meiosis and ultimately form secondary spermatocytes;
3. Spermiogenesis – the process of final differentiation into fully functional spermatozoa.

At the early stage, SSCs are large oval cells containing a large nucleus. As the cells progress through, they become smaller and round (Beullens et al., 1997). The primary spermatocyte is formed from proliferated spermatogonia (Schulz and Nobrega, 2011a, 2011b). In the leptotene and zygotene stages of the first meiotic division, the chromosomes spread at one end of the nucleus and the nucleolar membrane disappears. Each chromosome becomes thicker and shorter. Primary spermatocytes then develop into secondary spermatocytes by the second meiotic division and undergo the maturation process. In this stage, the secondary spermatocytes become a spermatids which will later develop into spermatozoa (Schulz et al., 2010). Sertoli cells, interstitial cells, and Leyding cells are somatic cells present in the testis. Sertoli cells provide nutrients for the germ cells while Leyding cells secrete sex steroids (Schulz and Nobrega, 2011a, 2011b). Spermatozoa have a large oblong head connected eccentrically to the basal end of the flagellum. The head of spermatozoa is elongated with a long narrow (sickle-like) and a rootlet projects from the neck (Marco-Jiménez et al., 2006). The construction of the flagellum is made up of outer dynein arms, radial spokes, and an absence of central structure. In contrast, the inner structure is similar to other vertebrates.

3.2.2 Development

The eel has a complex biological cycle that includes two metamorphoses (Figure 3; Cresci, 2020). The first metamorphosis is when the larvae turn into glass eel stage, and the

second one is when yellow eels transform into silver eels. The eel's lifecycle can be divided into four stages (Cresci, 2020; van Ginneken and Maes, 2005). The first stage is when the larvae travel for about a year across the Atlantic to the European coasts. The second stage is when the glass eels swim into the estuaries along the European coast. The third stage is the longest and is known as the feeding stage or yellow eel stage, which can last from 4 to 20 years in some cases. The fourth and final stage is the second metamorphosis from yellow eel stage to silver eel stage, which lasts about 6 months. During this period, the fish go back downstream into the seas and oceans for the spawning season.

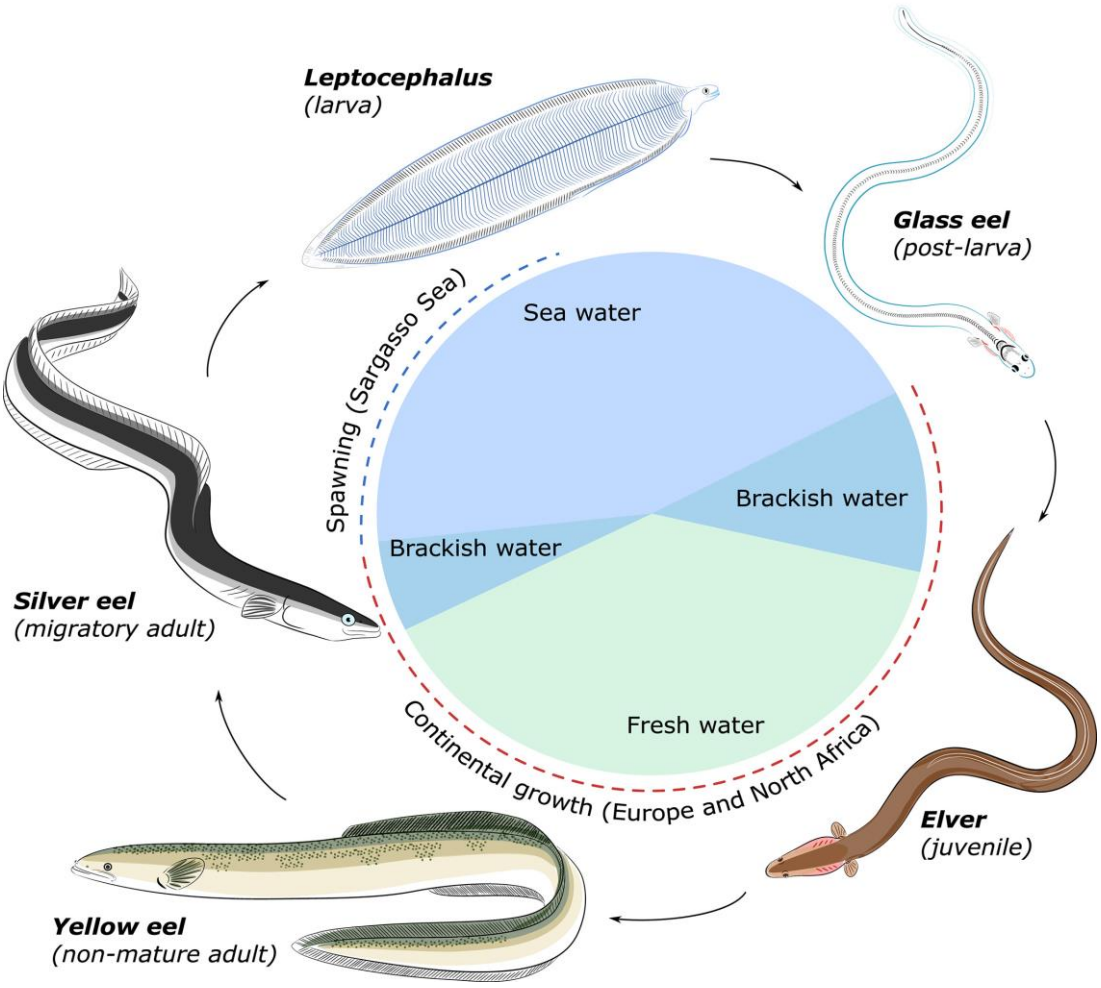

Figure 3. Life history of the European eel (*Anguilla anguilla*). Cited from Cresci (2020) with permission from the publisher.

Larvae stage

The European eel's larvae stage is called leptocephalus and is found only in the ocean. Leptocephali transport is the first mystery in the life cycle of eels. The complexity arises from the unknown and unstudied Sargasso Sea, including its physical environment, which

contributes to the Gulf Stream and its transportation through recirculation, meandering, or eddy formations (McCleave, 1993). Despite the first observations of the natural transportation of larvae by Schmidt in 1925 (cited from McCleave, 1993), there are still many unanswered questions that require further research. According to McCleave and Kleckner (1987), there are no vertical migrations for larvae smaller than 5 mm. They excluded active swimming based on their morphological parameters since, in that size, they are no more than 7 days old. Larvae measuring between 7-15 mm could be found at depths of 75-300 m, while those measuring 25 mm could be found up to 50 m deep. During the day, larvae have been found at deeper layers than during the night. This pattern of vertical migration indicates the need for larvae to dive deeper during the day, possibly to avoid light intensities (Tesch and Wegner, 1990).

The Sargasso Sea is a place where one more eel species (the American eel, *Anguilla rostrata*) spawns during the same time. This poses a mystery regarding the stream force and transportation of the larvae to two different continents for varying periods. There are two theories regarding the migration of the larvae; one suggests a passive oceanic transport, while the other explains it through active processes that include metamorphosis and take up to 9 months. Interestingly, both theories cannot explain the 1% finding of American eel larvae on the coasts of Europe. The area of the Sargasso Sea presents a considerable geographical overlap for both species. It provides an opportunity for hybridization (Als et al., 2011), especially after the discovery of hybrids in Iceland (Albert et al., 2006; Avise et al., 1990). The first molecular population genetic study of 21 microsatellite loci in larvae of both species revealed that the only confirmed fact is panmixia, based on genetic differentiation, isolation by distance, isolation by time, and assignment tests (Als et al., 2011).

Glass eel – elvers

The first metamorphosis in the eel life cycle occurs after the larval stage. The duration of this stage is not yet precisely determined, but it usually lasts between 4 to 8 weeks. Once they completely leave the area of the Sargasso Sea, the larvae undergo morphological and physiological changes. During their first metamorphosis, the eel larvae take on an elongated shape (eel-like shape), but their body stays transparent. This stage is known as the glass eel or elver stage. During this stage, the elvers become ready to swim upstream. The metamorphosis changes make them ready to feed, and as a result, larval teeth are replaced by adult teeth, and the digestive tract is reorganized (Dufour et al., 1993). During the later phases of this stage, their body goes through the process of pigmentation process.

Yellow eel

The yellow stage in the eel's biological cycle is also known as the adult or feeding stage. It is the longest phase of its life, lasting anywhere from 5 to 25 years (Cresci, 2020; Durif et al., 2009). During this period, the eel's body is yellow-green in color without any sheen. The color of the upper part of its body can vary from grey, brown, green, and yellow, while the lower parts are white or grey (Moriarty, 2003). The head of the yellow eel is small and has a large mouth and a couple of rows of teeth in its jaws. The gill gaps are tight and covered by small flaps. The eel may have two types of head forms depending on its feeding habits and habitat, either wide or narrow-headed (Moriarty, 2003). This stage is essential for the eel's growth and preparation for its future journey and silvering process. During this period, the eel grows to a size suitable for silvering and contains enough fat for the onset of the second metamorphosis, which will enable it to start its reproduction trip. The yellow eel can be found in both salty and fresh water and can spend varying amounts of time. The exact time spent in a certain ecosystem can be determined by the ratio of strontium and calcium in the otolith.

Silver eel – silverling

Silvering is a process that is considered to be a second metamorphosis, and it is a complex phenomenon. Silvering is a requirement and a trigger for beginning the reproduction cycle. Once the silvering begins, silver eels are ready for their downstream migration and reproduction journey. This return trip to the Sargasso Sea involves a long metamorphosis process that includes various physiological processes that provide all the necessities for the eel's body to survive and successfully mate.

During the silver stage, some changes can be grouped into substages. The first stage is the actively feeding silver eel, which occurs before the metamorphosis begins. Then there is the present developmental stage between the yellow and silver phases. After that, the adult pre-migration stage follows, during which eels undergo changes in eye size and the pectoral fin takes on a lance-like shape. Finally, the silver eel migrates to the spawning site, where they stop eating and are ready for spawning.

The silvering process in eels is a natural phenomenon that occurs between 4-20 years in females and 2-15 years in males. However, determining the exact age when metamorphosis commences is still challenging, as limited research has been conducted on this subject, and obtaining specimens during this period is difficult. The transitional stage between the yellow and silver stages is where the most significant changes occur, both externally and internally. Externally, the most obvious change is the transformation of the belly color to a silvery white

hue, which assists in adapting to the pelagic zone. During this stage, eels accumulate fat, mostly serving as an energy source during their long journey, with a small portion being used for gonadal development (Bergersen and Klemetsen, 1988). As a result, eels store fat as much as possible, mostly under the skin and in the liver. Another visible feature is the change in eye size and structure. To absorb light efficiently, the eye's volume and surface area increase, making absorption easier. Additionally, retinal pigments shift from porphyrin and rhodopsin to chrysopsin, allowing for scotopic vision (visible in black and white, as in deep-sea fishes). Lateral lines become more prominent, and sensory cells increase, while olfactory cells degenerate (Sorensen and Pankhurst, 1988; Zacchei and Tavolaro, 1988).

As mentioned, eels also undergo internal changes. During the silvering process, they stop feeding and this lasts until the end of their life cycle. This fasting period results in the shortening and partial degeneration of their intestine, as well as degeneration of their alimentary tract (Pankhurst and Sorensen, 1984). The swim bladder is also affected during this process, which is important for their movement. Silver eels have a swim bladder that helps them decrease their density by re-absorbing gas, thus aiding in buoyancy. This metamorphosis is a result of the development of the finest precapillary arterioles of the rete mirabilis in the swim bladder wall, which increases the size of the bladder and enhances gas exchange. Swim bladder wall is thicker during this process to prevent gas loss by diffusion (Yamada et al., 2001).

The process of adapting to salt water in eels is osmoregulatory and is particularly swift in silver eels (Cao et al., 2018). Even in the yellow stage, eels can survive in both fresh and salt waters. This is possible because eels are euryhaline, which means they can quickly adjust to changes in salinity. During the transition from fresh to salt water, eels lose a significant amount of water and their blood's ion concentration increases. However, the osmotic balance is maintained by a high absorption of water by the alimentary tract. The skin thickens to minimize water loss, while the intestines, kidneys, and gills work together to eliminate ions. When silver eels reside in freshwater, they undergo demineralization.

Hormonal control of silverling transition

The factors that trigger the silvering process in eels and the age and length at which it occurs are highly variable and not fully understood yet. While some eels start the process earlier even at a smaller size, others may not show any signs even at a larger size. The factors that lead to the best morphological and physiological condition of eels may be influenced by their habitat location. Age and growth rate are not necessarily correlated, and differences in habitat can affect both these factors, which can be calculated by the distance and remoteness of the

Sargasso Sea. Studies have shown that eels located upstream tend to increase body weight regularly, while those located farther away have longer bodies. During induced sexual maturation, eels over 70 cm in length have a higher gonad weight and body size, which contributes to their fertility success and is directly correlated with their body size. In some cases, females may start silvering even at 50 cm in length, as it can enhance their fertility (Durif and Elie, 2008; Palstra et al., 2009; Vollestad, 1992). However, males differ significantly from females in energy consumption during their journey, and their silvering usually occurs at a size of 30 cm and is not correlated with habitat and growth factors. Silvering is primarily controlled by hormones responsible for osmoregulatory changes in eels and marks the onset of sexual maturation.

During the silvering process in eels, there is no evidence that the thyrotropic axis is involved, as the levels of thyrotropin mRNA and thyroid hormone do not change. It is still unclear whether T4 treatments affect yellow eels, as it is known that thyroid hormones induce hyper-locomotor activity, which has not been observed in eels (Aroua et al., 2006). However, there is a difference in thyroid gland activity between the two stages of eel development. The ionic transport in gills plays an important role during the transition from freshwater to seawater, and it was expected that thyroid hormone levels would increase during the silvering of Japanese eels. However, in European eels, the levels of thyroid hormone remain the same. During the silvering process, the number of adrenal cells increases, and cortisol levels increase as well. This is due to the stimulation of the adrenal gland and synthesis of corticosteroids by Adrenocorticotropic hormone (ACTH). Once cortisol levels increase, the activity of Na^+, K^+ ATPase also increases, inducing an increment of water absorption in the gut. This may play a role in lipid breakdown during fasting for the journey to the Sargasso Sea.

The development and activity of gonads are regulated by two gonadotropin hormones (GtHs): luteinizing hormone (LH) and follicular-stimulating hormone (FSH). Both hormones are secreted by the pituitary gland. GtH stimulates the production of gametes and steroid hormones. In laboratory conditions, the artificial sexual maturation of carp or salmon can be induced by injecting pituitary extracts (in females) and human chorionic gonadotropins (hCG), which results in steroid hormone production and allows for yolk deposition (vitellogenesis) in oocytes. Vitellogenin synthesis occurs in the liver, supported by estradiol, while the incorporation of vitellogenin is controlled by GtHs in oocytes. Although GtHs do not play a significant role in the yellow eel reproductive cycle, there is some activity during the silvering stage. Sexual maturation in silver eel is correlated with insufficient production of GnRH and dopaminergic inhibition preventing LH production (Dufour et al., 1989). In the yellow stage,

gonads fail to respond to pituitary extract. However, the combination of a GnRH agonist and dopamine receptor antagonist results in an increase in pituitary LH, leading to the better locomotor activity of the eels (Dufour et al., 1988).

Another pituitary hormone, the growth hormone (GH), also plays a key role in controlling reproduction, but also in metabolism, and osmoregulation (Le Gac et al., 1993). It increases the secretion of Insulin-like Growth Factor 1 (IGF-I), which negatively impacts growth by inhibiting pituitary GH secretion. However, it has a positive effect on reproduction by activating LH production (Huang et al., 1999). The gonadotropic axis is controlled by the increase in estradiol and GH inhibition. Experiments conducted on yellow eel indicate that cortisol and increases in growth hormone positively impact the control of silvering in captivity. Environmental factors such as salinity and water pressure also trigger maturation in eels (Kagawa, 2003; Sébert et al., 2007). Salinity has been proven to positively affect GSI and oocyte diameter, while high-pressure triggers maturation by leveraging dopaminergic inhibition and increasing the rate of biosynthesis of dopamine (Sébert et al., 2008).

Silvering and maturation in eels may be influenced by swimming, although there is a negative correlation between the gonadosomatic index (GSI) and migration distance (Palstra and van den Thillart, 2010). Swimming may be a natural trigger for silvering, as it can impact lipid mobilization and early maturation. Lipolysis is expected to initiate once the fish begins its journey. In 2006, a new stream-gutter prototype was developed at Leiden University in the Netherlands to investigate the effects of swimming on both sexes during silvering and maturation. The prototype aimed to minimize the adverse effects observed in previous studies. The impact of swimming on silvering was confirmed by an increase in the eye diameter (eye index - EI) of all eels after two weeks of exposure to swim trials, with a more substantial effect observed after six weeks (Palstra et al., 2007). This effect was observed only in freshwater, not in salty water, and young farmed eels did not show any changes, indicating age sensitivity for maturation. No other visible external changes were observed during these trials.

The transformation of oogonia into functional oocytes in eels passes through four developmental stages, with oocytes becoming visible in the first stage. After swimming in Lake Balaton (Palstra et al., 2007), there was a significant increase in oocyte size and lipid droplets in just one week. After six weeks of swimming, the GSI and oocyte diameter were significantly higher, indicating that lipid incorporation in oocytes plays a role in swimming and maturation. While swimming in artificial conditions does not result in the fourth developmental stage of the oocyte, it has been shown that swimming in *Anguilla japonica* can increase diameter and some characteristics related to vitellogenesis in oocytes.

In males, swimming also has a positive effect on GSI. Typically, the GSI is less than 0.2%, but it significantly increases after 1.5 months of swimming. Swimming also increased the percentage of differentiated type A and late type B spermatogonia in males (Palstra et al., 2009). Swimming for three months yields spermatocyte appearance in the testis, while treatment with hCG in salty water can lead to earlier spermiation and higher sperm quality in volume and density. Although swimming has been shown to increase LH hormone secretion in the pituitary, it is still unclear whether this secretion is stimulated by swimming.

3.3 Conservation

The European eel, known for its adaptability, is currently classified as a red IUCN species (Pike et al., 2020). Despite early warnings, its population has decreased since the 1970s/1980s. Being a catadromous species, eels are more sensitive to harmful environmental factors. These factors are typically grouped under "Global Change" and can include climate changes, land overuse and land cover modifications, biogeochemical and hydrological cycles and pollution, biotic mixing (biological invasions), and overexploitation of natural resources.

3.3.1 Factors influencing European eel vulnerability

Global warming is a well-documented and widely-known aspect of global change. Many species, including eels, have been affected by it. This impact is visible in biogeochemical cycles, the length of seasons, the seasonality of water discharge, etc. Human activities, such as industrialization, land use, and modification, are just some of the main contributors to this issue. Anthropogenic factors are usually the primary cause of animal suffering in the wild. The high demand for urbanization, industries, and agricultural products has increased pollution, resulting in quicker global changes every year. One of the most significant challenges in ecosystem conservation and restoration is the fragmentation of aquatic ecosystems and habitat loss (Aarts et al., 2004). Biological invasion is another factor contributing to the decline in eel populations. Due to climate change, there has been an increase in the arrival of alien fish and other aquatic species that can affect native populations directly through predation or indirectly by spreading various diseases or modifying the ecosystem's composition) (Bernery et al., 2022). Another factor under human control is the overexploitation of natural resources, such as fisheries. This plays a significant role in the negative population trends of certain high-demand marine species, making them permanently endangered (Allan et al., 2005). Due to these negative factors, global change is rapidly occurring and modifying ecosystems. Several possible responses to these issues include micro-evolutionary adaptations, phenotypic

plasticity, and changing distribution areas. However, these responses are not a solution to the problem and will ultimately result in species' tragic loss and disappearance. It is clear that eels are currently on the edge of their possible survival, and without help, their population will disappear soon.

Impact of global change on leptocephalus drift and survival

The survival of eels depends significantly on the leptocephalus drift phase, which takes place during their life cycle (Cresci, 2020; Miller, 2009). This drift phase lasts about nine months, during which eel larvae are vulnerable to predation and other threats. The physical condition of the ocean, which has been negatively impacted by global change, has a solid link to the modifications that affect the eel population. For instance, global warming negatively correlates with sea surface temperature, leading to important shifts in the diversity, abundance, and distribution of larvae (van Ginneken and Maes, 2005). Due to the increase in sea surface temperature, the oscillation of salinity is affected, making it difficult for silver eels to detect spawning grounds. Additionally, there has been a decrease in larvae production in the Sargasso Sea due to climate change and global warming. This is mainly attributed to the lower production of carbohydrates, resulting in less food for larvae (Cresci, 2020; van Ginneken and Maes, 2005). Given these factors, natural recovery of the eel population may only be possible with adequate food and temperature.

The successful hatching and survival of larvae are directly correlated with the success of spawning migration. Any negative factors that affect migration will also impact the survival of larvae. River discharge and reduced discharge are two such factors that have adverse effects. River discharge speeds up migration, while reduced discharge postpones or stops it altogether. Humans partially control discharge influence due to our need for water extraction for agriculture and industry. Unfortunately, this negatively impacts the silver eel spawning migration and correlates negatively with the number of larvae and their survival rate (van Ginneken and Maes, 2005).

Dams

During its spawning migration and upstream migrations as elvers, this species meets another issue in their lifecycle that have a high impact on its natural recovery. Their reproduction trip is commonly stopped by various barriers (dams and weirs, fish ladders, pipelines, basins). Today in Europe, we can find over 24000 hydropower stations and 10000 pumping stations that need to be screened appropriately. All those obstacles and barriers make

eels suffer from blocked pathways, sometimes not even allowing them to find alternative routes around them. This brings eel as a species to a serious position and gives it a high possibility of extinction. Three main impacts on eel affected by dams: converted river habitat, blockage of upstream freshwater habitat, mortality during downstream spawning migration. Some countries, such as Canada, have stated the mortality of approximately 40% of downstream silver eel migration (Verreault et al., 2012), while in Europe, the success rate of overcoming dams and finding migration pathways was around 23%. European eel's turbine-related mortality rates are 9-60% (Pedersen et al., 2012). They stated that once they reached hydropower stations, European eels usually stopped the trip and hesitated to continue their migration. After a couple of tries, some will pass the station through the turbines, spillways, or bypass passages.

Pollution

Over the last few decades, significant global pollution, particularly in large cities, has been evident. This pollution extends into rural areas and natural habitats. Both humans and animals, including European eels, have been adversely affected by this pervasive issue. Currently, eels are suffering from the bioaccumulation of contaminants, rendering them increasingly unsuitable for human consumption. These contaminants primarily accumulate in fatty tissues, though significant accumulation also occurs in internal organs such as the liver and kidneys. This bioaccumulation leads to adverse effects on the fish's development, reproduction, and behavior. Pollution is widespread, affecting everything from the molecular to the organism level, and even extending its impact to community levels, with notable socioeconomic consequences (van der Oost et al., 2003). The impact of pollution is largely determined by the type of contaminants present, and it can be exacerbated by synergistic interactions with multiple substances or correlated with various environmental factors such as temperature, salinity, oxygen levels, and pH (Lawrence and Elliott, 2003). Pollutants mainly affect eels during their yellow stage but also impact all other life stages (Geeraerts and Belpaire, 2010). During the yellow stage, eels resist these conditions but continue to accumulate contaminants for years. By the time they reach the silvering stage, their fat content can increase to 27-29% (van Ginneken et al., 2009), leading to substantial accumulation of lipophilic xenobiotic contaminants. During this stage, eels accumulate contaminants through their gills, skin, and food intake, yet their rate of depuration remains extremely low (Daverat et al., 2006). Today, the most common chemical pollutants affecting eels include pesticides, heavy metals, and biphenyls, as well as polycyclic aromatic hydrocarbons (PAHs), perfluoro-octane sulfonic

acids (PFOS), brominated flame retardants (BFRs), dioxins, furans, and volatile organic compounds (VOCs). For example, extensive pollution in Dutch rivers from pesticides and insecticides, including those based on mercury and zinc, resulted in the loss of half a million eels and lasting damage to their habitats extending up to 650 km downstream (Christou, 2000, cited from Geeraerts and Belpaire, 2010).

European eels are often considered resilient, but evidence points to significant effects of pollution on their lifecycle. Eels are particularly vulnerable during various stages such as growth, silvering, migration, the development of reproductive cells, and the larval phase. Although less damage is observed during the yellow stage due to fat storage and growth, most harm occurs during the silvering stage as the eels undergo significant morphological and physiological changes. Belpaire et al. (2009) noted potential issues with migration and spawning due to the accumulation of contaminants during the yellow stage. The continuous burning of fat during migration intensifies the presence of contaminants, leading to elevated toxicity levels. This high toxicity severely impacts the endocrine, nervous, reproductive, and immune systems, establishing a correlation between pollutants and decreased resistance to infections from viruses and parasites. It is evident that chemicals influence the eel and its lifecycle. The effects of newer chemicals recently introduced to industry and agriculture on eels remain uncertain. Despite their known resilience to environmental fluctuations such as temperature, salinity, food availability, and oxygen levels, eels are also highly sensitive to pollutants.

Diseases

Diseases of European eel usually come together with some other harmful environmental factors, but not necessary. Mainly, diseases can be divided into groups belonging to wild individuals and groups belonging to farmed eel. The increase in demand for European eel during the last few decades brings more problems with maintaining health conditions in captivity and avoiding disease (Haenen et al., 2009). In nature, parasites usually occur in small groups (less density) and do not have a significant impact, as in captivity, where density is higher they can have serious health implications (Haenen et al., 2009; Haenen and Davidse, 2001). Some common parasites are trematode *Anguillicola grassland* (nematode), and *Myxidium giardia* (myxosporean) (Borgsteede et al., 1999). In Hungary, one of the most notable mass death events of European eel occurred at Lake Balaton where an estimated 250 tons of eel were found dead floating on the water surface. Further investigation proved that it was caused by the nematode *Anguillicola crassus* (Molnár et al., 1991). Some pathogenic

bacteria commonly infecting the European eel are *Vibrio vulnificus*, *Vibrio anguillarum, Pseudomonas anguilliseptica* and *Edwarsdsiella tarda* (Callol et al., 2015; Pirollo et al., 2023). The negative influence of those pathogens is usually in correlation with the fish condition, which means that any injury or stress will negatively affect the survival rate. Viruses commonly infecting the European eel and which can have serious implications are Eel Virus European (EVE), Eel Virus European X (EVEX), Herpesvirus anguillae (HVA) and andalloherpesvirus anguillid herpesvirus 1 (AngHV1) (Haenen et al., 2009; Van Beurden et al., 2012). Most of these infections are treatable. In captivity, findings of double infections such as herpesvirus and reovirus, EVEX and reovirus, HVA and EVE, and EVEX and EVE, are possible, but in natural conditions it is highly unlikely. Mortality ratio depends on the type of disease, fish conditions, and its environment.

3.3.2 Artificial propagation

Artificial propagation is an essential part of species conservation, and is one of the first technologies to be developed for endangered species. The artificial maturation of European eel has been a subject of research for over a century. To initiate artificial maturation, different hormones such as carp pituitary and hCG, have been commonly used. Other extracts, including prolanin, gestyl, ambion pregnil, thyrotroph hormone, and spleen extract, have also been used with varying degrees of success. Boëtius and Boëtius (1967) developed a successful protocol for artificial maturation by combining different concentrations of hCG. However, the duration of the process remained unchanged. Meske (1973) used Solcosplen (spleen protein), Synahorin (a mixture of gonadotropic hormone from the hypophyseal anterior lobe and animal placenta), and Cyren B (a synthetic estrogen, diethylstilbo-estrolddipropionate) to obtain sperm. Dollerup and Graver (1985) later developed a protocol that involved administering hCG 500 IU on days 0 and 7, followed by two more administrations on days 179 and 186, and on days 400 and 407. After the spermiation process was complete, the fish were given feed. The first trial resulted in a 91% success rate for maturation, while the subsequent two attempts had slightly lower success rates of 83% and 70%.

Furthermore, it was discovered that seawater and freshwater have an impact on artificial maturation. Specimens kept in artificial seawater produced better quality sperm. The protocol involved weekly hormone injections in different groups of fish based on their origin. The males were treated with 100 IU hCG/fish, testosterone propionate 5mg/fish, desoxycorticosterone 5mg/fish, and saline solution. Only hCG treatment had a positive effect, and only individuals

from seawater survived spermiation. Leloup-Hâtey et al. (1985) used 0.6 mg carp pituitary three times a week for five weeks, which resulted in an increase in gonad somatic index and gonadal tissue containing spermatozoa. Khan et al. (1987) implemented another technique, injecting males with 250 IU of hCG, which led to successful maturation after three months. Pérez et al. (2000) developed a protocol using hCG at a rate of 1.5 IU/body weight g per week for ten weeks. Fish began spermiation after the fourth week but could not survive past the tenth week. However, the result showed a 100% success rate of spermiation from the eighth week, and motility results improved progressively with each subsequent injection. It was also found that sperm density increased significantly in the first six hours after injection and decreased slowly thereafter. Lately, Peñaranda et al. (2018) started to use homologous recombinant LH and FSH obtained from transfection of mammalian cells of Chinese hamster ovary cells. This approach is showing much promise as the hormones of the European eel itself are used, therefore facilitating gametogenesis and gamete production.

3.3.3 Cryopreservation

Cryopreservation entails freezing biological material to the temperature of liquid nitrogen (-196 °C) at which metabolic process cease, and at which they can be held practically indefinitely (Ma et al., 2011). Once thawed, the cells survive and retain their physiological function. The technique involves a series of steps that include sample selection, selection of optimal cryomedium, cooling rate, storage, warming rate and other.

Cryopreservation and cryobanking are some of the most important tools in domestic animal breeding, population management, and human infertility programs. Germplasm cryobanking particularly has important implications in broodstock management, maintenance of important strains of laboratory fish, genetic selection programs, biodiversity preservation and assisted reproduction (Asturiano et al., 2017; Marinović, 2021; Martinez-Paramo et al., 2017). Among the germline, sperm cryopreservation is one of the most developed and most commonly used conservation strategies in fish. Furthermore, it has great potential in aquaculture setting as well. The most common benefits of sperm cryopreservation are that sperm can be stored in liquid nitrogen practically indefinitely, it allows storage of leftover sperm after artificial reproduction, broodstock maintenance is simplified, it enables efficient transport of sperm between laboratories or fish farms and other (Cabrita et al., 2010). Furthermore, these cells are convenient to freeze as they have a small surface/volume ratio, small size, and high chilling resistance. Protocols for eggs and embryos on the other hand have

not yet been developed as they are very large, have a large surface/volume ratio, a complex structure, and a very high chilling sensitivity (Isayeva et al., 2004; Tsai et al., 2009).

In European eel, sperm cryopreservation protocols have been developed. In a recent review by Herranz-Jusdado et al. (2019), two main sperm cryopreservation protocols have been identified; one developed by a Spanish team (Asturiano et al., 2004; Peñaranda et al., 2009), and one developed by a Hungarian team (Müller et al., 2004; Szabó et al., 2005). The two protocols differed in extenders, cryoprotectants, dilution ratios and straw sizes. The highest motility values obtained were around 40% (Peñaranda et al., 2009). Vitrification of European eel sperm was also attempted (Kása et al., 2017), however, the results were very poor compared to conventional freezing which is a common observation in fish sperm cryopreservation (Asturiano et al., 2017).

To fully preserve the European eel genetic resources and to be able to obtain a new generation when needed, female genetic resources need to be preserved as well. However, as previously mentioned, successful protocols for fish egg and embryo cryopreservation have not yet been developed, even though some progress has been made in the recent years (Khosla et al., 2017). To circumvent this limitation, protocols for cryopreservation of early-stage oocytes have been developed (Guan et al., 2010, 2008) as these oocytes are smaller, do not contain yolk and are therefore less complex, and have a more permeable membrane. However, the limitation to this approach is that *in vitro* growth protocols have not yet been successfully developed in fish, thus even though early-stage oocytes can be cryopreserved, their utility is negligible, and the next generation of fish still cannot be made.

The alternative was found in cryopreservation of germline stem cells (GSCs). These are the baseline cells of gametogenesis, and have the ability to reconstitute both spermatogenesis and oogenesis (Marinović, 2021). Furthermore, they are not difficult to cryopreserve as they are small, do not contain yolk and their membrane is relatively permeable to both water and solutes. Hence, protocols for GSC cryopreservation were developed for several fish species (Franěk et al., 2019b; Lee et al., 2016b, 2013; Marinović, 2021; Pšenička and Saito, 2020). As these cells remain functional after freezing, cryopreservation of GSCs and creation of cryobanks coupled with transplantation and production of donor-derived offspring from surrogate parents offer numerous possibilities and advances in conservation biology and broodstock management of the European eel.

3.3.4 Surrogate broodstock technology

As previously stated, the application of cryopreservation in preserving the genetic resources of fish is plausible only to an extent as only male gametes (spermatozoa) can be cryopreserved. An alternative has been found in cryopreservation of GSCs, however, these are the stem cells of the germline, hence, without further technologies they cannot be used for the creation of the next generation of fish. The main ways in which GSCs can be used for gamete production is through *in vitro* cell culture or through transplantation and surrogate broodstock technology.

Surrogate broodstock technology entails the production of donor-derived gametes and offspring from surrogate parents (usually belonging to a different strain or species) after transplantation of donor germline stem cells into recipients (Figure 4; Goto and Saito, 2019; Yoshizaki and Yazawa, 2019). Cell types that can be transplanted are either primordial germ cells (PGCs), spermatogonial stem cells (SSCs; male germline cells) or oogonial stem cells (OSCs; female germline cells). Initially, most studies focused on developing protocols for transplantation of PGCs (Ciruna et al., 2002; Saito et al., 2008). However, PGCs are very few in number, obtaining them from larvae is very laborious, and it is very difficult to identify them without the use of transgenic or reporter fish lines. Hence, it would be difficult to implement these protocols in which species in which it is not possible to obtain transgenic lines (such as the European eel). Therefore, the majority of researchers shifted their focus to SSCs which are the most numerous of the GSCs, that can be simply obtained, and which can be enriched through a variety of methods.

As mentioned, surrogate broodstock technology is based on isolation of GSCs from donor individuals and their transplantation into recipient individuals. Donors are usually species which have a high economic or conservation interest, while the recipients usually belong to another species which is commonly reared in an aquaculture or laboratory setting. An important aspect is that both species are closely related, i.e., that they are phylogenetically close. In case of a large phylogenetic distance, the physiology of the organisms can be very different, as well as the immunity markers, therefore, the transplanted donor cells would either not be able to survive within the recipient or they will be rejected by the recipient's immune system. In some cases it is possible to use immunocompromised recipients such as the zebrafish *rag2E450fs* mutant line (Tang et al., 2014), however, such transgenic fish are not always available and are difficult to rear, especially in an aquaculture setting. Furthermore, donor cells can be transplanted into recipient larvae or adult individuals. Most commonly, GSCs are

transplanted into larvae as they do not have a developed immune system (Yoshizaki et al., 2011), and the technique necessitates less cells then when transplanting into adults.

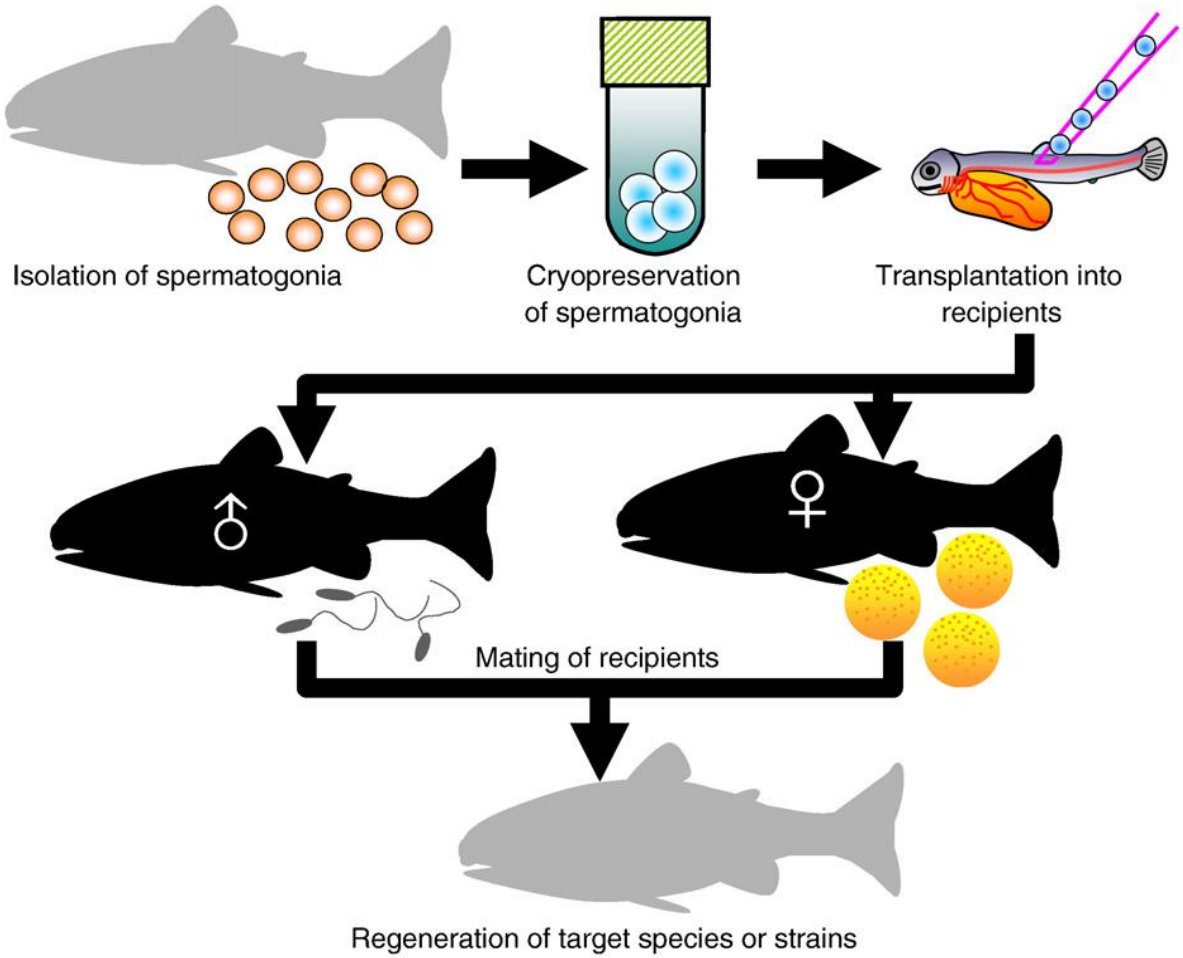

Figure 4. The baseline principle of the surrogate broodstock technology. Cited from Yoshizaki et al. (2011) with permission from the publisher.

Another important aspect of the surrogate broodstock technology is the sterility of recipient individuals. This is necessary to prevent the creation of endogenous recipient gametes and enable creation only production of donor-derived gametes. The most common ways of obtaining sterile recipients is triploidization, using sterile hybrids, or knock-down or knock-out of key genes involved in PGC migration (Franěk et al., 2022). Even though triploid and hybrid recipients are sterile, their sterility is cause by mismatches in chromosome segregation during meiosis, hence, these individuals contain endogenous GSCs but do not produce later-stage germline cells. This however could be a potential problem from the aspect of GSC transplantation as donor GSCs need to compete with the endogenous GSCs for the stem cell niches. Sterility caused by knock-downs is thus more favorable as in this case PGCs mis-migrate and end up in different organs where they go through apoptosis or transdifferentiate

(Gross-Thebing et al., 2017). This in turn leaves the gonads without germline cells and containing only somatic cells. Therefore, the stem cell niches are free for the donor cells to incorporate and proliferate. Knock-down of the *dead end* (*dnd*) through morpholino oligonucleotides (MOs) is the most common form of knock-down and has been applied in many different fish species to obtain sterile recipients (Franěk et al., 2019a; Marinović et al., 2019; Yoshizaki et al., 2016).

A significant advantage of the surrogate broodstock technology from the aspect of species conservation is that it can be combined very well with cryopreservation. Frozen GSCs can be thawed and transplanted once recipient larvae are available, and thus give rise to donor-derived gametes of both sexes, thus creating a new generation of donor individuals. Furthermore, GSCs from several different donors can be combined and transplanted into recipients thus increasing the genetic diversity. In the case of European eel, novel conservation and management strategies are desperately needed, hence, the development of GSC preservation and surrogate broodstock technologies can significantly contribute to the conservation efforts for this endangered species.

3.4 Farming of the European eel

European eel farming still relies on wild catches of glass eel individuals due to the lack of a fully successful protocol for its reproduction in captivity (Figure 5; Delrez et al., 2021). Recirculating systems are used to intensively produce captured elvers (Dalsgaard et al., 2013), while extensive eel culture is almost non-existent nowadays. The glass stage of European eels is reached during their natural migration from the Sargasso Sea to freshwater, with the highest abundance occurring from November to May. Eel captures are mainly conducted near the shores of France, Portugal, Spain, and the United Kingdom, with various techniques used by national and EU legislative preferences. Spain and Portugal mainly use scoop nets and traps, while France prefers tiny wing nets and trawls. The only legal way of capturing eels in the UK is through scoop nets. EU regulations limit the glass eel quota for future consumption to 40% of the total catch, with the remaining 60% used for restocking (Altmayer, 2024).

Intensive eel culture typically involves a recirculating system setup featuring circular or square fiberglass or concrete tanks with a surface area ranging from 25 to 200 square meters (Kirkegaard et al., 2010). These tanks are designed to provide controlled environmental conditions, with a continuous supply of fresh water, adequate aeration, appropriate filtration, and automated feeding systems. One of the critical requirements for successful eel cultivation

is maintaining optimal water temperature around 23-26 °C, which is achieved by using electric heating systems. In this system, eels are typically cultured at a density of 100 to 150 kg/m^3 (Kirkegaard et al., 2010).

Eel farming involves two traditional methods: extensive pond system and viticulture. Extensive ponds are used for juvenile and adult eels, with sizes ranging from 100 to 300 m^2 and 1000 to 1500 m^2, respectively. These ponds may have either static or flow water circulation systems. On the other hand, viticulture is an ancient practice that utilizes natural migratory routes for eels, particularly in the Adriatic region of Italy. Eels are kept in marine lagoons or brackish waters with a density of 4 to 15 kg/ha (Ottolenghi et al., 2004). Feeding is a crucial aspect of eel farming. Nowadays, formulated feeds in moist paste are commonly used for elves, while more prominent individuals are fed with steam-pressed or extruded pellets.

Eel farming is a complex process that involves various procedures, one of which is harvesting techniques. The stress on eels during harvest time can have a negative impact, which is why it is vital to maintain high oxygen levels. Feeding should be stopped 1-2 days before market transport to prepare for harvest (Ottolenghi et al., 2004). The harvest process involves using a grading machine and pipes that passively select eels based on market size. Any eels that do not meet the market size requirements are placed back in the tank for further growth (Ottolenghi et al., 2004). After selection, eels are kept in holding tanks without feed to eliminate any off-taste. During transport, eels are placed in plastic bags filled with water and oxygen to ensure sufficient moisture or in larger tanks if necessary. In Europe, the most popular system for eel farming is the recirculation system, which provides optimum conditions for eel growth and is cost-effective since the water can be reused many times. This system includes mechanical and biological filtration systems that consume low levels of energy.

Eel farming is prevalent in many European countries, with the Netherlands being the main center for eel production, followed by Germany and Denmark (Altmayer, 2024). Other countries, such as Italy, Greece, Sweden, Poland, and Portugal, also have smaller eel farms, producing smoked eels or exporting them to the Asian market for further growth.

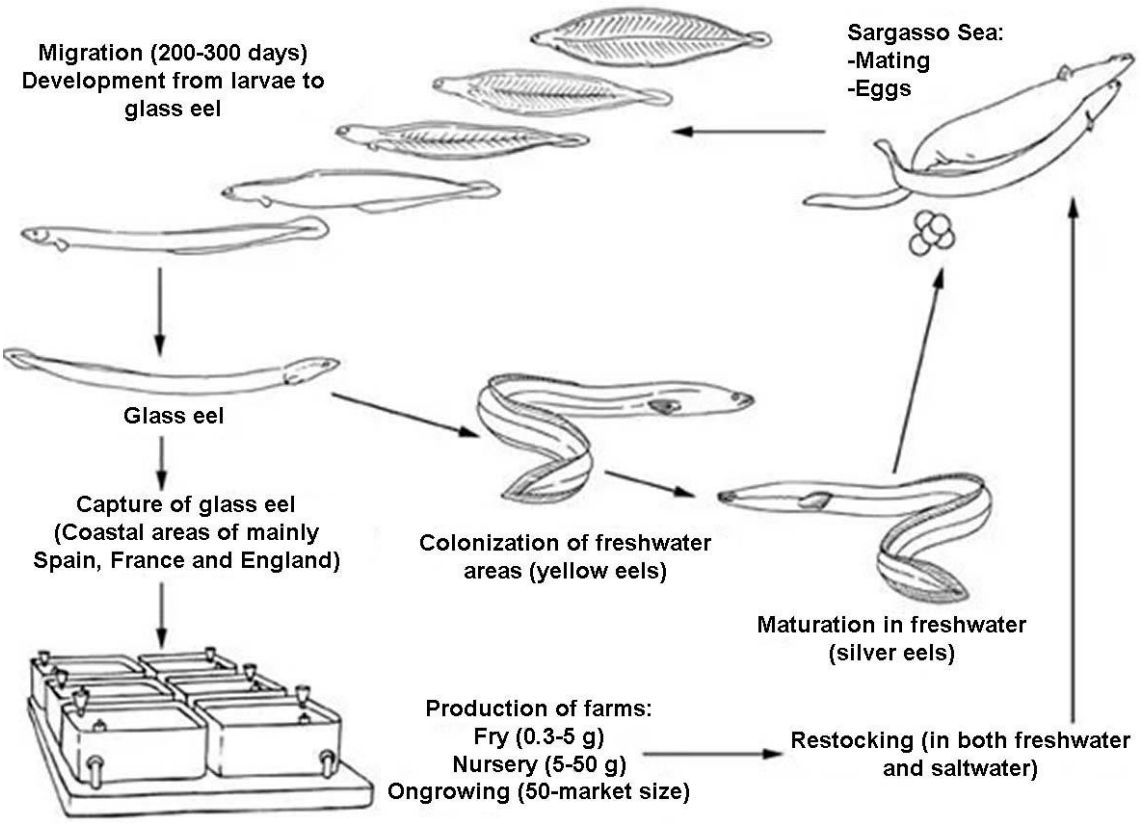

Figure 5. The production cycle of the European eel. (https://thefishsite.com/articles/cultured-aquatic-species-european-eel).

4 MATERIAL AND METHODS

4.1 Ethics

The study was conducted at the Hungarian University of Agriculture and Life Sciences, Institute of Aquaculture and Environmental Safety (Gödöllő, Hungary) and at the Polytechnic University, Institute for Animal Science and Technology (Valencia, Spain). All experiments performed in Hungary were conducted in accordance with the Hungarian Animal Welfare Law, Hungarian Government Directive 40/2013 on Animal Experimentation and the Directive 2010/63/EU of the European Parliament and of the Council. In addition, all experiments conducted in Hungary were approved under the Hungarian Animal Welfare Law (approval number: PE/EA/188-6/2016).

Experiments conducted in Spain were in accordance with European Union regulations concerning the protection of experimental animals (Dir 86/609/EEC) and with the recommendations given in the Guide for the Care and Use of Laboratory Animals of the Spanish Royal Decree 53/2013 regarding the protection of animals used for scientific purposes (BOE 2013). Protocols used were approved by the Experimental Animal Ethics Committee from the Universitat Politècnica de València and the final permission was given by the local government (Generalitat Valenciana, Permit Number: 2019/VSC/PEA/0073).

4.2 Chemicals

The list of chemicals used in this study is provided in Supplement 1.

4.3 Animals and handling

Experiments conducted on females were done at laboratories of the Hungarian University of Agriculture and Life Sciences, Institute of Aquaculture and Environmental Safety (Gödöllő, Hungary). Females were originating from Lake Balaton and were kept in controlled conditions in the recirculating system of the Institute. Fish were kept at a constant temperature of 16±1 °C. They were fasted at least one week prior to the experimental procedures and the aquaria were covered to maintain constant shade to reduce fish stress.

Experiments conducted on males were done at both laboratories of the Hungarian University of Agriculture and Life Sciences, Institute of Aquaculture and Environmental Safety (Gödöllő, Hungary) and the laboratories of the Polytechnic University, Institute for Animal Science and Technology (Valencia, Spain). Experiments done in Hungary were also done on

males from Lake Balaton which were kept in controlled conditions in a recirculation system at a constant temperature of 16±1 °C without feeding. Experiments in Spain were done on farmed immature males which were bought alive at a local supermarket and transported to the facilities of the Laboratory of Fish Reproduction of the Universitat Politècnica de València (Valencia, Spain). Fish were kept in a recirculation system at a constant temperature of 14±1 °C. Fish were fasted during the experiment, and the aquaria were covered to maintain constant shade to reduce fish stress.

For the experiments done in Hungary, fish were euthanized in an overdose of 2-phenoxyethanol for a minimum of 5 minutes followed by decapitation. For experiments done in Spain, fish were euthanized in an overdose of benzocaine followed by decapitation. After removing excess mucus from the body surface, the fish was positioned dorsoventrally and disinfected with 70% ethanol.

4.4 Dissection and isolation of the gonadal tissues

After euthanization, fish were carefully opened to expose the internal organs and to excise the gonads. To open the body, the skin and underlying muscle on the belly of the fish between the pectoral fins were horizontally snipped and the skin and underlying muscle were cut along the belly until the anal fin thus exposing the internal organs. Gonadal tissue was carefully extracted from the body, paying attention not to cause any unnecessary contamination by cutting the gastrointestinal tract or blood vessels. After excision, gonads were sterilized in a 0.5% bleach solution for 2 min, after which they were washed three times in phosphate buffered saline (PBS). Gonads were then placed in Petri dish filled with PBS and were cleaned from large blood vessels and excess connective tissue. Gonads were then kept in PBS on ice until further analyses (for maximum of 30 min).

4.5 Histological identification of GSCs

4.5.1 Classical histology analyses of the gonads

Histological analysis of the gonadal tissue was conducted in order to determine the developmental and maturation stage of the European eel individuals which would be used for subsequent GSC manipulations. Both male and female eels were dissected as described above (Section 4.4). Immediately after dissection, testes were cut into ~20 mg pieces, while ovaries were cut into ~50 mg pieces. Both testicular and ovarian tissues were immediately placed into

10% neutral-buffered formalin (equivalent of 4% formaldehyde) and kept for fixation overnight (~16 h) at 4℃. After fixation, tissues were washed three times with PBS, and were dehydrated in 30% EtOH for 30 min, 50% EtOH for 30 min, and were lastly stored in 70% EtOH at 4℃ until processing. Since the structure of tissues fixed in 10% NBF was not well preserved (Section 5.1.1), the fixation strategy was changed. This time, gonadal tissues were fixed in a modified Davidson's solution (30% of 37–40% formaldehyde, 15% ethanol, 5% glacial acetic acid, and 50% distilled water; Howroyd et al., 2005; Wang et al., 2016). Similarly as before, tissues were fixed overnight (~16 h) at 4 ℃, and afterwards were dehydrated in 30% and 50% EtOH series before finally being stored in 70% EtOH at 4 ℃ until further processing.

Tissue processing was done in a Shandon Citadel 2000 Automatic tissue processor (Thermo Fisher Scientific, Budapest, Hungary). Samples were put into plastic cassettes, labelled and put into the tissue processor. The protocol for processing is shown in Table 1.

Table 1. Tissue processing protocol.

Reagents	**Time (h)**
70% Patosolv	1:00
90% Patosolv	1:30
100% Patosolv	1:30
100% Patosolv	2:00
Patoclear	2:00
Patoclear	1:00
Xylol	2:00
Paraffin (58 ℃)	2:00
Paraffin (58 ℃)	2:00

After processing, tissues were embedded into paraffin blocks. This was done in a HistoCore Arcadia Embedding Station (Leica, Wetzlar, Germany). Subsequently, each block (sample) was cut into ~5 µm think sections on a Leica RM2245 rotary microtome (Leica, Wetzlar, Germany). Slices were then mounted on glass slides and placed in an incubator at 40 ℃ until further processing. Standard haematoxylin/eosin (H&E) staining was done in an automatic Shandon Varistain 24-4 (Thermo Fisher Scientific) staining machine. Protocol for staining is shown in Table 2. After slide preparation, sections were analyzed on a Nikon Eclipse 600 light microscope (Nikon Europe B.V., Amstelveen, The Netherlands) and photographed using a QImaging Micro Publisher 3.0 digital camera.

Table 2. Protocol for staining samples with H&E method.

Process	Chemical	Time
Clearing/Removal of paraffin	Patoclear	10 min
	Patoclear	5 min
	Patoclear	8 min
Rehydration	100% Patosolv	3 min
	70% Patosolv	3 min
	50% Patosolv	10 min
	H_2O	1 min
	H_2O	2 min
Staining	Haematoxylin	30 sec
Washing	H_2O	3 min
	H_2O	3 min
	H_2O	9 min
Staining	Eosin	30 sec
Dehydration	50% Patosolv	5 min
	70% Patosolv	3 min
	100% Patosolv	5 min
Clearing	Patoclear	5 min
	Patoclear	5 min
	Patoclear	10 min
	Xylol	~

4.5.2 Western blot

According to the study of Blanes-García et al. (2024), vasa is a good and reliable marker of SSCs in immature European eel. In this study, we wanted to confirm the presence and stage of the germline cells through immunohistochemistry. The verification whether the antibody is correctly binding to the chose antigen (vasa/DDX4 in this case) was done by western blot.

European eel testicular tissues were dissected as described above (Section 4.4). Three tissue pieces were placed in separate 2-ml Eppendorf tubes and were snap-frozen in liquid nitrogen. The tubes were stored in a -80 ℃ deep freezer until further analyses.

At the time of analysis, tissue samples were taken out of the deep freezer, and were allowed to thaw at RT. They were first weighed on an analytical scale and subsequently homogenized by using a Sample Grinding Kit (Cytvia, Budapest, Hungary). Shortly, samples were immediately placed in a 2-ml Eppendorf tube containing abrasive grinding resin and 100 µl of thiourea/urea lysis buffer (20 mM Tris, 7 M urea, 2 M thiourea, 5 mM $Mg(Ac)_2$, 4% CHAPS; pH 8.5) supplemented with 1% phosphatase and 1% protease inhibitor cocktail. They were then grinded using a motoric pestle and were further homogenized using sonication. Samples were then centrifuged at 15000 ×g for 5 min, and the supernatants were collected and used for subsequent analyses.

Protein concentrations were measured using a 2D-Quant kit (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions and with 5 µl of each sample. For reference we used BSA concentrations from 5 to 60 µg, and all measurements were done in triplicates.

For polyacrylamide gel electrophoresis (PAGE), a 15 %/10% separating and a 4% stacking gels were cast. 15 µg of each sample was added to 2× Laemmli sample buffer supplemented with 200 mM DTT and were heated for 5 min at 96 °C. Samples were then loaded onto the gel and the electrophoresis was conducted. Electrophoresis was carried out on a Mini-PROTEAN® Tetra System (Bio-Rad, Budapest, Hungary) using 150 V until the samples reached the top of the separating gel and 200 V until the end of the run. During the run, the electrophoresis system was cooled using ice.

After the PAGE run, separated proteins were transferred onto an immunoblot membrane. Namely, gels were placed in gel holder cassettes, on top of a sponge and filter paper (wetted using transfer buffer). An Immun-Blot® LF PVDF membrane (Bio-Rad, Budapest, Hungary) was activated using abs. MeOH and was soaked in transfer buffer. The gels were covered with a membrane, a wet filter paper and a wet sponge. The cassettes were closed and placed in the tank and were covered with transfer buffer. Proteins were transferred to the membrane using 20 V for 150 min (Mini-PROTEAN) while cooling using ice.

After the proteins were transferred onto the membrane, membranes were placed in a blocking solution (5% BSA TBS-T) for 1 h with continuous shaking (with proteins facing up) at RT. The membranes then were incubated in blocking solution containing primary antibody against the vasa antigen (DDX4; Abcam, cat. no. ab13840; Abcam, Cambridge, UK) at 1:1000 dilution at 4 °C. After primary antibody incubation, membranes were washed 5 times in TBS-T, and were agitated for 10 min in TBS-T. Subsequently, the membranes were incubated in TBS-T containing the secondary anti-rabbit antibody conjugated to Alexa 647 (Jackson ImmunoResearch; 711-605-152; Jackson ImmunoResearch, West Grove, PA, USA) at a 1:800 dilution for 2 h with gentle agitation at RT. Membranes were then washed 6 times in TBS-T and were agitated 4 times for 5 min in TBS-T. Lastly, stained membranes were scanned by using a Fujifilm FLA-5100 fluorescent image analyzer (Fujifilm, Budapest, Hungary).

4.5.3 Immunohistochemistry

As Western blot confirmed that the selected antibody was specifically binding to the vasa antigen, immunolocalization of this antigen has been done by immunohistochemistry.

Five-µm thick sections were made from samples previously processed for histological analyses. After cutting, sections were mounted on Superforst Ultra Plus glass slides (Thermo-Fisher, Budapest, Hungary). These slides are positively charged and allow for a firmer connection between the tissue and glass which is important during the harsh antigen retrieval process. After preparation, slides were left at 4 °C until the immunohistochemistry protocol. The same primary antibody against vasa (DDX4; Abcam, cat. no. ab13840; Abcam, Cambridge, UK) which was used for a western blot was used for immunohistochemistry as well. The secondary antibody was an anti-rabbit antibody conjugated with horseradish peroxidase raised in goat (Abcam, cat. no. ab6721; Abcam, Cambridge, UK). The signal was developed using the 3,3'-diaminobenzidine (DAB) immunoperoxidase visualization method. The immunohistochemistry protocol in its entirety is shown in Table 3.

Table 3. Protocol for immunohistochemistry staining.

Process	**Chemical/Procedure**	**Time**
Deparaffinization	Xylol	10 min
	Xylol	10 min
Rehydratation	100% EtOH	10 min
	100% EtOH	5 min
	90% EtOH	3 min
	70% EtOH	3 min
	ddH_2O	3 min
	ddH_2O	3 min
Heat-induced antigen retrieval (HIAR)	Cooking the slides in HistoVT One buffer (Nacalai Tesque, Tokyo, Japan) at 80-86 °C in a TintoRetriever Pressure Cooker (Bio SB; Santa Barbara, CA, USA)	20 min
Wash 3×	PBS	5 min
Inhibition of endogenous peroxidases	Incubation with 3% H_2O_2	30 min
Wash 3×	PBS	5 min
Blocking	Incubation with PBS supplemented with 10% FBS and 10% goat serum at RT	1 h
Primary antibody	Incubation with the anti-vasa antibody (1:200) diluted in PBS supplemented with 5% FBS and 5% goat serum at RT	1 h
Wash 3×	PBS	5 min

Table 3. Continued.

Secondary antibody	Incubation with the anti-rabbit antibody (1:500) diluted in PBS supplemented with 5% FBS and 5% goat serum at RT	30 min
Wash 3×	PBS	5 min
DAB staining	Incubation with a 0.05% DAB solution containing 0.015% H_2O_2 in PBS at RT	5 min
Wash 3×	PBS	5 min
Counterstaining	Hematoxylin	3 min
Wash 3×	ddH_2O	5 min
Dehydratation	70% EtOH	3 min
	90% EtOH	3 min
	100% EtOH	5 min
	100% EtOH	10 min
	Xylol	5 min
	Xylol	10 min
Mounting	DPX	-

4.6 Dissociation of the gonadal tissue

4.6.1 General approach to dissociation

Gonadal tissue pieces were dissociated by using an enzymatic treatment. Enzymatic medium consisted of L-15, 2 mg/ml collagenase, 3 mg/ml trypsin and 60 µg/ml DNase I (unless otherwise specified). The mentioned enzymes and concentrations were selected after optimizing the dissociation procedure (Sections 4.6.3 and 5.2). For every 50 mg of tissue (or up to 50 mg), 500 µl of the dissociation medium was used (Marinović, 2021). The dissociation was conducted in 2-ml Eppendorf tubes due to their conical bottom which allowed for more balanced shaking on the shaking plate. Firstly, the enzymatic medium was pipetted into the 2-ml Eppendorf tube, then the tissue pieces were inserted and minced into small pieces with scissors. Ovarian tissues were then incubated for 1 h, while testicular tissues were incubated for 1.5 h. Incubations were done on a shaking plate at room temperature (RT; ~24 °C). The difference in incubation times between the sexes is due to the structure of each tissue. From the previous experience of the lab in dissociating gonadal tissues of different species, ovaries were most commonly more sensitive, therefore, they needed shorter incubation times than testes. After incubation, the dissociation procedure was stopped by adding 10% (v/v) FBS and an equal volume of L-15. Samples were then filtered through 30 µm mesh-size filters and centrifuged at 300 ×g for 10 min at RT. Supernatants were discarded and the pellets were resuspended in an appropriate volume of fresh L-15 medium supplemented with 10% FBS.

4.6.2 Viability assessment

Viability was primarily assessed through trypan blue (TB) staining. TB staining is an exclusion test where live cells are not stained, while dead cells that have a permeable membrane uptake the TB dye and are stained blue. TB test was done by mixing cell suspensions with 0.4% TB at a 1:1 ratio. The suspensions were incubated for at least 1 min at RT and the viability was checked in a hemocytometer under a phase-contrast microscope. The number of live GSCs was counted in 10 fields of a Bürker-Türk hemocytometer for each sample under a light microscope with phase contrast (Nikon Eclipse E600) at 400× magnification.

During the cryopreservation and hypothermic storage experiments, final survival rates were determined as the proportion of live cells isolated from the cryopreserved/stored tissue compared to the number of live cells isolated from fresh tissue of the appropriate size. Final cell survival rate was assessed as: $Viability\ (\%) = (N_{cryopreserved} / N_{fresh}) \times CF \times 100$ while correcting for the tissue size with a correction factor: $CF = \frac{Weight_{fresh\ tissue}}{Weight_{cryopreserved\ tissue}}$ (Lujić et al., 2017).

4.6.3 Optimization of the dissociation procedure

To optimize the dissociation procedure for both testicular and ovarian tissues, the effectiveness of different enzymes and their combinations was tested. Gonadal tissues were excised as described above (Section 4.4). As immature European eel testes are generally smaller than the ovaries, testes were cut into ~10 mg pieces, while ovaries were cut into ~50 mg pieces. For both males and females, three different individuals were used as biological replicates. Two-ml Eppendorf tubes were filled with 1 ml of an appropriate enzymatic solution and tissue pieces were randomly placed into each tube. Prior to dissociation, each tissue piece was weighed; tissue pieces were gently tapped on sterile paper towels to remove excess of liquid and were weighed on an analytic scale (Mettler-Toledo AB204-S; Mettler-Toledo, Columbus, OH, USA). Tissues were then minced and incubated for an hour (ovarian tissue) or 1.5 h (testicular tissue) on a shaking plate at room temperature (RT). Dissociation was stopped by adding 100 µl of FBS and 200 µl of Leibovitz L-15 medium. The suspensions were then filtered through filters with 30 µm mesh size and centrifuged at 300 ×g for 10 min. Supernatant was discarded and pellets were resuspended in an appropriate volume of L-15 supplemented with 10% FBS and 1% antibiotics (10000 U/ml penicillin and 10 mg/ml streptomycin).

A total of six enzymatic groups containing different concentrations of collagenase and trypsin were tested: (1) 2 mg/ml collagenase (Marinović, 2021); (2) 6 mg/ml collagenase (Fernández-Díez et al., 2012); (3) 1.5 mg/ml trypsin (Marinović, 2021); (4) 3 mg/ml trypsin (Marinović et al., 2017); (5) 2 mg/ml collagenase + 1.5 mg/ml trypsin (Marinović, 2021); (6) 2 mg/ml collagenase + 3 mg/ml trypsin. All enzymatic groups contained L-15 and 50 µg/ml DNase I. Viability of cells was determined by trypan blue staining. As the vast majority of cells (>90%) were viable, we only counted the number of live cells. To control for the tissue size, the number of cells obtained from each group was then appropriated to the size of 10 mg for testicular tissue or 50 mg for ovarian tissue. This was done by using the following formula:

$$N_{corrected} = \frac{N_{observed} \times W_{fixed}}{W_{observed}}$$

where $N_{corrected}$ is the number of live (TB-) cells after the weight correction, $N_{observed}$ is the number of live (TB-) cells counted on the chamber, W_{fixed} is the controlled weight (10 mg for testicular tissue and 50 mg for ovarian tissue), and $W_{observed}$ is the weight of each individual tissue piece measured on the analytical scale. Once the optimal enzymatic medium was determined, all further dissociations were done by using this enzymatic treatment.

4.7 Germline stem cell preservation

In the current study, both long- and short-term GSC preservation techniques were developed. For the long-term preservation strategy, two cryopreservation methods were chosen: (1) slow-rate freezing and (2) ultra-rapid cooling or vitrification. For short-term preservation, a procedure for hypothermic storage at 4 °C was optimized.

4.7.1 Freezing

To optimize the freezing procedure, several consecutive trials were conducted where the best outcome of each trial was used in subsequent trials. Even though in each trial a different cryopreservation parameter was tested, the freezing procedure itself was similar in all trials. Namely, the gonadal tissues were excised as described above (Section 4.4) and the gonadal tissues were stored in PBS in ice for no longer than 30 min. In all trials, gonadal fragments were frozen in 1.8 ml cryotubes filled with 1 ml cryomedium (containing both the extender and permeable and external cryoprotectants). Tissues were cut into smaller fragments (size was dependent on the tissue and trial), placed into the filled cryotubes, and equilibrated for a set duration on ice (the duration depended on the tissue and trial). Cryotubes were then placed into

CoolCell (BioCision; Research Instruments Pte Ltd, Clementi West, Singapore) freezing containers and placed into a deep freezer (- 80 °C) to enable cooling rates of ~1 °C/min. After reaching the desired plunging temperature, the samples were taken out of the deep freezer and plunged into liquid nitrogen. In the case of testing different cooling rates, samples were frozen using a controlled-rate freezer (IceCube 14S programmable freezer; IceCube Series v. 2.24, Sy-Lab, Neupurkersdorf, Austria) with different cooling rates down to the desired plunging temperature before being plunged into liquid nitrogen. Samples were stored in liquid nitrogen storage containers for at least 24 h. Cryotubes were then thawed in a water bath (the conditions depended on the tissue and trial), and the gonadal tissues were rehydrated and washed in three changes of L-15. Gonadal tissues were then weighed, dissociated as described above (Section 4.6) and the viability was calculated as described in Section 4.6.2.

4.7.1.1 Freezing of the testicular tissue

Freezing of testicular tissue was conducted through five sequential trials where the best outcome of each trial was used in subsequent trials (Figure 6). Firstly, the effects of six different cryoprotectants at a concentrations of 1.5 M on the viability of SSCs were tested. The tested cryoprotectants were dimethyl sulfoxide (Me_2SO), ethylene glycol (EG), propylene glycol (PG), methanol (MeOH), 2-methoxyethanol (2ME) and glycerol (Gly). In the next step, three cryoprotectants from the previous trial with the optimal outcome were tested in three different molar concentrations (1 M, 1.5 M, 2 M). After defining the optimal permeable cryoprotectant and its concentration, the cryomedium was supplemented with different sugar and protein sources to determine the optimal external cryoprotectant. Firstly, three sugar (0.1 M of either trehalose, glucose or sucrose) and two protein (either 1.5% of BSA or FBS) sources were tested. Additionally, the effects of three amino-acids (glycine – Gly, glutamine – Glut and proline – Prol) in three concentrations (10, 20 and 40 mg/ml) on SSC viability were tested. Lastly, three different plunging temperatures were tested by cooling testicular tissue to -40, -60 or -80 °C before plunging into liquid nitrogen. The baseline medium in which the tested cryoprotectant components were dissolved was PBS supplemented with 25 mM HEPES. In each trial, testicular tissues of three different individuals were used as biological replicates.

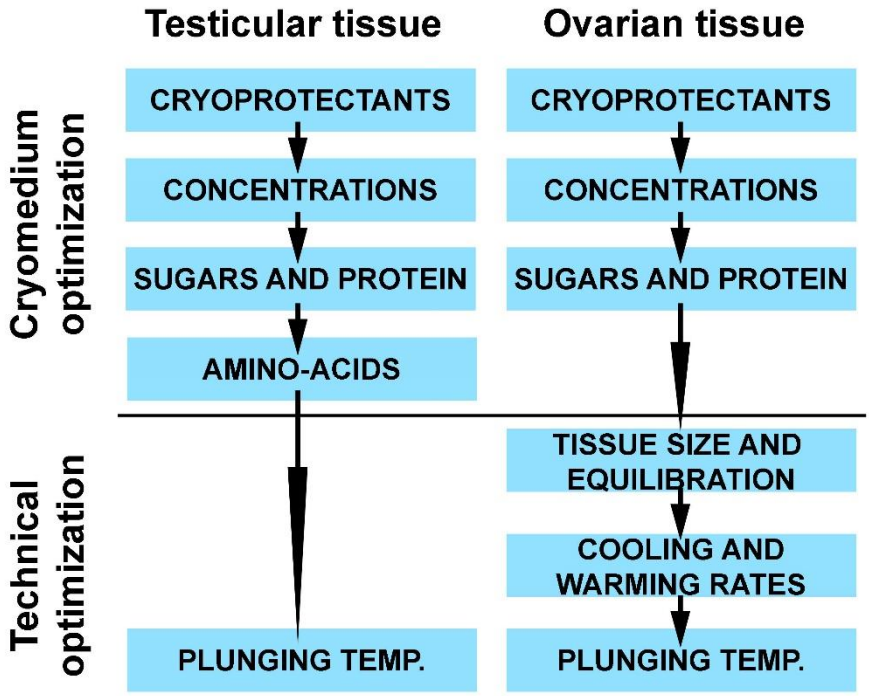

Figure 6. A flow chart displaying the sequence of freezing experiments conducted on testicular and ovarian tissues.

4.7.1.2 Freezing of ovarian tissue

Freezing of ovarian tissue was conducted through six sequential trials where the first three trials focused on optimizing the cryomedium, while the subsequent three trials focused on optimizing the technical aspects of the protocol (Figure 6). Cryomedium optimization was conducted by varying cryomedium components such as permeable and non-permeable cryoprotectants and their concentrations, while the technical development of the protocol included optimization of cooling and warming rates, tissue size, equilibration time and plunging temperatures.

In the first experiment of cryomedium optimization, each test group was composed of one of six cryoprotectants (Me_2SO, PG, EG, Gly, MeOH and 2ME) in a concentration of 1.5 M. Subsequently, the three cryoprotectants resulting with highest survival rate of the cells from the first experiment were used in 1, 1.5 and 2 M concentrations. Lastly, the effects of sugar (0.1 M of glucose – Glu, sucrose – Suc and trehalose – Tre) and protein (1.5% bovine serum albumin – BSA and 10% fetal bovine serum – FBS) supplementation were tested. The baseline medium in which the tested cryoprotectant components were dissolved was also PBS supplemented with 25 mM HEPES.

Upon these three experiments, cryomedium with the best outcome (1.5 M of Me_2SO, 0.1 M of glucose and 1.5% BSA diluted in PBS supplemented with 25 mM HEPES) was used for technical improvement in further experiments. Thus, the influence of different tissue sizes (25, 50 and 75 mg) and equilibration times (5, 15 and 30 minutes) in ice or at RT on cell survival was tested. Then, six cooling rates (-0.5, -1, -5, -10, -20 and -40 °C/min) were applied, each with two thawing approaches; one thawing approach was to thaw the samples in a 10 °C water bath, while the other was to thaw the samples at a controlled reciprocal rate to the cooling rate. This experiment was conducted using a controlled rate freezer (IceCube 14S programmable freezer; IceCube Series v. 2.24, Sy-Lab, Neupurkersdorf, Austria). Lastly, three different plunging temperatures were assessed by cooling ovarian tissue pieces at -1 °C/min down to -40, -60 and -80 °C before the tissue was plunged into lN_2. In each trial, ovarian tissues of three different individuals were used as biological replicates.

4.7.2 Vitrification

In the present study, needle-immersed vitrification (NIV) method was used, similarly to Lujić et al. (2017) and Marinović et al. (2018). With this method, testicular and ovarian tissue pieces are pinned to an acupuncture needle which is used for holding and manipulating the tissue pieces. The samples are then passed through an equilibration and vitrification solutions, and directly plunged into liquid nitrogen. The general methodological approach was the same for both males and females.

Firstly, the gonads were excised as described in Section 4.4 and cut into small pieces. Both testes and ovaries were cut into approximately 20 mg pieces and were stored in PBS on ice no longer than 30 min before the experiment was performed. Testicular or ovarian tissue pieces originating from three different (male or female) individuals were pooled together and three randomly chosen pieces were gently dried on a sterile wipe and pinned on an acupuncture needle without touching each other. Each acupuncture needle represented one test group and three tissue pieces represented replicates for the particular test group. Each needle was then placed in an equilibration solution (ES) for 5 min and subsequently in a vitrification solution (VS) for 30 sec. Excess liquid was carefully absorbed from the tissue by a sterile paper towel and the needles were plunged in liquid nitrogen. Needles were then placed into 5 ml cryotubes and stored in a storage dewar. After at least one day of storage, tissues were warmed in three sequential warming solutions (WS) at RT for different periods of time. All warming solutions contained L-15 supplemented with 10% FBS and various concentrations of trehalose (WS1 –

0.3 M; WS2 – 0.1 M; WS3 did not contain sucrose). Tissues were then weighed, dissociated (Section 2.6), and viability was determined as described above (Section 2.6.2).

Protocol optimization for vitrification was conducted by testing the effects of three equilibration solutions (ES1 – ES3) and three vitrification solutions (VS1 – VS3) on SSC and OSC viability. Different combinations of three cryoprotectants (Me_2SO, MeOH and EG) made up the equilibration solutions and the vitrification solutions (Table 4). The extender used consisted of L-15 supplemented with 10% FBS, 25 mM HEPES and 0.5 M trehalose.

Table 4. Test groups for the vitrification of European eel gonads with three equilibration (ES1 – ES3) and three vitrification solutions (VS1 – VS3) containing different combinations and concentrations of methanol (MeOH), propylene glycol (PG) and dimethyl sulfoxide (Me_2SO).

	Equilibration solution			Vitrification solution		
	ES1	ES2	ES3	VS1	VS2	VS3
MeOH	1.5 M	1.5 M	-	1.5 M	1.5 M	-
PG	1.5 M	-	1.5 M	4.5 M	-	3 M
Me_2SO	-	1.5 M	1.5 M	-	5.5 M	3 M

4.7.3 Hypothermic storage

During the hypothermic storage trials, effectiveness of storing tissue pieces or cell suspensions in maintaining germline stem cell viability was tested. Cells were stored at 4 °C. The experimental setup was very similar in both sexes, but with slight differences, so they will be described separately. In each trial, three males or three females were used as biological replicates.

In males, we firstly compared the effectiveness of storing testicular tissue pieces to the effectiveness of storing isolated testicular cell suspensions. Testes were dissected as described above (Section 4.4). Part of the testes was cut into ~20 mg pieces, placed in 1 ml storage medium in 24-well plates and refrigerated. The remainder of the testes was dissociated as described above (Section 4.6). After centrifugation, cells were resuspended in the storage medium, and approximately 200 µl of the suspension was placed in 1.5 ml tubes and refrigerated. Storage medium consisted of L-15 supplemented with 10% FBS, 25 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin. Initially, both tissues and cells were stored for 48 h, and viability was regularly checked every 12 h. Viability of SSCs in cell suspensions was done by trypan blue staining, counting the number of live cells, and comparing them to the initial cell number. Viability of testicular tissue pieces was checked by dissociating the tissue

pieces and comparing the number of obtained SSCs to the number of SSCs isolated from the fresh tissue (at 0 h; Section 4.6.2). As storage of cell suspensions displayed significantly higher viability, hypothermic storage of cell suspensions was extended to 144 h and viability was verified every 24 h. Testicular tissues of three different individuals were used as biological replicates.

In females, we also firstly compared the effectiveness of storing ovarian tissue pieces compared to storing isolated ovarian cell suspensions, however, with few minor modifications. The experiment was 24 h longer, i.e., it lasted 72 h. In addition, possible size effect of the tissue pieces on the survival rate of the cells was tested. Ovaries were dissected as described above (Section 4.4) and cut into 20, 50 and 100 mg pieces. Tissue pieces were placed in 1 ml storage medium in 24-well plates and refrigerated. The remainder of the ovarian tissue was dissociated as described above (Section 4.6) and after centrifugation, cells were resuspended in the storage medium and approximately 200 µl of the suspension was placed in 1.5 ml tubes and refrigerated. Similarly to the trials with testicular tissues, the storage medium consisted of L-15 supplemented with 10% FBS, 25 mM HEPES, 100 U/ml penicillin and 100 µg/ml streptomycin. The viability of OSCs stored in tissue pieces was checked every 12 h by dissociating the ovarian tissue pieces and comparing the number of obtained OSCs to the number of OSCs isolated from the fresh tissue (at 0 h; Section 4.6.2). Viability of OSCs in cell suspensions was done by trypan blue staining, counting the number of live cells, and comparing them to the initial cell number. As storage of cell suspensions displayed significantly higher viability, hypothermic storage of cell suspensions was extended to 312 h and viability was verified every 24 h until 144 h, and one additional time at 312 h (13 days). Ovarian tissues of three different individuals were used as biological replicates.

4.8 Transplantation of spermatogonial stem cells

To test whether GSCs are functional after cryopreservation, transplantation of frozen/thawed European eel SSCs was conducted. Common carp was chosen as a recipient species as it is a part regular broodstock at the Institute of Aquaculture and Environmental Safety (MATE, Gödöllő).

4.8.1 Preparation of cells

European eel males were dissected as described above (Section 4.4) and the testicular tissue pieces were frozen according to the optimal protocol (Section 4.7.1.1 and Section

5.3.1.1). One day before transplantation, testicular tissues were thawed and dissociated according to the optimal protocol (Section 4.6). Once a monodisperse cell suspension was obtained, donor cells were fluorescently labelled to enable visualization and tracking and transplanted cells within the recipients. Cells were stained with a fluorescent linker dye PKH-26 which intercalates into cell membranes. Three µl of dye was used for staining 1 million cells (Lujić et al., 2018b) and the staining was done by following the manufacturer's instructions. In short, isolated cells were washed twice in PBS, stained with 3 µl of dye / 1 million of cells for 5 min, and afterwards washed three times with L-15 supplemented with 10% FBS. Cells were finally resuspended in an appropriate volume of L-15 supplemented with 10% FBS.

4.8.2 Preparation of recipients

Diploid common carp larvae were used as recipients. Larvae were obtained by artificial spawning of the regular broodstock of the Institute of Aquaculture and Environmental Safety (MATE, Gödöllő). Three females and two males were selected and were injected with Ovopel according to the manufacturer's instructions. Namely, Ovopel is distributed in pellets (of approximately 25 mg) containing a mammalian GnRH analogue (D-Ala^6, Pro^{9}NEt-m-GnRH; ~20 µg/pellet) and a dopamine receptor antagonist metoclopramide (~10 mg/pellet) (Horváth et al., 1997). As suggested, the full dose was 1 pellet per 1 kg of body weight. Females were firstly injected with a priming dose (10% of the full dose) 22 h before spawning, and 12 h later with a resolving dose (90% of the full dose – 10 h before spawning). Males were injected with a single (full) dose 24 h before spawning.

Fish were checked for gamete production at the appropriate time by an abdominal massage. Once two out of three females were ready to spawn, eggs were collected by abdominal massage and mixed. Immediately after, milt was collected from the two injected males and mixed. Milt was added to the eggs together with a small volume of water in order to activate both eggs and spermatozoa, and was stirred for a short period. Subsequently, eggs were treated with the Wojnarowicz solution and tannic acid to prevent adhesion of eggs to plastic surfaces. This was an important step so that embryos can be injected with morpholino oligonucleotides (MOs). Namely, after a short initial stirring, an appropriate volume of the Wojnarowicz solution (4 g/l urea + 3.5 g/l NaCl) was added to the fertilized eggs and was stirred for 30 min. Afterwards, eggs were exposed twice to 5 g/l of tannic acid for 15 sec, with rinsing in system water between the two treatments, and after the second treatment. As the exposure to the Wojnarowicz solution was short (it is usually 90 min) since the embryos need

to be injected with MOs by the 8-cell stage, embryos were returned into the Wojnarowicz solution and were stirred until 90 mins have passed. During the second Wojnarowicz solution incubation, part of the embryos was continuously placed in a plastic Petri dish with a low volume of Wojnarowicz solution, injected with MOs, and returned into the Wojnarowicz solution until 90 min has passed.

In order to sterilize the common carp recipients and to prevent the development of the endogenous germline cells, recipient embryos were injected with MOs against the *dead end* gene (*dnd*-MO; 5'-CCTGCTGTAGCTGCTGTCCCTCCAT-3'). The *dnd* gene is the main gene involved in guiding PGCs during their migration into the genital ridge; the knock-out of knock-down of this gene causes mis-migration of PGCs which usually migrate into other organs and either undergo transdifferentiation or apoptosis (Gross-Thebing et al., 2017). Embryos at 2-8 cell stage were injected with approximately 3 ng of *dnd*-MO resuspended in nuclease free water. Injections were done by using a MINJ-1 microINJECTOR^{TM} System (Tritech Research, Los Angeles, CA, USA). Needles were obtained by pulling glass capillaries (Narishige GD-1; Narishige International Limited, London, UK) with a Narishige PN-31 needle puller and the edges of the needles were broken off with forceps to create a sharp edge. Injection volume was set by measuring the diameter of spheres created after injecting nuclease-free water into mineral oil. As described above, after injection embryos were transferred to the Wojnarowicz solution until 90 min has passed. Afterwards, embryos were incubated in the recirculation system until transplantation.

4.8.3 Transplantation of SSCs

Transplantation was done in 7 day-old MO-sterilized larvae. Recipient larvae were anesthetized in 0.03% 2-phenoxyethanol and transferred into a petri dish coated with 2% agar. Transplantations were also conducted by using a MINJ-1 microINJECTOR^{TM} System (Tritech Research). As described above, needles were obtained by pulling glass capillaries (Narishige GD-1) with a Narishige PN-31 needle puller and the edges of the needles were broken off with forceps to create a sharp edge. The approximate edge diameter of the needles was around 50 µm. Approximately 3000 frozen/thawed PKH-26-labelled cells were injected into the abdominal cavity of each recipient by inserting the tip of the needle at one of the two possible entry points: (1) between the swimming bladder and the intestines , or (2) behind the swimming bladder (Marinović, 2021). After injection, larvae were returned to the system water, and were reared for 60 days until the first checking of transplantation success by fluorescence

visualization. Furthermore, the recipients were reared for 2 more years until sexual maturity to observe whether progression of gametogenesis occurred. Control groups of intact fish and MO-sterilized larvae were exposed to the same rearing conditions as the experimental individuals were; however, no operations were conducted on them.

4.8.4 Verification of transplantation success

To confirm the colonization of donor-derived (frozen/thawed European eel) cells into recipient gonads, recipients were dissected 60-days post-transplantation (60 dpt) to localize the PKH-26-labelled cells through fluorescence microscopy. Recipients were euthanized in an overdose of 2-phenoxyethanol and were placed dorsally on a dissecting mat. Fish were then opened ventrally to expose their internal organs; the digestive organs were removed thus exposing the gonads. Gonads were checked for a fluorescent signal under a Leica M205FA stereomicroscope and were subsequently sampled in RNAlater for qPCR analyses.

After the initial verification, recipient individuals and controls were reared further. Fish were reared in a recirculation system at the Institute of Aquaculture and Environmental Safety (MATE, Gödöllő). Fish were housed under a 12 h light/12 h dark cycle at 24 ± 1 °C and were fed twice per day with a low-fat diet of appropriate size. At the age of six months, 30 recipients and 11 control fish were sampled. Fish were euthanized in a 2-phenoxyethanol overdose, dissected as described above (Section 2.4) and placed on a dissection mat. Their gonads were sampled in mDF for histological analyses (Section 4.5.1) and Trizol for qPCR analyses.

The remaining fish were reared for two more years until sexual maturity. By the age of 2+ years, both males and females in normal conditions become fertile. Therefore, the fish were checked for gamete production and gonadal development. Recipient fish were anesthetized in a 0.4 ml/l solution of 2-phenoxyethanol before handling. All recipients and control fish were stripped by an abdominal massage. In the case the fish gave some gametes, the gametes were collected, analyzed under the microscope, and sampled in Trizol for qPCR analyses. The recipients were then sacrificed by an overdose of 2-phenoxyethanol and a blow to the head. Fish were dissected as described above (Section 4.4) and their gonads were sampled in mDF for histological analyses (Section 4.5.1) and Trizol for qPCR analyses.

4.8.5 Molecular verification of transplantation success

Total RNA from preserved gonads from recipient and control individuals was isolated using phenol/chloroform extraction method in the Trizol Reagent (Life Technologies, Inc.,

Carlsbad, CA, USA). RNA concentration and 280/260 and 280/230 ratios were determined using NanoDrop One Spectrophotometer (Thermo Fisher Scientific). DNase I treatment and first-strand complementary DNA (cDNA) synthesis were performed from 500 – 1000 ng of total RNA from gonads from recipient and control individuals using Random Hexamer Primer and RevertAID Reverse Transcriptase enzyme (Thermo Fisher Scientific) following the manufacturer's instructions.

Quantitative real-time Polymerase Chain Reactions (qPCR) were carried out using specific qPCR primers for common carp *dnd1* gene (*Fw*: 5'-CGGCCGGCCGGAGAGATGAG-3'; *Rv*: 5'- GATCTGGATAACCCCGCACA-3') (Franěk et al., 2019a) and European eel *dnd1* gene (*Fw*: 5'- CGGGACATCTACGAGGACAA-3'; *Rv*: 5'-TTCATCATCAGGCGGAACTC-3') (Blanes-García et al., 2024). Prior to the analyses, the primers were tested and were found to be species-specific and did not cross-react. Primers were purchased from Integrate DNA Technology Inc. (IDT, Coralville, IA, USA).

Expression of common carp and European eel *dnd1* in gonads of control and recipient common carps were measured by performing qPCR assays using a StepOnePlus real-time PCR System (Step One Plus Real-Time PCR Systems, Thermo Fisher Scientific) with 5× Hot FIREPol EvaGreen qPCR Mix Plus (Solis Biodyne, Tartu, Estonia). qPCR program was performed as an initial step of 95 ºC for 10 min and 40 cycles of 95 ºC for 15 s, 60 ºC for 30 s, and 95 ºC for 15 s. To evaluate assay specificity, the qPCR instrument performed a melting curve analysis directly after PCR by slowly (0.3 ºC/s) increasing temperature from 60 to 95 ºC, with continuous registration of any changes in fluorescent emission intensity. A total volume for each qPCR reaction was 10 µl with 2.5 µl of diluted cDNA (1:20) template, forward and reverse primers (250 nM each) and SYBR Green/ROX Master Mix (2 µl). Target genes in samples were run in duplicate PCR reactions. A non-template control (cDNA replaced by water) and a negative control using genomic DNA (25 ng/µl) was run in all plates for each primer pair.

5 RESULTS

5.1 Histological analyses of the gonadal tissue

5.1.1 Histology

Histological samples were first fixed in 10% neutral buffered formalin. It was possible to recognize that both male and female gonads were immature, however, it was also very clear that the samples were not properly fixed in this solution. Testicular cells (especially SSCs) seemed to have shrunk compared to their normal size which was evident by the presence of vacuolization along the cell's edges (Figure 7A-A''). Nuclear details were also not detectable as the nuclei seemed very condensed. Within the ovarian tissue, oocytes appeared very scattered with a lot of vacuolization between them (Figure 8A, A'). Almost no germline cells below the size of stage V oocytes were observed. Therefore, we have decided to change the approach and fix the cells in a modified Davidson's solution (mDF).

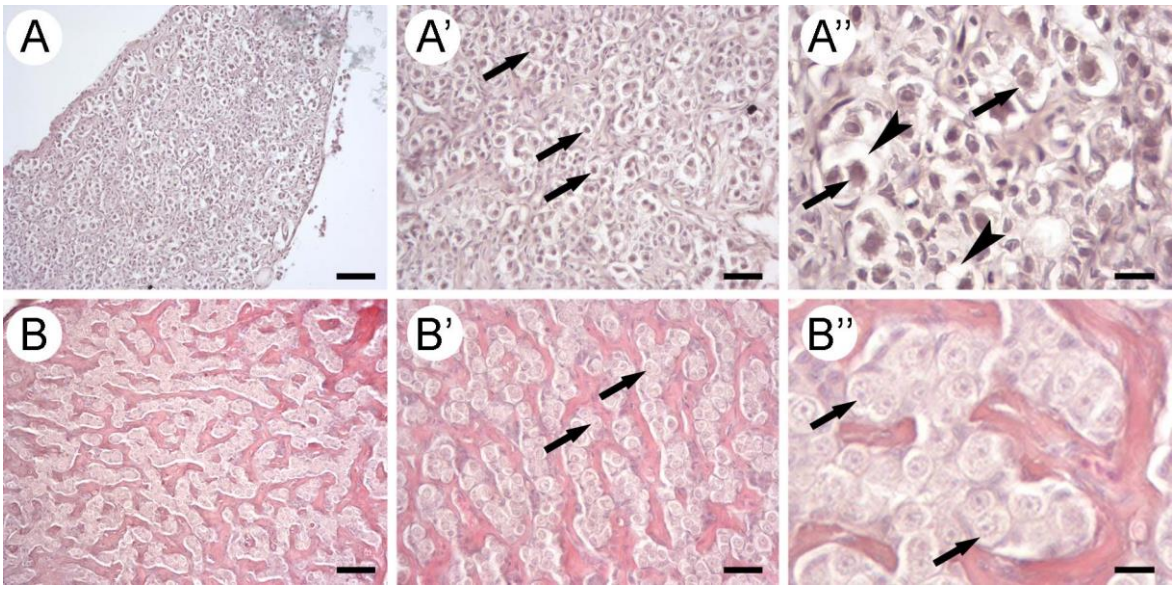

Figure 7. Histological observations of European eel testicular tissue fixed in 10% normal-buffered formalin (10% NBF; A-A'') or modified Davidson's fixative (mDF; B-B''). Arrows – spermatogonial stem cells (SSCs); arrowheads – vacuolizations. A, B – samples under 20× magnification; A', B' – samples under 40× magnification; A'', B'' – samples under 100× magnification. Scale bars: A, B – 50 µm; A', B' – 25 µm; A'', B'' – 10 µm.

Fixation in the mDF substantially improved the quality of the samples. Histological observations displayed that both male and female gonadal tissues were immature. Testicular tissue was composed of spermatogonial stem cells (SSCs) that were the only germline cells present in the tissues, and the supporting somatic cells (Figure 7B-B''). Nuclear details were also very clear. Within the germline cysts, SSCs were characterized by their large and round

nuclei containing a large nucleolus, while Sertoli cells were characterized by a more condensed and oval nucleus. Ovarian tissue contained more germline cells (Figure 8B, B'). Namely, oogonial stem cells (OSCs) were not the only germline cells within the ovarian tissue; a predominant presence of early-stage oocytes, ranging from the smaller leptotene/zygotene oocytes (stage I and II) to larger mid-vitellogenic oocytes (stage V) was also observed. However, even in mDF it was difficult to distinguish smaller than early-mid vitellogenic oocytes, and vacuolizations were observed as well.

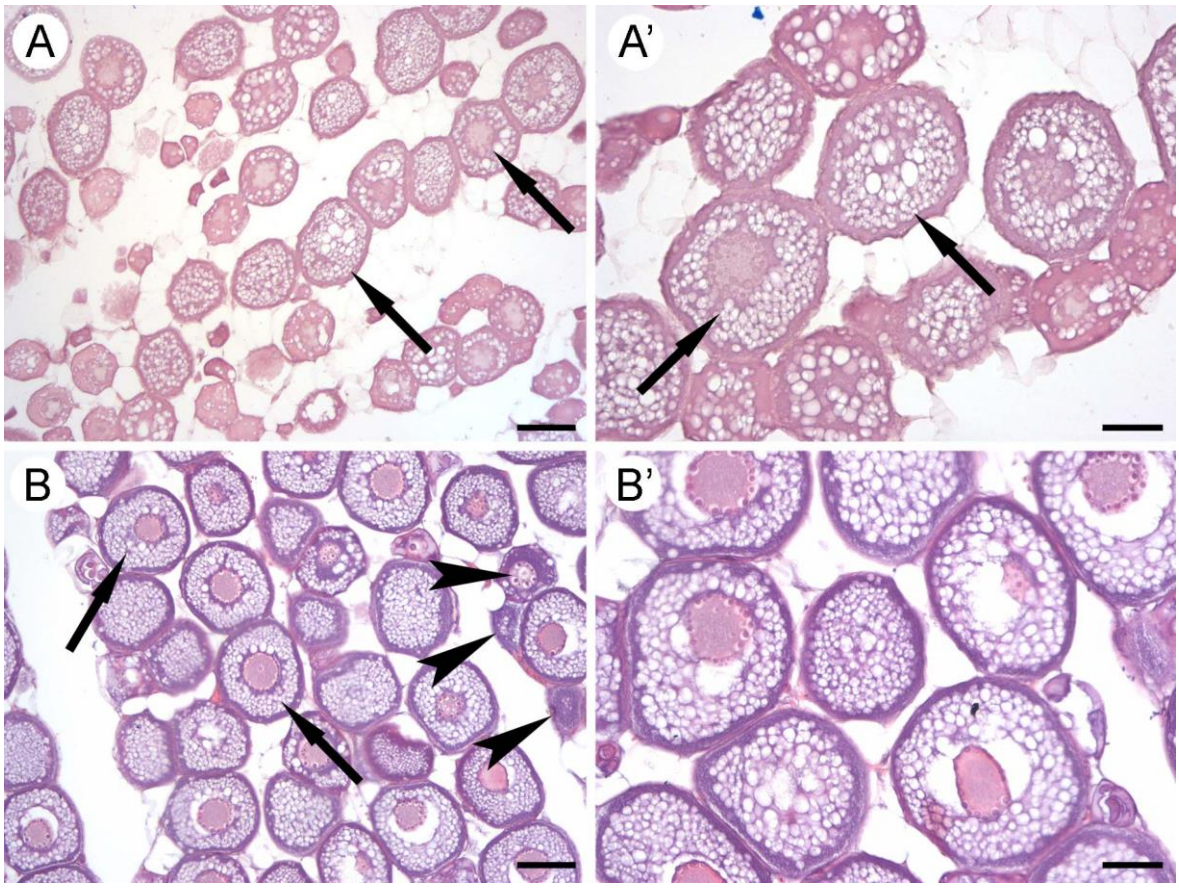

Figure 8. Histological observations of European eel ovarian tissue fixed in 10% normal-buffered formalin (10% NBF; A, A') or modified Davidson's fixative (mDF; B, B'). Arrows – mid-vitellogenic oocytes (stage V); arrowheads – leptotene/zygotene oocytes (stage I and II). A, B – samples under 10× magnification; A', B' – samples under 20× magnification; Scale bars: A, B – 50 µm; A', B' – 25 µm.

5.1.2 Western blot

The aim of western blot was to determine the specificity and capability of the antibody used against the vasa (DDX4) to correctly bind to its target antigen. After the procedure, two bands of approximately 76 kDa and 65 kDa were identified (Figure 9). These two bands correspond to the vasa1 and vasa2 proteins identified in the European eel. According to

database insertions and bioinformatic predictions, the molecular weight of vasa1 (XP_035234983.1) is 78 kDa, while the molecular weight of vasa2 (XP_35248267.1) is 69 kDa. Based on these results, a conclusion can be drawn that the antibody against vasa used in this study successfully identifies both vasa isoforms and can be used further in immunohistochemistry.

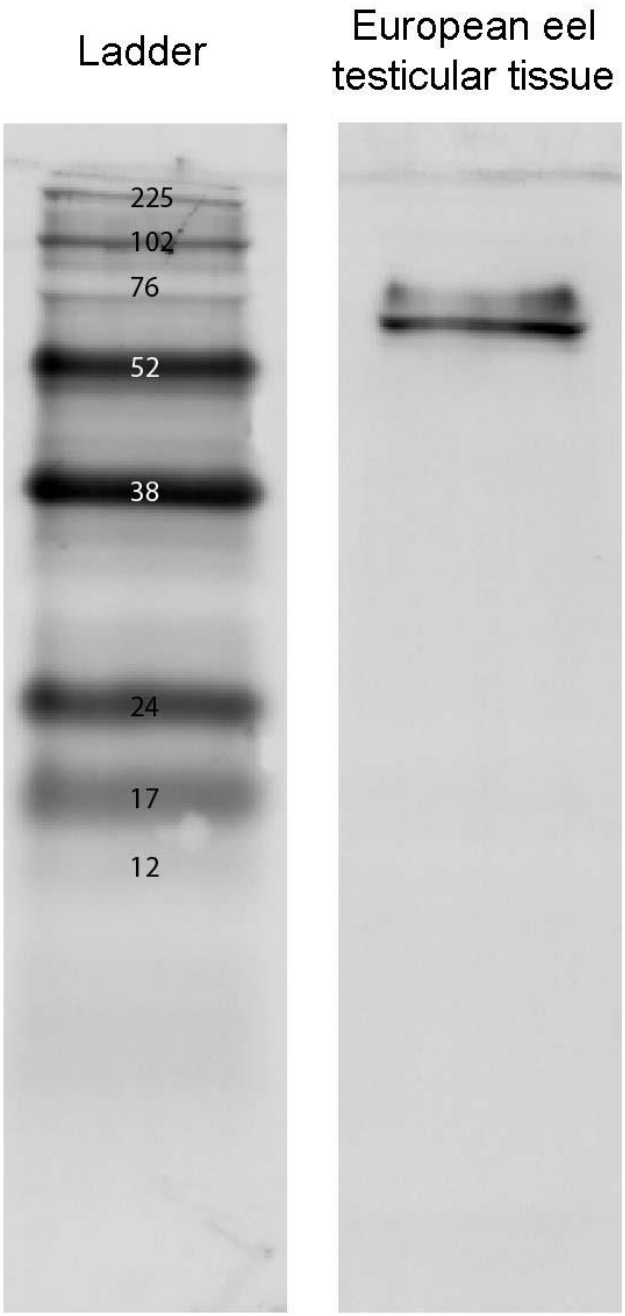

Figure 9. Western blot analyses of the binding capacity of the anti-vasa antibody (DDX4; Abcam, cat. no. ab13840) in the European eel testicular tissue.

5.1.3 Immunohistochemistry

After confirming that the anti-vasa antibody specifically labels the vasa antigen, samples fixed in mDF were labelled for the immunolocalization of this antigen through immunohistochemistry. Within the testicular tissue, only SSCs displayed a positive DAB signal (Figure 10). These cells were identified by their large size, and large nuclei with pronounced nucleoli. All other cells did not display a DAB signal. Furthermore, a signal was not observed in the secondary antibody control (samples stained only with the secondary antibody) nor in the DAB control (samples not exposed to any of the antibodies but stained with DAB).

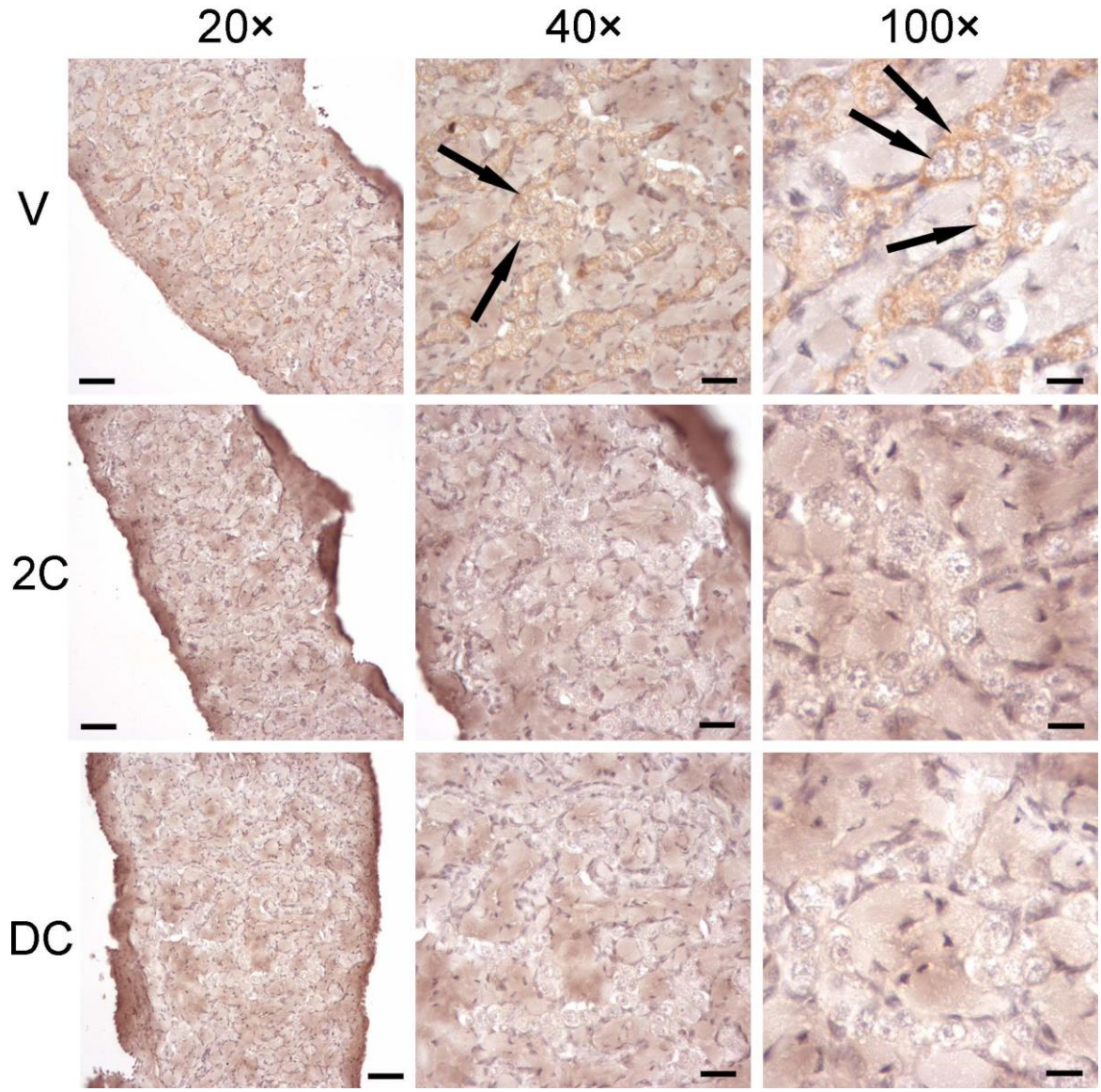

Figure 10. Immunohistochemical localization of the vasa (DDX4) protein in immature European eel testicular tissue. V – staining for the vasa protein; 2C – secondary antibody control; DC – DAB control. Arrows – SSCs displaying positive DAB signal. Samples were photographed at 20×, 40× and 100× magnification. Scale bars (vertically through panels): 20× – 50 µm; 40× – 25 µm; 100× – 10 µm.

In ovarian tissue, only germline cells expressed the vasa antigen. It was detected in all germline cells, including the early-stage oocytes (Figure 11). The signal was the strongest in the cytoplasm of the vitellogenic oocytes, while in stage I oocytes it was diffusely spread in the cytoplasm, and was also concentrated around the nucleus. Stage I oocytes and OSCs displayed the signal evenly throughout the cytoplasm. On the other hand, somatic (follicular) cells did not display a positive signal. A signal was also not observed in the secondary antibody control (samples stained only with the secondary antibody) nor in the DAB control (samples not exposed to any of the antibodies, but stained with DAB).

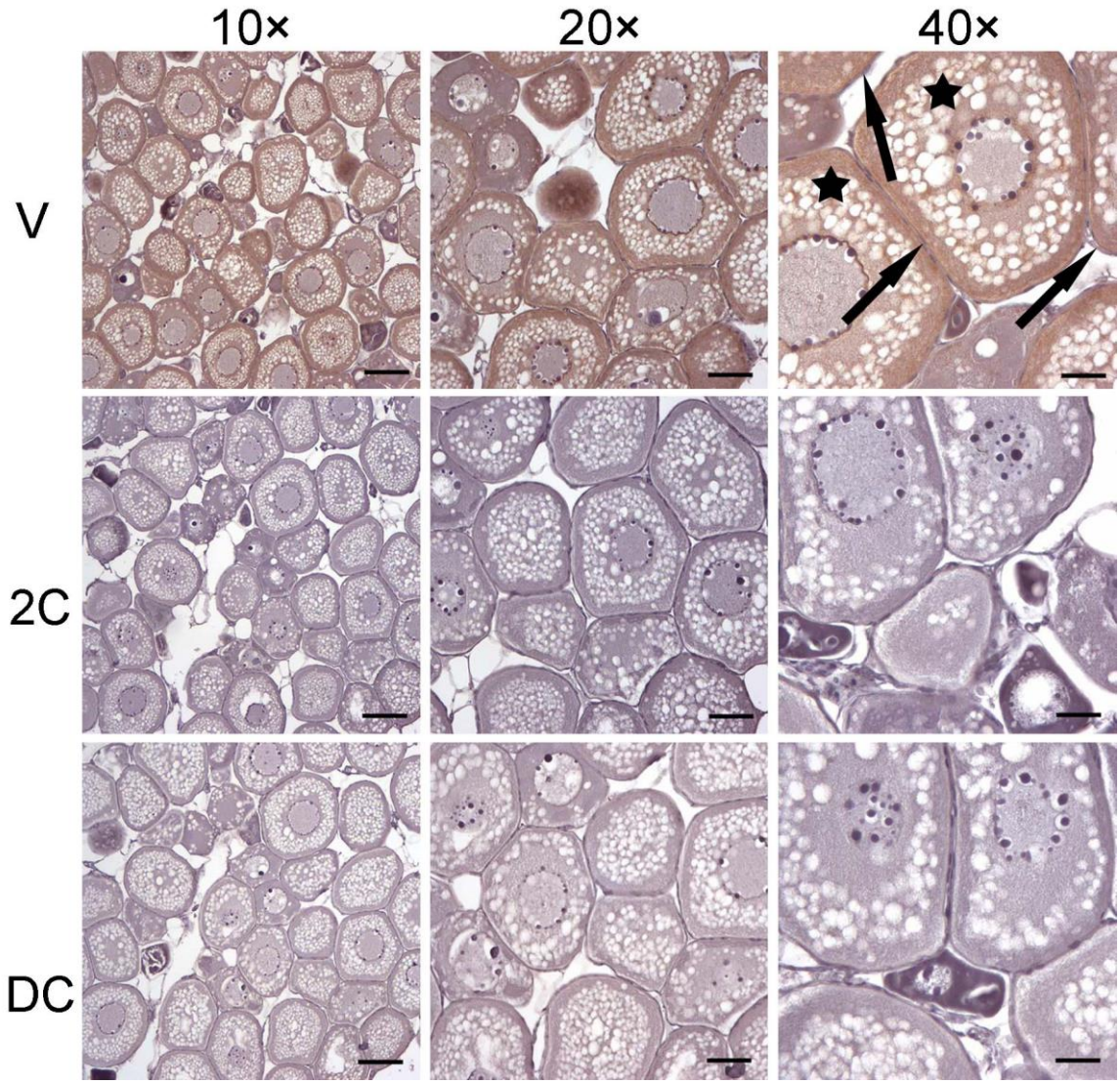

Figure 11. Immunohistochemical localization of the vasa (DDX4) protein in immature European eel ovarian tissue. V – staining for the vasa protein; 2C – secondary antibody control; DC – DAB control. Asterisk – DAB-positive oocytes; arrows – DAB-negative follicular cells. Samples were photographed at 20×, 40× and 100× magnification. Scale bars (vertically through panels): 10× – 100 µm; 20× – 50 µm; 40× – 25 µm.

5.2 Isolation of GSCs

During the dissociation of testicular tissue, dissociation media did not have a significant effect on the number of isolated SSCs (One-way ANOVA; $p > 0.05$). In addition, there were no differences between the tested groups (Tukey's HSD post-hoc; $p > 0.05$). However, the highest average viability was observed when using dissociation medium 6 (Figure 12A; 2 mg/ml collagenase + 3 mg/ml trypsin), therefore, in subsequent trials we have used this enzymatic combination.

During dissociation of the ovarian tissue on the other hand, a significant effect of the dissociation medium on the number of isolated OSCs was observed (One-way ANOVA; $p < 0.05$) highlighting the concentration-dependent efficiency of the enzymes. However, statistical delineation between individual groups was not pronounced as only the medium with the highest yield was significantly different from the group with the lowest yield. Similarly to the testicular tissue, the highest yield was obtained by using dissociation medium 6 (Figure 12B; 2 mg/ml collagenase + 3 mg/ml trypsin), which was used in subsequent trials for dissociation of ovarian tissue as well.

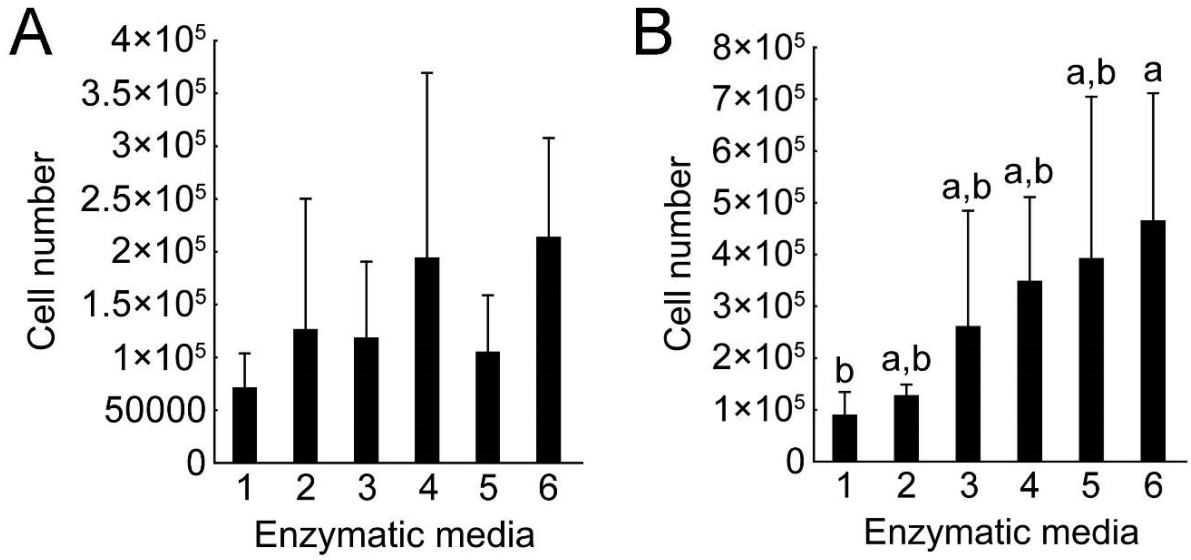

Figure 12. Dissociation of European eel testicular (A) and ovarian (B) tissues with different enzymes and their combinations. Enzymatic media contained various concentrations and combinations of collagenase and trypsin: 1 – 2 mg/ml collagenase; 2 – 6 mg/ml collagenase; 3 – 1.5 mg/ml trypsin; 4 – 3 mg/ml trypsin; 5 – 2 mg/ml collagenase + 1.5 mg/ml trypsin; 6 – 2 mg/ml collagenase + 3 mg/ml trypsin. All values are presented as mean±SD. *N*=3. Different letters above the SD bars indicate statistical significance (Tukey's HSD, $p < 0.05$); the lack of letters indicates no significant differences.

5.3 Preservation

5.3.1 Freezing

5.3.1.1 Freezing of testicular tissues

Post-thawing procedure displayed a superiority of Me_2SO in comparison to other cryoprotectants and resulted in approximately 45 % of viable cells (Figure 13A). In the second trial, Me_2SO and PG provided the highest cell survival rate at 1.5 M concentration (compared to 1 M and 2 M), while 2 M was the optimal concentration for EG (Figure 13B). For further trials, 1.5 M Me_2SO was used as it yielded the highest average viability. Sugar and protein supplementation did not display significant differences between the tested groups (Figure 13C). Similar was observed for amino-acid supplementation (Figure 13D) as the addition of none of the amino-acids increased the viability of SSCs post-thaw. When testing different plunging temperatures, plunging at -80 °C resulted in superior viability rates compared to the other two groups (Figure 13E). The optimized cryomedium contained 1.5 M of Me_2SO supplemented with 0.1 M glucose and 1.5% BSA with the highest average viability reaching ~ 50%.

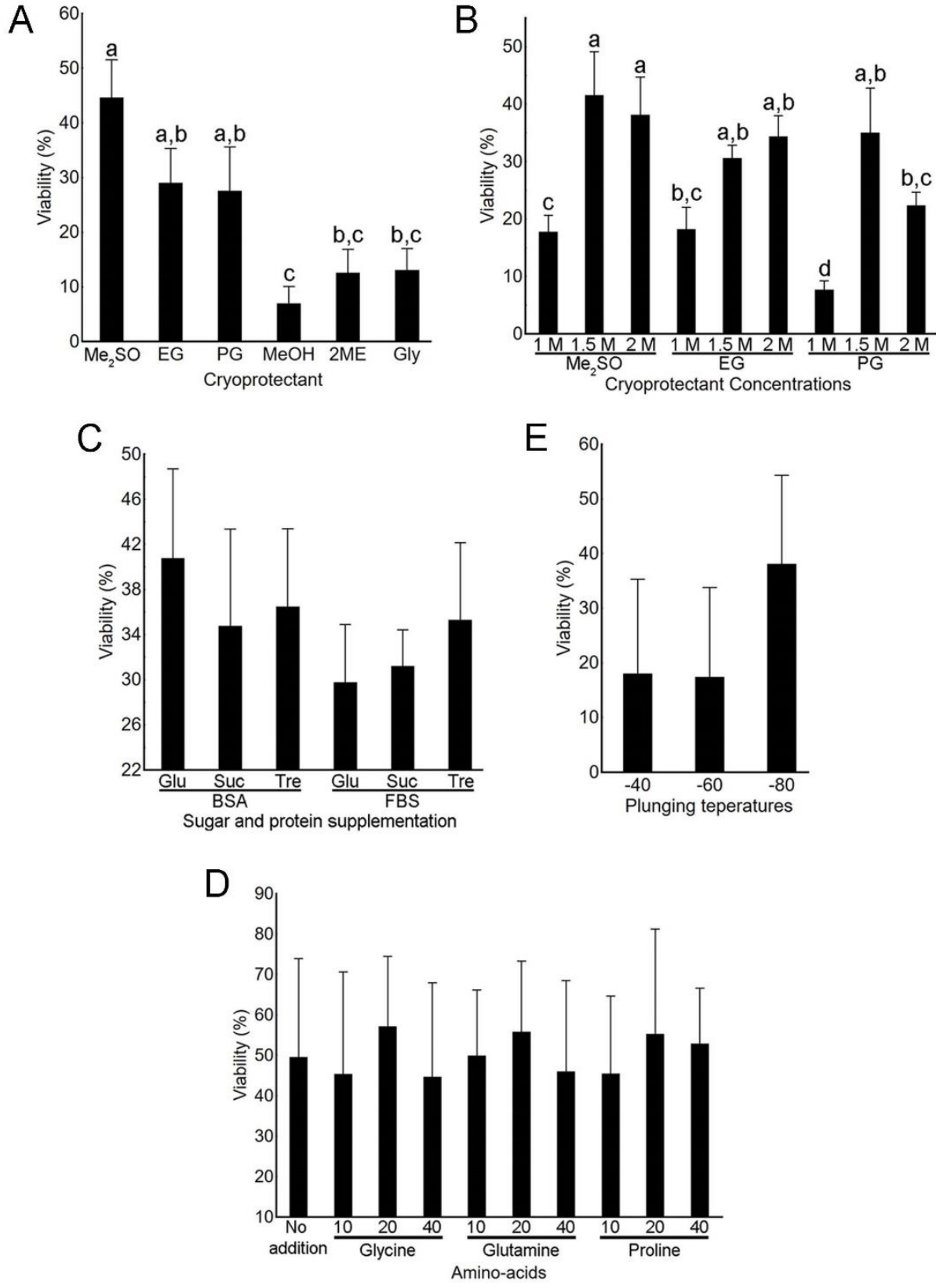
A
Viability (%)
a
a,b
a,b
c
b,c
b,c
Me2SO
EG
PG
MeOH
2ME
Gly
Cryoprotectant
B
Viability (%)
c
a
a
b,c
a,b
a,b
d
a,b
b,c
1 M 1.5 M 2 M
Me2SO
EG
PG
Cryoprotectant Concentrations
C
Viability (%)
Glu
Suc
Tre
Glu
Suc
Tre
BSA
FBS
Sugar and protein supplementation
E
Viability (%)
-40
-60
-80
Plunging teperatures
D
Viability (%)
No addition
10
20
40
Glycine
Glutamine
Proline
Amino-acids

Figure 13.

Figure 13 (continued). Optimization of the freezing protocol for the European eel spermatogonial stem cells (SSCs). (**A**) Viability of SSCs after freezing with 1.5 M dimethyl sulfoxide (Me_2SO), ethylene glycol (EG), propylene glycol (PG), methanol (MeOH), 2-mercaptoethanol (2-ME) and glycerol (Gly). (**B**) Viability of SSCs after freezing with either 1, 1.5 or 2 M Me_2SO, EG or PG. (**C**) The effects of sugar (glucose – Glu, Sucrose – Suc and trehalose – Tre) and protein (1.5% BSA and 10% FBS) supplementation on SSC viability. (**D**) Effects of amino-acid (glycine, glutamine and proline) supplementation at concentrations of 10, 20 or 40 mg/ml on SSC viability. (**E**) Effect of plunging temperatures on SSC survival. All values are presented as mean±SD. *N*=3. Different letters above the SD bars indicate statistical significance (Tukey's HSD, $p < 0.05$); the lack of letters indicates no significant differences.

5.3.1.2 Freezing of ovarian tissue

The first experiment in optimization of the cryopreservation of European eel OSCs displayed the superiority of three cryoprotectants: Me_2SO, EG and GLY (Fig 14A). In the subsequent experiments, these three cryoprotectants were used in different molar concentrations (1, 1.5, and 2 M) where they displayed similar results. However, the highest average viability was observed when using 1.5 M of Me_2SO (Fig 14B), therefore this cryoprotectant was chosen for the subsequent experiments. Varying sugar and protein supplementation did not display significant differences in cell survival (Fig 14C), therefore cryomedia with 1.5 M Me_2SO supplemented with 0.1 M glucose and 1.5% BSA was used for the technical optimization of the cryopreservation protocol.

No significant differences were observed between the tested cooling rates when thawing in a 10 ℃ water bath (warming procedure 1), while reciprocal thawing rates (warming procedure 2) yielded slightly lower viability rates (Fig. 14D). Since freezing at a cooling rate of ~1 ℃/min does not necessitate expensive and dedicated equipment such as a controlled-rate freezer and can be done in simple CoolCell boxes in dry ice (or a deep freezer), this cooling rate was chosen for further trials. Similarly, as warming procedure 1 does not necessitate a controlled-rate freezer, and can be done in a water bath, this thawing procedure was selected for subsequent trials. When comparing the effects of different tissue sizes and equilibration techniques, equilibration of 50 mg tissue pieces for 30 min on ice resulted in the highest viability (Fig 14E). Only equilibration time had a significant effect on viability ($p < 0.05$), while Tukey's HSD post-hoc test displayed no significant differences among the tested groups. Lastly, plunging temperature of -80 ℃ was superior (~85%) to the other plunging rates (Fig 14F).

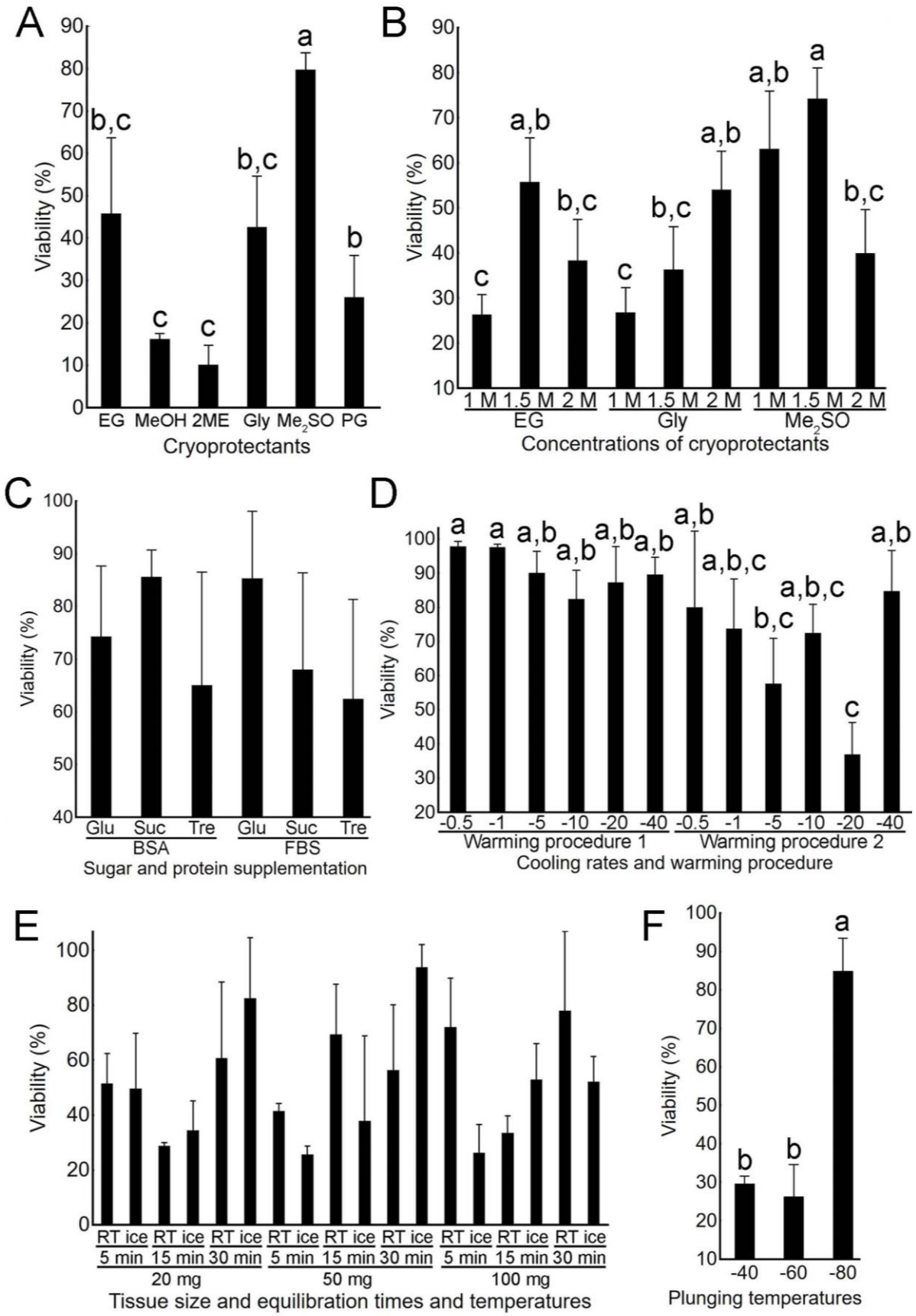
A
Viability (%)
b,c
c
c
b,c
a
b
EG MeOH 2ME Gly Me2SO PG
Cryoprotectants
B
Viability (%)
c
a,b
b,c
c
b,c
a,b
a,b
a
b,c
1 M 1.5 M 2 M
EG
Gly
Me2SO
Concentrations of cryoprotectants
C
Viability (%)
Glu Suc Tre Glu Suc Tre
BSA
FBS
Sugar and protein supplementation
D
Viability (%)
a a a,b a,b a,b a,b a,b a,b,c b,c a,b,c c a,b
-0.5 -1 -5 -10 -20 -40 -0.5 -1 -5 -10 -20 -40
Warming procedure 1
Warming procedure 2
Cooling rates and warming procedure
E
Viability (%)
RT ice 5 min
RT ice 15 min
RT ice 30 min
20 mg
50 mg
100 mg
Tissue size and equilibration times and temperatures
F
Viability (%)
b b a
-40 -60 -80
Plunging temperatures

Figure 14.

Figure 14 (continued). Optimization of the freezing protocol for the European eel oogonial stem cells (OSCs). (**A**) Viability of OSCs after freezing with 1.5 M ethylene glycol (EG), methanol (MeOH), 2-mercaptoethanol (2-ME), glycerol (Gly), dimethyl sulfoxide (Me_2SO) and propylene glycol (PG). (**B**) Viability of OSCs after freezing with either 1, 1.5 or 2 M of EG, Gly and Me_2SO. (**C**) The effects of sugar (glucose – Glu, Sucrose – Suc and trehalose – Tre) and protein (1.5% BSA and 10% FBS) supplementation on OSC viability. (**D**) The effects of six cooling rates (-0.5, -1, -5, -10, -20 and -40 ℃/min) and warming in a 10 ℃ (procedure 1) or warming at reciprocal warming rates (procedure 2) on OSC viability. (**E**) Influence of different tissue sizes (25, 50 and 75 mg) and equilibration times (5, 15 and 30 minutes) in ice or at room temperature (RT) on cell survival. (**F**) Effect of plunging temperatures on OSC survival. All values are presented as mean±SD. *N*=3. Different letters above the SD bars indicate statistical significance (Tukey's HSD, $p < 0.05$); the lack of letters indicates no significant differences.

5.3.2 Vitrification

In males, both ES and VS had a significant effect on SSC viability (two-factor ANOVA, $p < 0.01$). VS3 yielded significantly higher viability than the other two VS except when combined with ES3, while ES3 demonstrated generally higher viability rates with each VS compared to other ES solutions (Figure 15A). The highest average viability was observed when combining VS3 with ES2, however, these results were not significantly higher than the combinations of VS2 and VS3 with ES3. The highest average viability reached ~ 70%.

Both ES and VS had a significant effect on OSC viability as well (two-factor ANOVA, $p < 0.01$). The highest viability was obtained by using the combination of ES3 and VS3 (~ 80%; Figure 15B). All other ES/VS combinations expect ES2/VS3 had a significantly lower viability. Therefore, an optimized protocol that would yield high viability of both SSCs and OSCs would be to firstly expose the pinned gonadal tissues to an ES containing 1.5 M Me_2SO and 1.5 M PG for 15 min, then to a VS containing 3 M Me_2SO and 3 M PG for 30 sec, wipe the excess of cryoprotectants and plunge the tissues into liquid nitrogen.

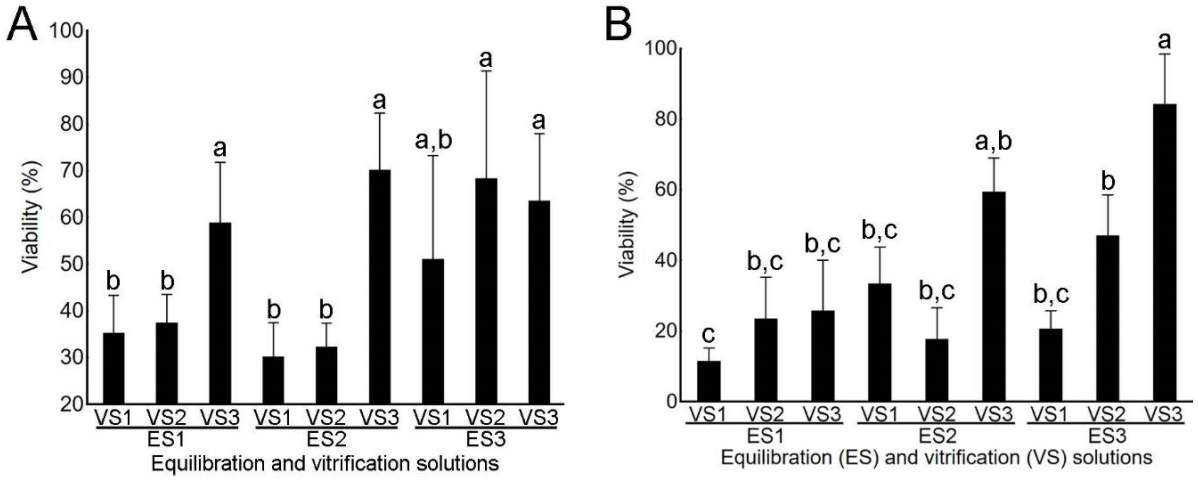

Figure 15.

Figure 15 (continued). The effects of different equilibration (ES) and vitrification (VS) solutions on germline stem cell viability after needle-immersed vitrification in (A) males and (B) females. All values are presented as mean±SD. *N*=3. Different letters above the SD bars indicate statistical significance (Tukey's HSD, $p < 0.05$).

5.4 Hypothermic storage

In males, when comparing the effectiveness of hypothermically storing tissue pieces or cell suspensions, storage of isolated cells displayed significantly higher viability at each time point during the 48-hour trial (Figure 16A). At the end of the trial, viability of SSCs isolated from the stored tissue was significantly lower than the fresh control, while there were no differences between cell suspensions and fresh control. Therefore, storage of isolated cells was prolonged to 144 h. No significant differences in viability were observed at any time point during the trial. By the end of the trial (144 h), the average SSC viability was around 75% (Figure 16B).

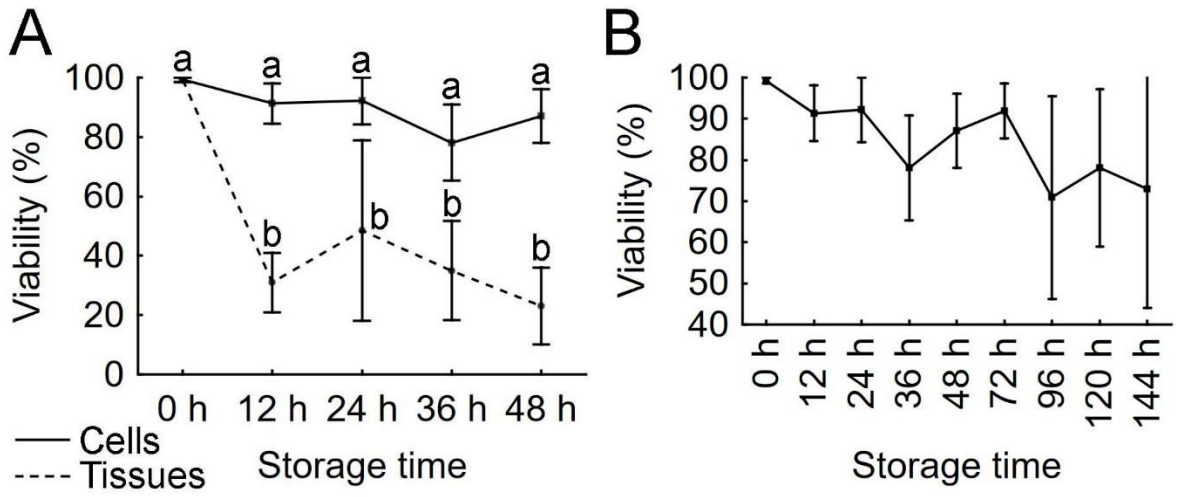

Figure 16. Hypothermic storage of European eel spermatogonial stem cells (SSCs). (**A**) The effects of storing testicular tissue pieces vs testicular cell suspensions on SSC survival. (**B**) The effects of 144 h storage of cell suspensions on SSC survival. All values are presented as mean±SD. *N*=3. Different letters above the SD bars indicate statistical significance (Tukey's HSD, $p < 0.05$); the lack of letters indicates no significant differences.

In females on the other hand, a different viability pattern was observed. At all time points, the viability of 20 and 50 mg tissue pieces was similar to that of cell suspensions (Figure 17A). At the 72-hour mark, the viability of OSCs stored in cell suspensions was higher than in tissue pieces, however, the differences were not statistically significant. The viability of cells stored in 100 mg tissue pieces was significantly lower at each time point. Since the viability of cell suspensions was higher on average, storage of cell suspensions was continued until 312 h.

A significant viability reduction compared to the control was observed at 48 h of storage. However, after that point, there were no more significant reductions in viability. By the end of the trial (312 h), the average OSC viability was slightly above 60% (Figure 17B).

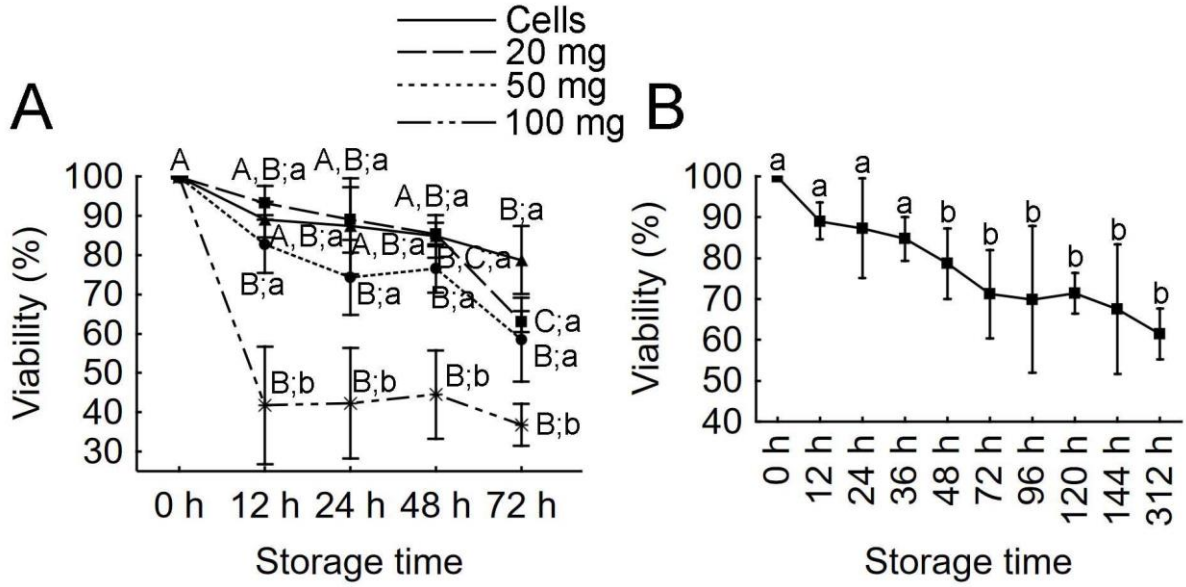

Figure 17. Hypothermic storage of European eel oogonial stem cells (OSCs). (**A**) The effects of storing ovarian tissue pieces vs testicular cell suspensions on OSC survival. (**B**) The effects of a 312 h storage of cell suspensions on OSC survival. All values are presented as mean±SD. *N*=3. Different letters above the SD bars indicate statistical significance (Tukey's HSD, $p < 0.05$). (A) Capital letters indicate significant differences from the control during storage, while lowercase letters indicate significant differences between tissue sizes at a specific time points.

5.5 Transplantation of SSCs

Transplantation of SSCs was conducted to verify the functionality of SSCs after freezing. Frozen/thawed European eel SSCs were transplanted into *dnd*-MO sterilized common carp larvae. 60 days post-transplantation, the viability of recipients (~80%) was similar to the controls (~85%). Upon fluorescence inspection, 8 out of 25 recipients (32%) have displayed red fluorescence within their gonads (Figure 18A-B'). This fluorescence was originating from the PKH-labelled donor (European eel) SSCs and was indicative of successful incorporation of donor-derived SSCs into the recipient gonads. None of the control individuals have displayed red fluorescence within their gonads (Figure 18C, C').

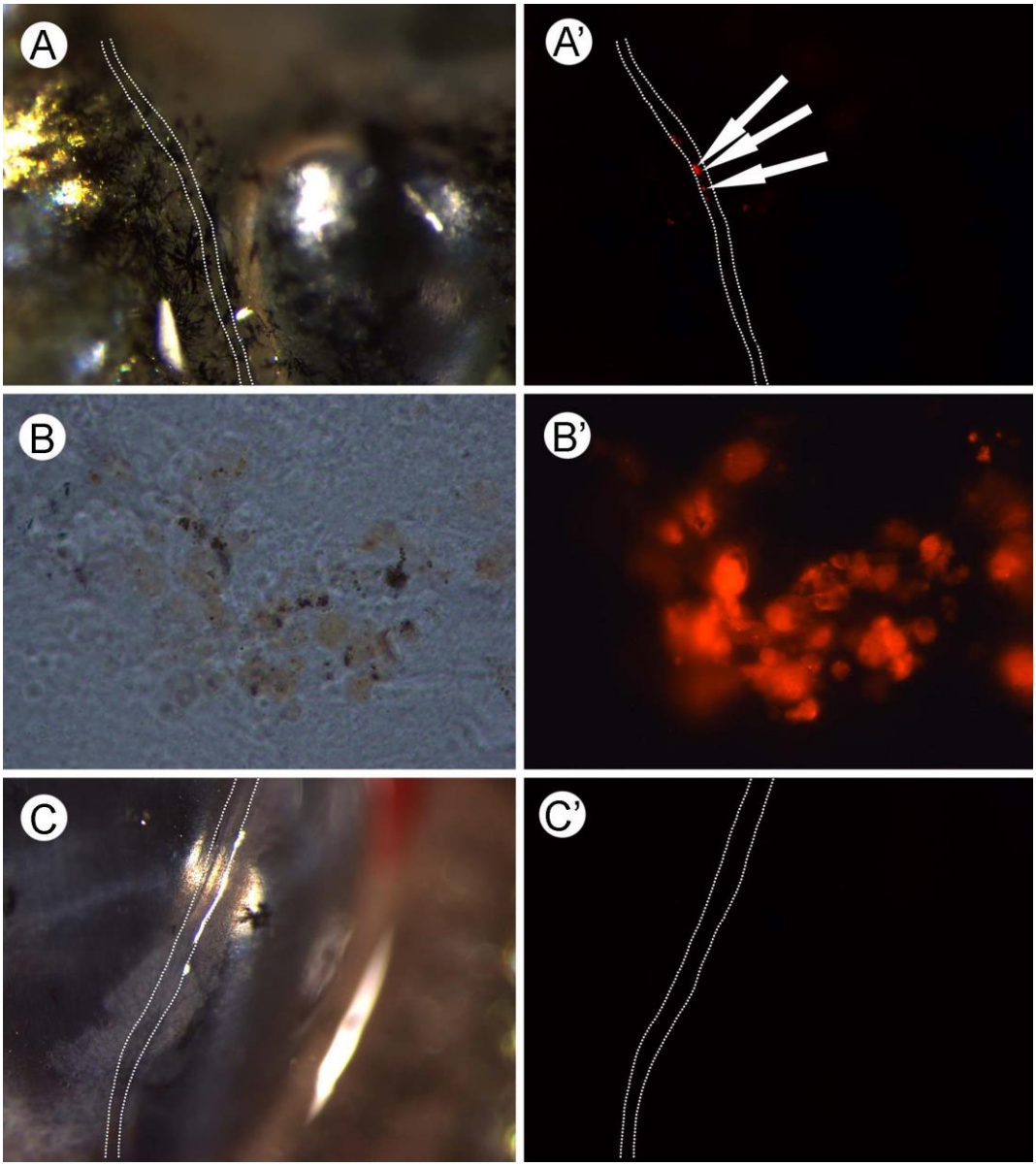

Figure 18. Fluorescent verification of incorporation of donor European eel SSCs into recipient common carp gonadal ridges. (**A**, **A'**) Observation of the recipient gonads under a fluorescent stereomicroscope showing a positive PKH-derived signal in recipient gonads. (**B**, **B'**) Observation of an isolated gonad under a fluorescent microscope displaying the presence of PKH-labelled cells within recipient gonads. (**C**, **C'**) Observation of gonads of control fish under a fluorescent stereomicroscope showing a lack of a fluorescent signal.

Recipient fish were dissected again 6 months after transplantation, and the gonads were sampled for histological and qPCR analyses. Histological observations displayed a lack of germline cells within the recipient fish (Figure 19A, A'). Similar was observed in control fish (Figure 19B, B') except for 1 fish in which we observed the presence of stage I oocytes (Figure 19C, C'). Furthermore, a lack of germline cells was demonstrated by immunohistochemical staining against the vasa antigen (Figure 20A, A'). A positive vasa signal was observed only in one control gonad (Figure 20B, B') in which we identified germline cells through H&E

histology. A positive signal was also not observed in the secondary antibody control (Figure 20C, C'), even though the sections showed a slightly higher background staining, most likely due to the sample structure. Further qPCR analyses displayed a lack of expression of European eel *dnd1* gene in all recipient and control fish, while an expression of common carp *dnd1* gene was observed in one recipient and one control fish.

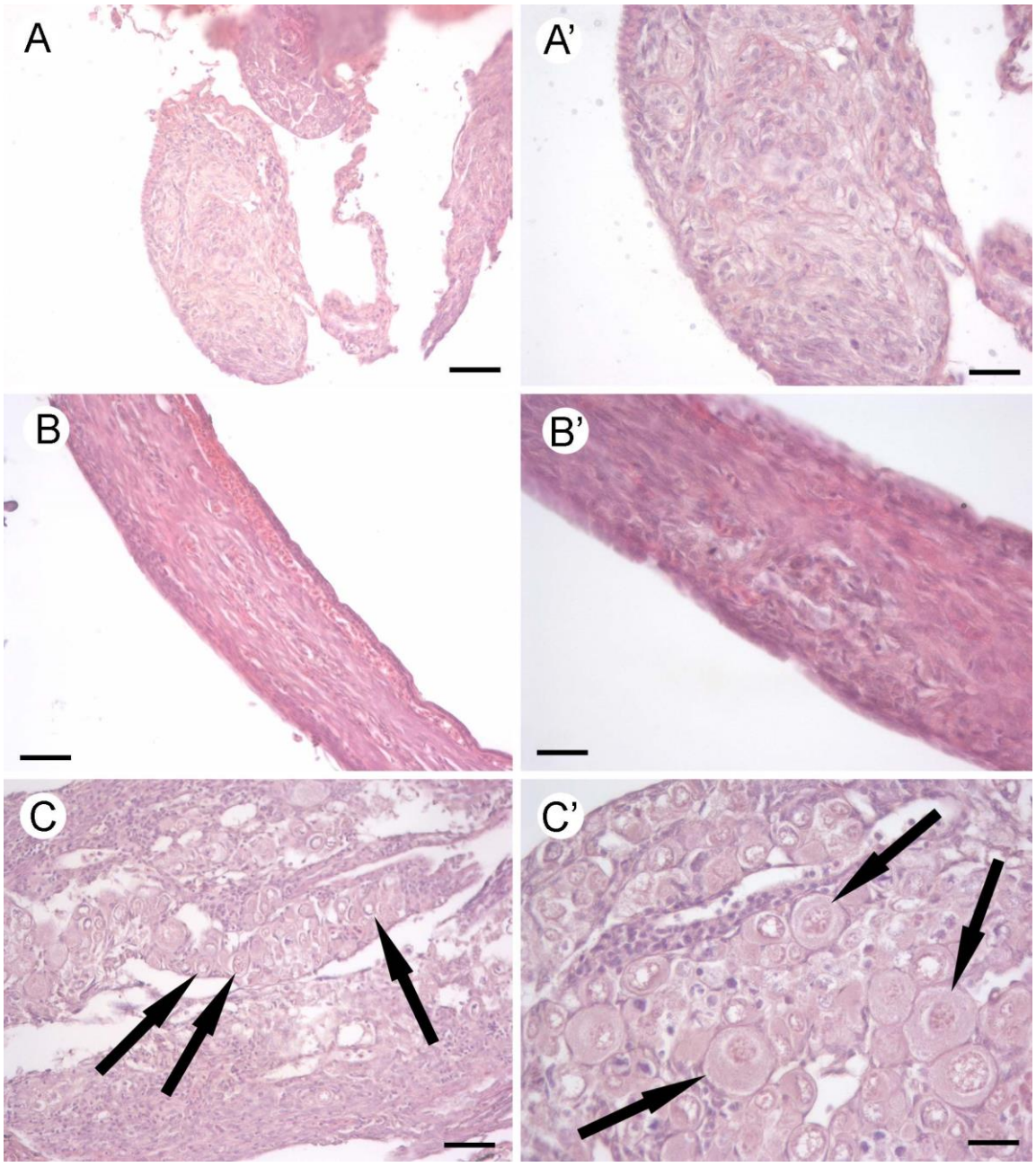

Figure 19. Histological observations of recipient (A, A') and control gonads at 6 months post-transplantation (B-C'). Histological observation of recipient gonads displayed no presence of germline cells (**A**, **A'**). The majority of control gonads displayed no presence of germline cells (**B**, **B'**) except for one control individual in which a progression of oogenesis was observed (**C**, **C'**). Arrows – early-stage oocytes. (A, B, C) The images were taken under a 20× objective. (A', B', C') The images were taken under a 40× objective. Scale bars: A, B, C – 50 µm; A', B', C' – 25 µm.

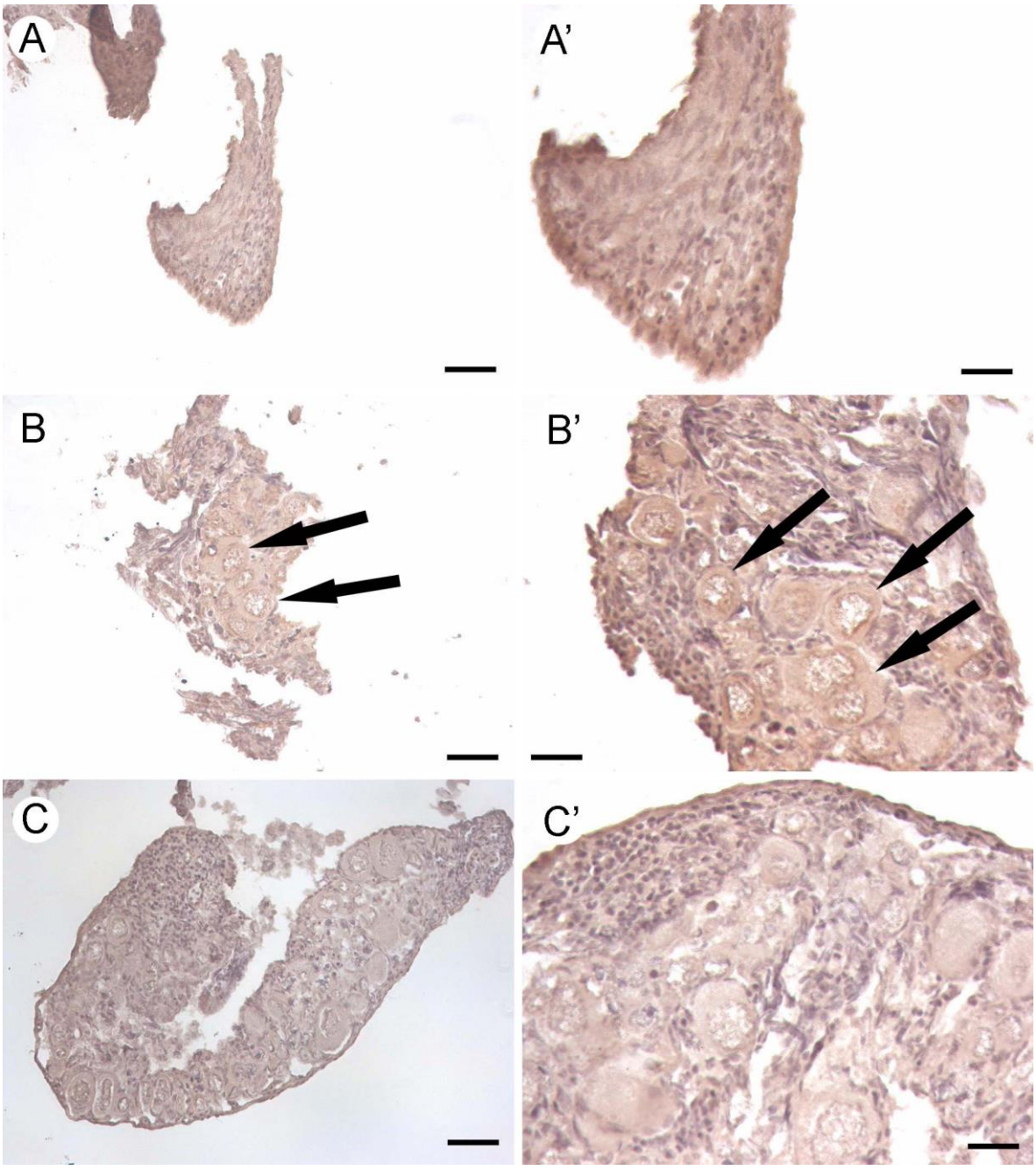

Figure 20. Immunohistochemistry observations of recipient (A, A') and control gonads at 6 months post-transplantation (B-C'). (**A**, **A'**) Gonads of recipient individuals displaying a lack of a vasa signal. (**B**, **B'**) One control individual which displayed progression of oogenesis has clearly displayed a positive vasa signal in the perinuclear region of Stage I oocytes (arrows). (**C**, **C'**) Secondary antibody control showing no vasa signal. (A, B, C) The images were taken under a 20× objective. (A', B', C') The images were taken under a 40× objective. Scale bars: A, B, C – 50 µm; A', B', C' – 25 µm.

After more than two years of rearing, all recipient and control fish were checked for gamete production. Two recipients and one control fish produced milt which was collected and analyzed morphologically. All spermatozoa had a round head which is characteristic for

common carp (Figure 21A, A') and not for the European eel which has a sickle-like head shape (Marco-Jiménez et al., 2006). In addition, the gonads of all remaining recipients and 10 controls were sampled for histological observations. Only fish that produced milt displayed a progression of gametogenesis (Figure 21B, B'), while all other fish (recipient or control) displayed a lack of germline cells. Furthermore, certain differences were observed in the germ-less gonads. Part of them displayed a tubular structure where the interstitial compartment encloses a tubular structure containing basally placed cells with large oval nuclei typical for Sertoli cells. Therefore, these gonads were identified as germ-less testes (Figure 21C, C'). The other part of the individuals displayed a more disperse interstitial compartment not forming a tubular structure but being dispersed throughout the gonadal tissue. In between the interstitial compartment, two types of cells were identified – one with large more rounded nuclei (characteristic of theca cells) and the other with smaller but very elongated nuclei (characteristic of granulosa cells). Therefore, these gonads were identified as germ-less ovaries (Figure 21D, D'). The qPCR analyses further corroborated the morphological observations. Only common carp *dnd1* was expressed in the testes of fish that produced milt, while neither European eel nor common carp *dnd1* were expressed in remaining samples.

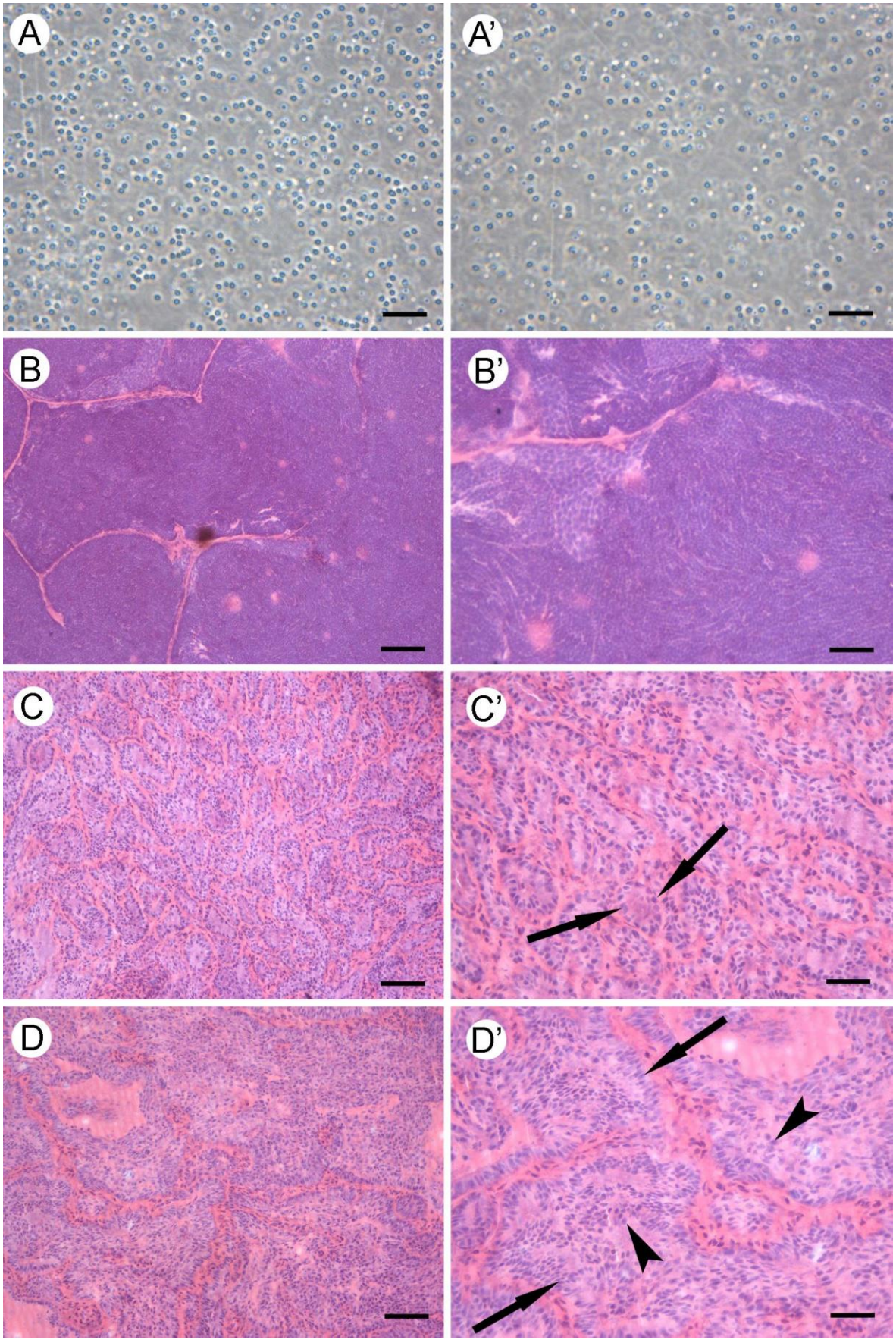

Figure 21.

Figure 21 (continued). Gamete production (A, A') and the gonadal structure (B-D') of recipient and control individuals after transplantation of European eel SSCs into common carp recipients. Spermatozoa produced by recipient (**A**) and control (**A'**) fish displaying a typical common carp morphology. (**B**, **B'**) Histological sections of gonads from individuals producing milt showing a progression of gametogenesis. (**C**, **C'**) Histological sections of gonads showing a germ-less testis-like tubular structure containing basally placed cells with large oval nuclei typical for Sertoli cells (arrows). (**D**, **D'**) Histological sections of gonads showing a germ-less ovarian-like structure with cells containing very elongated nuclei characteristic for granulosa cells (arrows) and cells containing large rounded nuclei characteristic for theca cells (arrowheads). (B, C, D) The images were taken under a 20× objective. (B', C', D') The images were taken under a 40× objective. Scale bars: B, C, D – 50 µm; A, A', B', C', D' – 25 µm.

6 DISCUSSION, CONDLUSION AND RECOMMENDATIONS

6.1 Histological analyses of the gonadal tissue

Histological analyses of the European eel were conducted to determine the maturation stage of individuals that were used for the development of GSC manipulation protocols. This step is very important in order to correctly choose further steps and GSC manipulation strategies as the approach might differ based on the maturation stage. For example, the GSC enrichment strategies are very different in the immature or mature testicular tissue, as different strategies need to be used to exclude somatic cells or to exclude spermatozoa from the cell suspensions.

In the current study, both male and female European eel individuals were immature, and their gonads contained only early-stage germline cells and the supporting somatic cells. In testicular tissues, SSCs were the only germline cells identified. They were characterized by their large oval shape and a large oval nucleus containing one centrally located nucleolus. In addition, these cells were positively stained with the vasa antibody further confirming their germline origin.

In females on the other hand, OSCs were not the only germline cells present within the ovarian tissue as large numbers of early-stage oocytes could be identified. The most dominant were the early- and mid-vitellogenic oocytes indicating that the process of oogenesis has progressed much further than spermatogenesis. In fact, OSCs could hardly be identified on the histological sections as mostly small previtellogenic oocytes (stage I) have been observed. However, this does not indicate that oogenesis has progressed too far, and that such tissues cannot be used for isolating OSCs. Namely, the most striking difference between ovarian germline cells is in their size. Even early-stage oocytes such as early diplotene oocytes are at least twice as big as the OSCs. Therefore, utilization of simple size selection methods such as filtering can enrich the cell suspensions for small early-stage ovarian germline cells such as OSCs.

The optimal methodology to confirm the germline origin of detected cells is to utilize molecular markers that are specific to a certain cell type. The most commonly used germline cell markers are *vasa* (*ddx4*), *oct4*, *dnd*, *amh*, *nanos2*, *nanos3* and other (Begum et al., 2022). *Vasa* is the most commonly used germline cell marker in most fish species. Blanes-García et al. (2024) described that *vasa* was the optimal germline cell marker for European eel SSCs among *vasa*, *dnd* and *nanos2*. Therefore, this marker was used in the current study as well. In testes, vasa antigen was specifically expressed in germline cells, specifically in SSCs as they

were the only germline cells present within this tissue. In ovaries, vasa antigen was also specifically expressed in germline cells, however, it was expressed in all germline cells. Therefore, a positive signal was observed in OSCs as well as in oocytes. Thus, vasa is not a suitable marker to differentiate between GSCs and other germline cells, and more specific stem cell-specific markers should be developed to distinguish GSCs in more developed gonads. However, in the case of immature gonads where GSCs are the only germline cells within the tissue, vasa can be a good marker for GSC identification.

During the histological procedure, tissues need to be fixed in order to stop their degradation during storage. This is most commonly done by using cross-linking agents that can form covalent bonds with proteins within the tissues, dehydration, heat, and salt formation (Suvarna et al., 2019). All of these approaches have their advantages and disadvantages, and some approaches can even be combined. However, a universal fixative that will work with guarantee for any application does not exist. Therefore, the selection of the fixation approach and fixative itself depends on the use case scenario and their ability to produce a final product needed to demonstrate a specific feature of a specific tissue (Grizzle et al., 2001). Hence, the fixation methodology should be optimized on a case-to-case basis.

The most commonly used fixative for most applications is formaldehyde either in its buffered form (formalin) or in its crystalline form (paraformaldehyde). Other fixing agents such as the acetic acid or picric acid can be added to form special formulations such as the Bouin's fixative. However, these fixatives do not always keep the intact tissue morphology, and differences between them can be observed in some tissues. In the present study, fixation in 10% neutral-buffered formalin led to creation of artifacts such as vacuolization and poor nuclear fixation. Poor testicular and ovarian fixation with formalin has been reported by other studies as well (Howroyd et al., 2005; Latendresse et al., 2002; Wang et al., 2020). In particular, the most problematic are poorly fixed nuclei which have a condensed appearance as different germline cells can be identified primarily based on their nuclear structure. Therefore, in addition to formalin, it is preferred to include a coagulant fixative such as acetic or picric acid which will be able to coagulate nucleic acids, thus preserving their native structure. Both acids are in the formulation of the Bouin's fixative, however, many studies have reported that fixation in this fixative, especially any fixative containing picric acid, can have a negative effect on immunohistochemistry and could impair antibody/antigen binding (Ananthanarayanan et al., 2005; Lenz et al., 2022). Thus, the fixation capability of an alternative fixating solution was tested. Modified Davidson's fixative (mDF) in addition to formaldehyde contains acetic acid and EtOH. Fixation in this fixative provided an improved preservation of morphological detail,

leading to less vacuolization and significantly improved the nuclear detail. In addition, it did not interfere with immunohistochemical staining for the vasa antigen. Similar was also reported by other studies (Howroyd et al., 2005; Latendresse et al., 2002; Wang et al., 2020) thus demonstrating that mDF leads to superior fixation of testicular and ovarian tissues and that it should be the preferred fixative when analyzing European eel gonadal tissue.

6.2 Isolation of GSCs

Obtaining a monodisperse cell suspension from testicular or ovarian tissue is the first step towards downstream applications of GSCs. GSCs are usually embedded deeply into the gonadal tissue within the stem cell niche, and their release necessitates either mechanical or enzymatic treatment. However, the structure of the testicular and ovarian tissues is different as the ovarian tissue is much looser than the testicular tissue. Therefore, the approaches towards releasing the cells are different. The ovarian cells can be released by simple mechanical manipulation such as tissue mincing and pipette trituration. Usually, the process is timely and necessitates pipettes of different diameter to release progressively smaller cells. However, this approach is effective only until a certain point, and smaller germline cells such as the very early oocytes or OSCs cannot be liberated only by mechanical stimulation. Further isolation necessitates the use of enzymes. As for the testicular tissue, it is much more compact and SSCs are much more tightly integrated into the tissue, therefore, simple mechanical dissociation is not possible, and it necessitates dedicated tissue dissociation equipment (e.g., GentleMACS from Miltenyi Biotec). Such equipment is rarely used, and testicular tissues are most commonly dissociated using enzymatic treatments.

Enzymatic dissociation is most commonly done by exposing the tissue to a solution containing a certain enzyme or enzymatic mixture. The tissue is dissected, transferred into the enzymatic solution, and minced into smaller pieces to increase the effective surface on which the enzymes can act. The enzymes then cleave the extracellular matrix thus releasing the cells into the medium. The efficiency of the enzymatic reaction depends on the type of enzyme used and its concentration. Enzymes can be either specific or non-specific. An example of a specific enzyme is collagenase which specifically cleaves the collagen triple helix at a defined site near the N-terminus (Gravallese and Monach, 2015), or hyaluronidase that specifically breaks down the hyaluronic acid by cleaving its glycosidic bonds (Jung, 2020). Trypsin on the other hand would be an example of a non-specific protease as it cleaves peptide chains at the carboxyl side of lysine and arginine, regardless of the peptide itself (Rawlings and Barrett, 1993). Usually,

during tissue dissociation, DNase I is used as well. Cells that die during dissociation release their contents into the cell suspension. As DNA is charged, it can bind charged extracellular proteins of cells thus creating clumps of even forming gel-like precipitates. The use of DNase I cleaves the DNA and releases the cells facilitating the formation of a monodisperse cell suspension.

In fish, most commonly used enzymes are trypsin, collagenase and DNase I. They are used either independently or in different combinations in different concentrations. Trypsin has been successfully utilized for isolation of germ cells in tench (Linhartová et al., 2014; Marinović et al., 2017), goldfish (Marinović et al., 2017), rainbow trout (Shikina et al., 2013), Nile tilapia (Lacerda et al., 2006) and sturgeon species (Pšenička et al., 2015). Collagenase was also successfully utilized in isolation of fish germ cells (Lacerda et al., 2010; Pšenička et al., 2015) and it clearly outperformed trypsin in dissociation of brown trout testicular tissue (Lujić et al., 2018b). The combination of these two enzymes was also successfully applied for the dissociation of zebrafish and common carp testicular tissue (Marinović, 2021).

In the present study, efficiency of different concentrations of trypsin and collagenase, as well as their combinations, was tested in dissociating ovarian and testicular tissues of the European eel. During the dissociation of testicular tissues, there were no statistical differences in the number of viable SSCs obtained by different enzymatic treatments. However, more cell clumps were noticed when using solely collagenase without trypsin. This observation was not made in the groups containing trypsin. As the highest average yield was obtained by using the combination of collagenase and trypsin, this enzymatic medium would be recommended for the dissociation of European eel testicular tissue. Similar was also observed by Marinović (2021) for zebrafish and common carp.

During the dissociation of ovarian tissue an effect of enzymatic media was observed. Even though significant differences were mostly not observed between the groups, higher average yield was noticed when using trypsin. The highest average yield was observed when using the same combination of collagenase and trypsin as in testicular tissue. Therefore, this enzymatic combination is recommended for the dissociation of European eel ovarian tissue as well.

6.3 Germline stem cell preservation

6.3.1 Freezing

Freezing is the most commonly used preservation technique for biological material. Freezing is usually done by cooling samples to the temperature of liquid nitrogen (-196 °C) at a slow rate (most commonly ~1 °C/min). Samples are placed in a cryomedium containing cryoprotectants that lower the freezing point, are equilibrated for a set time period, and are then frozen. Hence this form of cryopreservation is also known as equilibration freezing as it allows for the exchange of fluids between the intra- and extracellular spaces, thus mitigating harsh osmotic changes (Rezazadeh Valojerdi et al., 2009). In addition, freezing requires lower cryoprotectant concentrations (usually up to 2 M) which mitigates potential toxic effects of the cryoprotectants.

Cryopreservation of GSCs can be done through two approaches: (1) cryopreservation of isolated testicular/ovarian cell suspensions or enriched SSCs/OSCs, or (2) cryopreservation of the whole testicular/ovarian tissue. In the current study, the focus was solely on cryopreservation of gonadal tissue pieces as it offers several advantages in context of the current study. Cryopreservation would primarily serve to enable efficient storage of valuable European eel genetic resources. As the tissue will potentially be frozen by many different researchers at different countries and/or localities, there is a high potential that in many cases transport to the laboratory would not be feasible, and that freezing would have to be done on the field. Cryopreservation of tissue pieces is a much faster process that can be done in field conditions as it postpones the lengthy process of cell isolation prior to cryopreservation and does not require specialized equipment or chemicals for tissue dissociation. Therefore, cryopreservation media can be prepared beforehand, and the tissues can be equilibrated right after the fish are sacrificed and frozen in dry ice. Even from the aspect of further application in spermatogonia/oogonia transplantation or cell culture, freezing tissues has an advantage over freezing cell suspensions as the tissue dissociation process happens after thawing. As described above, when using non-specific enzymes during tissue dissociation, these enzymes lyse the transmembrane proteins thus allowing extracellular water to enter the cells which subsequently swell and burst. In this way, cell suspensions are enriched for live cells; when cryopreserving cell suspensions, dead cells remain in the suspension after thawing, and further steps of dead cell and debree removal such as MACS sorting are needed. Lastly, the studies of Pšenička et al. (2016) and Marinović et al. (2017) displayed that freezing of tissue pieces results in higher SSC viability that when freezing cell suspensions. Therefore, when cryopreserving European

eel GSCs for conservation purposes, cryopreservation of the gonadal tissue should be the preferred approach.

In the current study, freezing protocols for the European eel testicular and ovarian tissue (SSCs/OSCs) were optimized for the first time. The freezing procedure has been optimized in several steps. Sequential trials were conducted where in each trial one or two cryopreservation parameters were varied, and the optimal outcome from one trial was used in the subsequent trials. Both cryomedium composition and technical aspects such as the cooling/thawing rates, equilibration times or tissue sizes have been optimized. In such manner, stepwise improvements of the protocols were made, which in turn after several trials granted the development of an optimized cryopreservation protocol for the gonadal tissue of both sexes.

The first step in optimization of both testicular and ovarian tissues was the determination of the optimal permeable cryoprotectant and its concentration. For both testicular and ovarian tissues, the optimal cryoprotectant was Me_2SO followed by EG and PG. The superiority of Me_2SO was displayed in many other fish species; it was an optimal permeable cryoprotectant for freezing of zebrafish testicular tissue (Marinović et al., 2019), common carp testicular (Franěk et al., 2019a) and ovarian tissues (Franěk et al., 2019b), brown trout testicular tissue (Marinović, 2021), European and African catfish testicular tissue (Marinović, 2021), rainbow trout testicular (Lee et al., 2013) and ovarian tissue (Lee et al., 2016b), goldfish and tench testicular tissues (Marinović et al., 2017), and others. On the other hand, EG was optimal for freezing of Siberian sturgeon testicular tissue (Pšenička et al., 2016). It is interesting to note that MeOH displayed the lowest viability rates in both testicular and ovarian tissues as it is very efficient and most commonly used for cryopreservation of fish spermatozoa (Asturiano et al., 2017). Regarding concentrations, results of the current study were in line with many of the previously mentioned studies where optimal concentrations ranged from 1.3 to 2 M, depending on the species.

Once the permeable cryoprotectants were optimized, various external cryoprotectants such as different sugars, protein, and even amino acids were tested. As previously mentioned, these cryoprotectants act like osmotic stabilizers as they mitigate rapid water influx or efflux in or from the cells, therefore maintaining cell integrity. In the current study, none of the external cryoprotectants had a significant effect on GSC survival neither in testicular nor ovarian tissues. This was also observed in many of the previously mentioned studies (Franěk et al., 2019a, 2019b; Marinović, 2021; Marinović et al., 2019). On the other hand, external cryoprotectants can have a crucial role in freezing of fish spermatozoa (Asturiano et al., 2017). The most likely reason is the approach of freezing gonadal tissues instead of cell suspensions;

the uptake of such substances by complex matrices like tissues is questionable, therefore, the exposure of each cell within the matrix is questionable. In cell suspensions this is not the case as all cells are exposed to sugars and protein in the cryomedium. In addition, tissues already contain significant levels of sugars and protein in the blood, therefore, supplementation with sugars and protein when freezing gonadal tissue pieces does not offer a significant osmo-protective role.

With regard to technical optimization of the freezing procedure, the most important are cooling and warming rates, plunging temperatures, equilibration times and the size of tissue pieces. Since protocol optimization for ovarian tissues was predominantly done in Hungary, while protocol optimization for testicular tissues was predominantly done in Spain, testing of different cooling and warming rates was done only for ovarian tissues due to technical restrictions. Warming rates predominantly had an effect on OSC viability, while cooling rates from -0.5 °C/min to -40 °C/min yielded similarly high viability rates (under warming procedure 1). This result is not particularly in line with other studies, as mostly other studies reported significantly higher SSC viability rates at slower cooling rates (-0.5 to -1 °C/min) (Franěk et al., 2019a; Lee and Yoshizaki, 2016). The exact reason for this distinction is difficult to determine, however, it might be since in the present study freezing was done on ovarian tissues contrary to the other two studies were testicular tissues were frozen. Nonetheless, further trials were conducted with a cooling rate of ~ -1 °C/min since this is the most commonly used cooling rate, but it is also simple to replicate as it can be done without the need for expensive equipment such as a controlled-rate freezer, but can be done with simple CoolCell boxes and frozen in dry ice. As for the warming rate, warming in a 10 °C water bath was superior to warming by a reciprocal rate of 1 °C/min. The most likely reason is that the warming rate of 1 °C/min is far too slow, and it enabled ice recrystallization during thawing. Recrystallization (or uncontrolled ice growth) during cooling, but especially during thawing, is one of the most common mechanism of lethal cryo-injury (Baust, 2004; Chaytor et al., 2012). Therefore, warming rates need to be higher in order to avoid excessive recrystallization and excessive cell death during thawing. Similar was also reported by Seki and Mazur (2012, 2008) where they established that warming is the most important cryopreservation parameter during vitrification and hypothesized that if the warming rate is high enough, cells will survive the procedure irrespectively of the cooling rates. Lastly, thawing in a water bath is much simpler and cost-efficient as it does not necessitate expensive controlled-rate freezers, hence, it is more convenient in practice. However, thawing temperature should not be above the physiological temperature range for the species (in this case European eel), especially in several tissue pieces

are frozen in the same cryotube, as not all parts of the solution will thaw equally, and exposure to higher temperatures might lead to overexposure of some fragments to unnaturally high temperatures compared to others leading to excessive cell death.

When freezing gonadal tissues, special attention needs to be given to the size of the tissue and the equilibration time (i.e., time the tissue spends submerged in the cryomedium). Both ovaries and testes of European eel are fairly large, therefore, they cannot be frozen whole and need to be cut into smaller pieces in order to be frozen. The size of the tissue on the other hand may dictate the equilibration time needed for the permeable cryoprotectants to permeate the whole tissue. Hence, the time needed for a cryoprotectant like Me_2SO to permeate a 20 mg tissue is different than the time needed to completely permeate a 100 mg tissue. In this optimization trial, the effects of tissue size, equilibration time, and the equilibration temperature on the survival of OSCs were tested. Only equilibration time had a significant effect on the OSC viability which can be also noticed as a general trend that equilibration at 30 min had higher average viability for each tissue size. Furthermore, generally higher viabilities were noticed for 50 mg tissue pieces, however, clear statistical delineations were not observed. As the highest average viability was observed for 50 mg tissue pieces equilibrated on ice for 30 min, these conditions would be recommendable for freezing the gonadal tissues of European eel.

6.3.2 Vitrification

Contrary to freezing which is considered a traditional form of cryopreservation and in which samples are cooled relatively slow, vitrification is a more novel and alternative approach to cryopreservation. Vitrification is also known as ultra-rapid cooling. As the name suggests, cooling rates achieved during this method are much higher; compared to traditional freezing which is commonly conducted at ~1 °C/min, vitrification can be done at cooling rates up to 12000 °C/min (Panagiotidis et al., 2013) or even 70000 °C/min (Mazur and Seki, 2011). Such cooling rates enable the transition of both intra- and extracellular milieu into an amorphous glassy state without crystalizing into ice (Mazur and Seki, 2011; Seki and Mazur, 2012). When warming rates are equally fast as the cooling rates, the viability of vitrified/warmed cells is frequently higher than the viability of frozen/thawed cells due to circumvention of ice formation (Seki et al., 2014). However, vitrification necessitates high concentrations of cryoprotectants; compared to average concentrations of 1-2 M used during freezing, vitrification necessitates concentrations as high as 6 M. Such high cryoprotectant

concentrations can be toxic to cells and cause cell death, thus exposure to the cryoprotectants as well as their removal need to be carefully considered and optimized. To mitigate the negative effects of high cryoprotectant concentrations, there are reports on vitrification without the use of permeating cryoprotectants (Isachenko et al., 2004, 2012; Slabbert et al., 2015). Such studies are limited to vitrification of spermatozoa, and they usually utilize non-permeating cryoprotectants such as different sugars and protein which can stabilize the osmotic shocks caused by the vitrification procedure. However, such attempts are not realistic for complex matrices such as tissues since non-permeable cryoprotectants have a limited (if any) action in such conditions. Therefore, such an approach was not attempted in this study.

To enable high cooling and warming rates needed for vitrification, different devices have been designed and developed. These have mostly been developed for cell suspensions and include open pulled straws (Vajta et al., 1998), cryoloops (Lane et al., 1999), cryotops (Succu et al., 2007), or solid-surface vitrification (Xing et al., 2010). Such devices most commonly enable direct exposure of cell suspensions to liquid nitrogen, therefore maximizing the cooling rates. However, such devices cannot be readily used when vitrifying complex matrices such as whole tissue pieces. Therefore, alternatives have been developed. They include open vitrification systems such as the copper electron microscope grids (Rahimi et al., 2004), solid surface vitrification (Lin et al., 2008) or needle-immersed vitrification (NIV; Liu et al., 2012), or closed vitrification systems such as common plastic cryotubes (Isachenko et al., 2007) or metal containers (Bos-Mikich et al., 2012). The advantage of the open vitrification systems is that they enable direct exposure of the sample to liquid nitrogen which greatly maximizes cooling and warming rates. On the other hand, such direct exposure to liquid nitrogen can lead to a high risk of bacterial (Bielanski et al., 2003) or viral infections (Bielanski et al., 2000). Closed vitrification systems mitigate the risk of contamination; however, plastic cryotubes do not conduct heat well, thus the cooling rates are much lower than needed for vitrification. On the other hand, metal containers are a good alternative. They have also been utilized in vitrification of zebrafish ovarian tissue (Marques et al., 2015). However, such containers are usually custom made and are not easily accessible to all laboratories.

In the present study, needle-immersed vitrification (NIV) method was used. This method relies on pinning tissue pieces on an acupuncture needle as a carrier (Wang et al., 2008) and has been developed and adapted for the vitrification of testicular and ovarian tissues in several mammalian (Andrae et al., 2021; Demirel et al., 2018; Macente et al., 2022; Wang et al., 2008) and bird species (Liu et al., 2013, 2012). Recently, it was also adapted for the vitrification of testicular and ovarian tissue in various fish species such as the brown trout

(Lujić et al., 2017), zebrafish (Marinović et al., 2019, 2018), common carp (Franěk et al., 2019a, 2019b) and sturgeons (Lujić et al., 2023). As previously mentioned, NIV is an open vitrification system; thus, the main advantage of NIV compared to straws and metal containers is the direct exposure of tissues to liquid nitrogen with minimal volumes of cryoprotectants being attached to them, hence maximizing the cooling and warming rates, but also reducing the concentrations of cryoprotectants needed and thereby their toxicity. In addition, by being pinned to the needle, all tissue pieces are exposed to the cryoprotectants and liquid nitrogen synchronously.

The vitrification method was optimized by testing equilibration (ES) and vitrification (VS) solutions containing different cryoprotectants in different concentrations. In both males and females, generally higher viability percentages were obtained when using ES3 among the ES solutions, and VS3 among the VS solutions. The most plausible reason why VS3 consistently yielded higher viability rates in both females and males is the concentration of cryoprotectants; VS3 contained a lower concentration of each individual cryoprotectant (3 M of PG and 3 M of Me_2SO), while VS1 and VS2 contained higher concentrations of one individual cryoprotectant (4.5 M of PG or 5.5 M of Me_2SO). High concentration of one cryoprotectant can be toxic to cells and can cause cell death. Thus, the utilization of two cryoprotectants at equal, but lower, concentrations most likely enhanced cell survival by negating the toxic effects of high concentrations of one cryoprotectant. Similar was observed in other studies as well (Franěk et al., 2019a; Liu et al., 2013; Lujić et al., 2017; Marinović et al., 2019; Marques et al., 2015; Wang et al., 2008).

Compared to other fish species, viability rates obtained in this study for European eel were relatively high as they reached up to 70-80%. Similar was observed when vitrifying brown trout ovarian (Lujić et al., 2017) and testicular tissue (Marinović, 2021), and zebrafish testicular tissue (Marinović et al., 2019), while much lower viability rates (up to 20%) were observed when vitrifying common carp testicular tissue (Franěk et al., 2019a) and European and African catfish testicular tissue (Marinović, 2021). The main differences that could cause such a drastic difference between species could be in the maturity status of the fish, and subsequently of their gonads. Namely, the gonadal tissues of the European eel and brown trout were immature, therefore, they contained only germline stem cells and supporting somatic cells. Zebrafish, common carp and catfish species were mature, and their gonads (testes in this case) contained various germline cells from GSCs to gametes (spermatozoa). However, when comparing the structure of mature testicular tissues of the mentioned species, there are also apparent differences; testes of common carp and the two catfish species contain a far larger proportion

of spermatozoa compared to zebrafish (Marinović, 2021). Since vitrification of fish spermatozoa generally leads to very poor viability and motility rates after warming (Asturiano et al., 2017; Kása et al., 2018, 2017), a high proportion of spermatozoa in the testicular tissues was the most probable reason for such low vitrification success. Hence, the vitrification of immature testicular tissue such as the one observed in the European eel would lead to a much higher survival of SSCs. Similar can be extrapolated to ovarian tissues, as immature ovarian tissues contain only OSCs, early-stage ovarian follicles and supporting somatic tissues, while mature ovaries also contain later-stage oocytes, especially fully-grown vitellogenic oocytes. Large vitellogenic oocytes cannot yet be successfully cryopreserved due to their very large size, high yolk content, large surface to volume ration, and a pronounced sensitivity to chilling (Isayeva et al., 2004; Tsai et al., 2009). Therefore, when combining the very low number of OSCs present in mature ovaries, and the pronounced sensitivity of later-stage oocytes, vitrification of immature ovarian fragments should be preferred. Ultimately, the application of freezing or vitrification in cryobanking of fish gonadal tissues mostly depends on the structure of the gonadal tissue itself, and the cells present within the tissue. Since most of the European eel individuals caught in European freshwaters are immature, both cryopreservation methods (freezing or vitrification) can be successfully applied, and the choice of the method would depend on other factors such as the location (field or laboratory), presence of dedicated equipment, cost-effectiveness and labor-effectiveness.

6.3.3 Hypothermic storage

Hypothermic or cold storage is one of the most important short-term preservation strategies for organs or cells. Hypothermic storage is defined as storing biological material at temperatures below its normal physiological temperatures, but higher than the freezing point of the storage solution (Tovar et al., 2008). Most commonly, biological material is stored at 4 °C. Storage at temperatures above the freezing point alleviates the potential damage caused to biological material by thermal shock during cryopreservation, potential creation of ice crystal, or the toxic effects of cryoprotectants (Mazur et al., 2008). However, storage at these temperatures does not completely cease physiological processes in cells, therefore, all cells remain metabolically active at about 10-12% of the normal activity (Yang et al., 2010). This can lead to accumulation of harmful metabolites and cell death. Therefore, optimal conditions for different cell types need to be developed.

This study is the first to demonstrate successful short-term hypothermic storage of European eel GSCs. In both males and females, storage of GSCs in cell suspensions was superior to storage of tissue pieces (even though clear statistical differences were not obtained for females). Similar was observed by Lujić et al. (2018a) for carp SSCs where the viability after 24 h of storage did not change in cell suspensions, while viability dropped to ~50% after storage of tissue pieces. Yang and Honaramooz (2010) and Yang et al. (2010) reported a similar finding for porcine testicular cells; storage in cell suspension after 6 days yielded ~88% viability, while storage of tissue pieces for the same period yielded around 26% viability. The most likely cause is the diffusion rate of nutrients and excretion of metabolic waste which is most likely hindered in complex matrices such as a tissue piece, while both nutrient uptake and waste excretion are direct in the case of cell suspensions. Furthermore, as the hypothermic storage slows the metabolism down to approximately 1/10 of its normal capacity, this also might hinder the transfer of metabolites in tissue pieces.

In females, the size of the ovarian tissue had an influence on the survival of OSCs. Namely, the bigger the tissue was, the lower OSCs survival was. The most likely reason is the same as to the superiority of storage in cell suspensions. As metabolism is slowed, transfer of nutrients and metabolites is slower, therefore it is harder for them to reach the central part of the tissue pieces. Most commonly, the main causes of cell death during hypothermic storage are inactivation of membrane pumps and subsequent disruption of ion homeostasis, osmotic shock, apoptosis, necrosis, or accumulation of harmful metabolites from dead cells (Falahatkar et al., 2017). Therefore, cell death is most likely initiated at the core of the tissue pieces, which then slowly progresses towards the periphery.

After 6 days, viability of SSCs was approximately 75%, while the viability of OSCs after 13 days was ~65%. These viability rates are similar to the ones observed after cryopreservation. These results have great practical implications since storage of germ cells is needed for almost any type of GSC manipulation. As cryopreservation commonly necessitates costly equipment such as deep freezers, controlled-rate freezers, liquid nitrogen, and have a labor-demanding sample preparation, hypothermic storage is a cost- and labor-effective alternative. Furthermore, it does not expose the cells to strong thermal shock, nor does it necessitate the use of potentially toxic cryoprotectants. Therefore, hypothermic storage is a valuable alternative to cryopreservation for periods shorter than two weeks, especially in the cases of routine cell manipulation intervals or transportation between laboratories.

6.4 Transplantation

This study was the first to develop optimal procedures for the preservation of European eel GSCs through verifying cell viability after testing different cryopreservation/hypothermic storage protocols. However, viability does not necessarily translate into good functionality of cells after such protocols. The most obvious example is the one of spermatozoa. Viability tests usually test the integrity of cell membranes of spermatozoa after cryopreservation. However, this does not necessarily translate into good functionality of these cells such as motility, as the actin filaments of spermatozoa tails can get damaged during cryopreservation. Therefore, even though the cells have survived displayed by an intact membrane, they will not be functional (i.e., they will not be able to swim) if their tails are damaged (Tamburrino et al., 2023). Similar stands for other cell types as well since cryopreservation can lead to different kind of damage, including alterations in the transcriptional patterns, oxidative stress and oxidative damage, DNA damage, and other (Rodrigues et al., 2021; Thomson et al., 2009). In such conditions, even though cell membranes remain intact, the functionality of the cell can be impaired. Hence, functionality is an important factor that needs to be tested during the development of cell preservation protocols.

The functionality of SSCs and OSCs can be primarily tested in two different ways: (1) through transplantation into suitable recipients and testing their ability to migrate, incorporate and proliferate, or (2) through *in vitro* cell culture and testing their ability to proliferate. In the current study we have tested the functionality of SSCs through transplantation into common carp recipients. Cell culture was not conducted as currently no protocols for the *in vitro* cultivation of European eel have been developed.

European eel SSCs were transplanted into larvae of common carp. Larvae were chosen as they still do not have a developed immune system, hence they have less ability to reject donor cells after transplantation (Yoshizaki et al., 2011). Furthermore, since European eel individuals are immature, their testes are small and do not contain a large number of SSCs, even though they are the only germline cells present within the testes. Transplantation into adult recipients would necessitate a far larger number of cells (from 10^5 (Majhi et al., 2009) to 10^7 cells (Lacerda et al., 2010) per recipient) compared to transplantation into larvae which necessitates only ~3000 cells per recipient (Marinović, 2021). Hence more recipients, and subsequently donor-derived progeny, can be obtained after using larvae as recipients compared to adults.

During the initial colonization verification, fluorescently labelled cells were identified within the recipient genital ridges at 60 days post-transplantation. This indicates that the

frozen/thawed SSCs are indeed functional and retain their ability to migrate and incorporate into the recipient genital ridges. The colonization rate of approximately 30% is similar to some published results such as transplantation of brown trout and grayling SSCs into rainbow trout larvae as recipients (~25%; Lujić et al., 2018b). However, it is a lower incorporation rate compared to the ones reported in zebrafish (50-60%; Marinović et al., 2019), common carp and goldfish (50%; Franěk et al., 2019a), rainbow trout (60-70%; Yoshizaki et al., 2016), brown trout (50%; Fernández-Díez et al., 2012) or rainbow trout and masu salmon (~70%; Lee et al., 2016a). A possible cause for a lower incorporation rate is the high phylogenetic distance between the European eel and the common carp. In all other cases the donor and recipient species are phylogenetically much closer, therefore the physiological conditions are more similar, thus facilitating incorporation and proliferation of donor cells. Another potential reason might be the fact that some of the cells were indeed damaged during cryopreservation, and therefore have lost their ability to incorporate. However, in cases of zebrafish (Marinović et al., 2019) and common carp (Franěk et al., 2019a) there was no difference in incorporation between the fresh and frozen/thawed cells. Given that only a small number of cells (usually 2-10) initially incorporate into the gonads, the presence of physiologically impaired but viable cells should not make a difference in the final incorporation rates. Furthermore, the most important conclusion of this trial is that it confirms that frozen/thawed SSCs are functional and are able to incorporate into recipient gonads, thus the developed preservation protocols can be used in practice and for the conservation of important European eel genetic resources.

After the initial incorporation, recipient fish were reared firstly for 6 months, and subsequently for 2 more years in order to check whether a proliferation of SSCs and initiation of gametogenesis would occur. At 6 months after transplantation, gametogenesis was observed in one recipient and one control fish. Molecular analyses displayed that both individuals expressed common carp *dnd1*, and not European eel *dnd1*. After two more years of rearing, two recipients and one control fish produced milt. Upon histological inspection, we confirmed that only these three individuals displayed a presence of germline cells within their gonads, while all remaining recipient and control individuals showed a lack of germline cells within their gonads. The qPCR analyses again confirmed that the three gonads displaying continuation of gametogenesis expressed common carp *dnd1*, and not European eel *dnd1*. Spermatozoa obtained had a typical cyprinid morphology, and none of the spermatozoa displayed the typical European eel sickle-like morphology. Therefore, conclusion can be drawn that the germline cells and milt produced by the recipients were of the endogenous common carp origin, and not of the donor origin. These results display that the sterilization procedure was not 100%

effective as shown in many other studies (Franěk et al., 2019a; Marinović et al., 2019). However, the study of Yoshizaki et al. (2016) also displayed that one of 13 masu salmon recipients also produced endogenous spermatozoa, and not only donor-derived (rainbow trout) spermatozoa. There are a few potential reasons that might explain this result. Embryos might have been injected at a later stage than the 8-cell stage at which point MOs do not equally enter all cells, and some of the cells will continue to express *dnd*. Technical omissions such as incorrect injections or skipping of individuals during injection can also occur. It is not possible to know the exact reason for this result, but it has to be taken into consideration that not every time 100% of recipients will indeed be sterile, and that the gametes produced by the surrogates need to be checked by molecular methods and/or morphological methods when possible.

In this study, surrogate production of European eel gametes by the common carp recipients was not observed. This however is not surprising, as one of the most important prerequisites for a successful surrogate production is that the donor and recipient species are phylogenetically closely related. Even though successful inter-family transplantation has been reported (Zhou et al., 2021), this is very rare, and to the best of our knowledge, there is only one such report in current literature. Similarly to this study, Saito et al. (2011) have observed that Japanese eel PGCs can migrate to the genital ridges after transplantation into zebrafish, however, they did not continue to proliferate. Therefore, even though transplantation of European eel SSCs into common carp recipients has demonstrated the functionality of frozen/thawed SSCs, common carp is not a suitable recipient for the surrogate production of European eel gametes and progeny. A better candidate would be a phylogenetically closely related species such as some other anguillid species. A good candidate would be the Japanese eel as the life cycle has been closed in aquaculture conditions. However, finding a good recipient candidate was not the aim of this study, and will be the topic of further studies.

7 NEW SCIENTIFIC RESULTS

1. Through histological analyses, modified Davidson's fixative was identified as a more suitable fixative for European eel gonads than the 10% neutral-buffered formalin. *Vasa* was identified as a suitable marker for germline cells in the European eel, however, it cannot effectively discriminate between germline stem cells and other germline cells.
2. Optimal protocol for isolation of GSCs was developed by using enzymatic dissociation of European eel testicular and ovarian tissues. The use of 2 mg/ml collagenase, 3 mg/ml trypsin and 50 μg/ml DNase I in L-15 yielded the highest number of isolated GSCs in both sexes.
3. Long-term preservation protocols for GSCs were developed by optimizing freezing and vitrification protocols for European eel testicular and ovarian tissues. The optimal cryomedium for freezing gonadal tissues of both sexes contained 1.5 M Me_2SO supplemented with 0.1 M glucose and 1.5% BSA (or 1.5% FBS) dissolved in PBS supplemented with 25 mM HEPES, while the optimal cooling rate was -1 °C/min, the optimal plunging temperature was -80 °C, and the optimal warming was in a 10 °C water bath. For vitrification, needle-immersed vitrification was used. The optimal equilibration solution for both sexes contained 1.5 M Me_2SO and 1.5 M PG in an extender solution (L-15 supplemented with 10% FBS, 25 mM HEPES and 0.5 M trehalose), while the optimal vitrification solution for both sexes contained 3 M Me_2SO and 3 M PG in L-15 in an extender solution.
4. Short-term preservation protocols were developed through hypothermic storage of isolated testicular or ovarian cell suspensions. Storing isolated gonadal cells in a solution of L-15 supplemented with 10% FBS, 25 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin retained ~75% viability of SSCs after 144 h and ~60% viability of OSCs after 312 h or storage.
5. Frozen/thawed European eel SSCs were functional as demonstrated by successful incorporation into recipient gonads after transplantation into common carp larvae. However, common carp is not a suitable recipient species for the surrogate production of European eel most likely due to a large phylogenetic distance between the two species.

8 SUMMARY

The European eel (*Anguilla anguilla*) is currently listed as critically endangered by the International Union for Conservation of Nature (IUCN). Over the past few decades, the population of this species has experienced a dramatic decline, estimated at over 90%. This alarming decrease is largely attributed to a combination of factors including overfishing, habitat loss, pollution, and barriers to migration such as dams and waterway modifications which hinder their natural lifecycle of spawning and returning to the sea. In response to the critical endangerment of the European eel, numerous conservation efforts have been initiated across various levels. These include strict regulations on fishing, habitat restoration projects, and the installation of eel ladders and passageways that facilitate their migration across water barriers. However, the European eel populations are still facing a decline, and development of novel conservation and management strategies are needed in order to bring this species back from its endangered status.

The aim of this dissertation was to develop and adapt the surrogate broodstock technology for the conservation of the European eel. This was done through developing several critical steps for the manipulation of both male and female germline stem cells (GSCs) – spermatogonial stem cells (SSCs) and oogonial stem cells (OSCs). These critical steps included (1) identification of SSCs and OSCs within the gonadal tissue through histological methods, (2) development of the optimal isolation techniques for both SSCs and OSCs, (3) development of optimal short-term (hypothermic storage) and long-term (cryopreservation through slow-rate freezing and vitrification) preservation methods for both male and female gonadal tissues, and (4) testing of the functionality of frozen/thawed SSCs through transplantation into common carp *Cyprinus carpio* recipients.

During histological analyses, we observed that fixation in the commonly used 10% neutral-buffered formalin did not lead to proper fixation of the ovarian and testicular tissues. Therefore, the fixative was changed to the modified Davidson's solution which in addition to formaldehyde contains acetic acid to coagulate the nucleic acids and ethanol to dehydrate the tissues. The use of this fixative led to improved morphology of the tissues, less vacuolization and preserved nuclear structure which is very important in identifying different germline cell stages.

Both testicular and ovarian tissues were immature. Tin addition to somatic cells, testicular tissue contained only SSCs as germline cells, while the ovarian tissue contained OSCs but also oocytes up to the mid-vitellogenic stage (stage V). In addition, the vasa antigen

as a marker for GSCs was tested. Through Western blot a suitable antibody was detected. Immunohistochemical labelling of the vasa antigen displayed its expression in the SSCs within the testicular tissue, and in all female germline cells within the ovarian tissue. Thus, this marker can be used for the identification on GSCs only when they are the only germline cells present within the tissue.

Subsequently, a protocol for the isolation of GSCs was optimized. As the enzymatic dissociation was chosen as a preferred method of GSC isolation, different concentrations and combinations of collagenase and trypsin were tested. In both males and females, the largest number of viable GSCs was obtained when using 2 mg/ml collagenase, 3 mg/ml trypsin and 50 µg/ml DNase I in L15, however, statistical delineations from other groups were not clear. It is important to note that trypsin is a non-specific protease, therefore, a prolonged treatment with this enzyme could lead to cell damages, and ultimately cell death.

In order to enhance conservation efforts, this study focused on developing GSC preservation protocols. Both short-term and long-term preservation strategies were explored. Cryopreservation through freezing and virification of gonadal tissue was selected for long-term preservation strategy. Freezing protocols were optimized by testing different cryoprotectants, their concentrations, cooling rates, sugar and protein supplementation, tissue size and equilibration time, and plunging temperatures. In both sexes, dimethyl sulfoxide (Me_2SO) at a concentration of 1.5 M was the optimal cryoprotectant. Supplementation with different sugars and protein did not have a significant effect, most likely because tissues are complex matrices that already contain sugar and protein in the blood vessels. Optimal cooling rates was -1 °C/min, optimal warming method was a 10 °C water bath, while the optimal plunging temperature was -80 °C. Using this protocol, viability of approximately 50% was obtained for SSCs and approximately 90% for OSCs.

In addition to freezing, vitrification was also tested. This method utilizes ultra-fast cooling rates, but in return, it necessitates high cryoprotectant concentrations (up to 5 – 6 M). In both sexes, both equilibration (ES) and vitrification solutions (VS) had a significant effect on GSC viability. In both sexes the optimal VS was VS3 containing 3M Me_2SO and 3 M propylene glycol (PG), while the ES varied. Utilization of a VS containing cryoprotectants in equal concentrations is favorable as it contains a lower concentration of each individual cryoprotectant which will not cause toxic effects on its own, but the cumulative concentration is still high to enable vitrification. The highest viability obtained by vitrification was ~70% for SSCs and ~80% for OSCs.

Cryopreservation is considered an efficient strategy for long-term preservation of genetic resources as biological samples can be kept in liquid nitrogen for indefinite periods. However, during transplantation, storage of cells is necessary only for short durations (usually over night to few days) during which cryopreservation is not an effective strategy due to the complexity of procedure and exposure of cells to thermal and solute shock that can lead to damage. Therefore, in this study we developed the optimal protocol for hypothermic storage of GSCs at 4 °C. In both sexes, higher GSC survival was obtained when storing isolated cell suspensions rather than gonadal tissue pieces. Furthermore, viability of ~75% was obtained for SSCs after 144 h of storage, and ~60% was obtained for OSCs after 312 h of storage. As this number was similar to the percentage of live cells obtained after cryopreservation, hypothermic storage could be a valuable alternative to cryopreservation for short-term storage.

Cryopreservation protocols were developed by testing the effects of different cryopreservation parameters on GSC viability. However, since cryopreservation can cause oxidative stress and oxidative damage, DNA damage, mitochondrial damage, viability does not always translate into cell functionality. Therefore, in this study we tested the functionality of SSCs by transplanting them into common carp *Cyprinus carpio* larvae. Recipients were firstly sterilized by injecting morpholino oligonucleotides against the *dead end* gene (*dnd*-MO) into 2-8 cell stage embryos. Seven days later, approximately 3000 fluorescently-labelled (by PKH-26) European eel SSCs were transplanted into sterilized larvae. After 60 days, recipients were checked for SSC incorporation by visual inspection under a fluorescent microscope. Fluorescent signal originating from donor cells was observed in ~30% of recipients. Therefore, frozen/thawed European eel SSCs were functional and retained their ability to colonize recipient gonads after transplantation. After 2+ years of rearing, gametes were obtained from 2 recipients and one control fish. However, spermatozoa had the characteristic morphology of common carp spermatozoa, and the testes they were derived from expressed only common carp *dnd1* and not European eel *dnd1*. This indicates that these individuals produced endogenous common carp gametes, and not donor-derived European eel gametes. Therefore, care must be given that not all *dnd*-MO sterilized individuals are sterilized and that technical omissions can occur. Furthermore, common carp is not a suitable recipient for the surrogate production of European eel gametes and progeny. This is most likely to a large phylogenetic distance between the two species. Therefore, a more suitable recipient species, most likely belonging to the anguillid family, should be found for the surrogate production of European eel. However, protocols developed in this study significantly contribute to the development of the surrogate broodstock technology for the European eel, and can contribute to preservation of important

European eel genetic resources until novel techniques for either surrogate broodstock production of *in vitro* germline cell culture for this species.

9 ÖSSZEFOGLALÁS

Az európai angolna (*Anguilla anguilla*) jelenleg a Természetvédelmi Világszövetség (IUCN) által kritikusan veszélyeztetett fajként van besorolva. Az elmúlt évtizedek során ennek a fajnak a populációja drámai csökkenést mutatott, amelyet meghaladta a több mint 90%-ot. Ez az aggasztó csökkenés nagyrészt több tényező együttes hatásának tulajdonítható, beleértve a túlhalászatot, az élőhelyek elvesztését, azok szennyezését, valamint az olyan vándorlási akadályokat, mint a gátak és a vízi útvonalak változásai, amelyek akadályozzák természetes életciklusukat, az ívás és a tengerbe való visszatérés során. Válaszul az európai angolna kritikus veszélyeztetettségére számos megőrzési kezdeményezés indult meg különböző szinteken az elmúlt időszakban. Ezek közé tartoznak a halászatot szigorító szabályozások, élőhely-rehabilitációs projektek, valamint az angolna-lépcsők és átjárók telepítése, amelyek elősegítik vándorlásukat a vízi akadályokon keresztül. Ezen erőfeszítések ellenére az európai angolna populációi továbbra is csökkenést mutatnak, és az állomány helyreállításához új megőrzési és kezelési stratégiák kidolgozására van szükség.

A dolgozat célja az volt, hogy kifejlesszen és alkalmazzon egy új típusú szaporodási technológiát az európai angolna megőrzésére. Ez a hím és női ivarsejt-törzssejtek (GSC-k): spermatogónia törzssejtek (SSC-k) és oogónia törzssejtek (OSC-k) manipulálásának több kritikus lépésén keresztül történt. Ezek a kritikus lépések a következők voltak: (1) az SSC-k és az OSC-k azonosítása a gonádszövetben hisztológiai módszerekkel, (2) az SSC-k és az OSC-k optimális izolálási technikáinak kifejlesztése, (3) a hím és női gonádszövetek optimális rövid távú (hipotermikus tárolás) és hosszú távú (lassú fagyasztással és vitrifikációval történő kriokonzerválás) megőrzési módszereinek kifejlesztése, és (4) a fagyasztott/kiolvasztott SSC-k funkcionalitásának tesztelése közönséges ponty (*Cyprinus carpio*) recipiensekbe történő beültetésével.

Hisztológiai elemzések során megfigyeltük, hogy a gyakran használt 10%-os semleges pufferelt formalin alkalmazása nem vezetett megfelelő rögzítéshez a petefészek- és hereszövetek esetében. Ezért új rögzítő oldatot alkalmaztunk, mely a módosított Davidson-oldat volt, amely a formaldehid mellett ecetsavat is tartalmaz a nukleinsavak koagulálásához és etanolt a szövetek dehidratálásához. Ennek a rögzítőanyagnak a használata javította a szövetek morfológiáját, kevesebb vákuolizációt okozott és megőrizte a mag szerkezetét, ami nagyon fontos a különböző fejlődési szakaszokban lévő ivarsejtek azonosításában.

Mind a hereszövet, mind a petefészekszövet éretlen volt. A szomatikus sejteken kívül a hereszövet csak SSC-ket tartalmazott ivarsejtként, míg a petefészekszövet OSC-ket, valamint

oocitákat tartalmazott a középső vitellogén szakaszig (V. szakasz). Emellett a vasa antigént is teszteltük GSC-k markereként. Western blot segítségével a kutatási munkához megfelelő antitestet azonosítottunk. Az immunhisztokémiai jelölés során a vasa antigén kifejeződése kimutatható volt a SSC-kben a hereszövetben, és az összes női ivarsejtben a petefészekszövetben. Így ez a marker csak akkor használható GSC-k azonosítására, ha ezek az egyedüli ivarsejtek a szövetben.

Ezt követően optimalizáltuk a GSC-k izolálásának protokollját. Mivel az enzimatikus disszociációt választottuk a GSC-k izolálásának preferált módszeréül, különböző koncentrációkat és kombinációkat teszteltünk a kollagenáz és tripszin esetében. Mind a hímeknél, mind a nőstényeknél a legnagyobb számú életképes GSC-t 2 mg/ml kollagenáz, 3 mg/ml tripszin és 50 µg/ml DNáz I alkalmazásával nyertük ki L15-ben, azonban a statisztikai megkülönböztetések más csoportoktól nem voltak egyértelműek. Fontos megjegyezni, hogy a tripszin egy nem specifikus proteáz, ezért a hosszantartó kezelés ezzel az enzimmel sejtkárosodáshoz, és végül sejthalálhoz vezethet.

A disszertáció a GSC-k megőrzési protokolljainak kifejlesztésére összpontosított. Mind rövid távú, mind hosszú távú megőrzési stratégiák a vizsgálat tárgyát képezték. A gonádszövet fagyasztással és vitrifikációval történő kriokonzerválása lett kiválasztva hosszú távú megőrzési stratégiaként. A fagyasztási protokollokat optimalizáltuk, különböző krioprotektánsok, koncentrációik, hűtési sebességek, cukor- és fehérje-adalékok, szövetméretek és ekvilibrációs idő, valamint a felolvasztási hőmérsékletek tesztelésével. Mindkét nemnél a dimetil-szulfoxid (Me2SO) 1,5 M koncentrációja volt az optimális krioprotektáns. A különböző cukrok és fehérjék kiegészítése nem mutatott szignifikáns hatást, valószínűleg azért, mert a szövetek komplex mátrixok, amelyek már tartalmaznak cukrot és fehérjét az erekben. Az optimális hűtési sebesség -1 °C/perc volt, az optimális felolvasztási módszer 10 °C-os vízfürdő volt, míg az optimális fagyasztási merítési hőmérséklet -80 °C volt. Ezzel a protokollal körülbelül 50% életképességet sikerült elérni az SSC-k esetében, és körülbelül 90% -ot az OSC-k esetében.

A fagyasztáson kívül a vitrifikáció is tesztelésre került. Ez a módszer ultragyors hűtési sebességeket használ, de cserébe magas krioprotektáns-koncentrációkat (akár 5–6 M) igényel. Mindkét nemnél az egyensúlyi oldatok (ES) és a vitrifikációs oldatok (VS) jelentős hatást gyakoroltak a GSC-k életképességére. Mindkét nem esetében az optimális VS a VS3 volt, amely 3 M dimetil-szulfoxidot (Me2SO) és 3 M propilénglikolt (PG) tartalmazott, míg az ES változó volt. Előnyös a krioprotektánsok egyenlő koncentrációit tartalmazó VS használata, mivel ez egyes krioprotektánsok alacsonyabb koncentrációját tartalmazza, amely önmagában nem okoz toxikus hatásokat, de a kumulatív koncentráció még mindig elég magas a vitrifikáció

lehetővé tételéhez. A vitrifikációval elért legmagasabb életképesség körülbelül 70% volt az SSC-k és körülbelül 80% az OSC-k esetében.

A kriokonzerválás hatékony stratégiának számít a genetikai erőforrások hosszú távú megőrzésére, mivel a biológiai mintákat folyékony nitrogénben korlátlan ideig tárolhatják. Azonban a transzplantáció során a sejtek rövid ideig történő tárolása szükséges (általában egy éjszakától néhány napig), ebben az időszakban a kriokonzerválás nem tekinthető hatékony stratégiának a eljárás bonyolultsága és a sejtek hő- és oldat-sokk általi expozíciójának következtében, amely károsodáshoz vezethet. Ezért ebben a tanulmányban kifejlesztettük az optimális protokollt a GSC-k hipotermikus tárolására 4 °C-on. Mindkét nemben magasabb GSC-túlélési arányt értek el, amikor izolált sejtszuszpenziókat tároltak, nem pedig gonádszövet-darabokat. Továbbá az SSC-k esetében 144 óra tárolás után körülbelül 75%-os, míg az OSC-k esetében 312 óra tárolás után körülbelül 60%-os életképességet értek el. Mivel ez az arány hasonló volt a kriokonzerválás után kapott élő sejtek százalékához, a hipotermikus tárolás értékes alternatívája lehet a kriokonzerválásnak a rövid távú tárolás esetében.

A kriokonzerválási protokollokat a GSC-k életképességére gyakorolt különböző kriokonzerválási paraméterek hatásainak tesztelésével fejlesztettük ki. Mivel azonban a kriokonzerválás okozhat oxidatív stresszt és oxidatív károsodást, továbbá DNS-károsodást, mitokondriális károsodást, az életképesség nem mindig jelenti a sejtek funkcionalitását. Ezért a disszertációban az SSC-k funkcionalitását azzal teszteltük, hogy ponty (*Cyprinus carpio*) lárvákba ültettük át őket. A fogadókat először sterilizáltuk a *dead end* gén (*dnd*-MO) elleni morfolino oligonukleotidokkal a 2-8 sejtes szakaszú embriókba történő injektálásával. Hét nappal később körülbelül 3000 fluoreszcensen jelölt (PKH-26-tal) európai angolna SSC-t ültettünk be sterilizált lárvákba. 60 nap múlva a fogadókat fluoreszcens mikroszkóp alatt vizuálisan ellenőriztük az SSC beépülésére. A donor sejtekből származó fluoreszcens jel körülbelül 30%-ban volt megfigyelhető a recipiensekben. Tehát a fagyasztott/kiolvasztott európai angolna SSC-k funkcionálisak voltak és megőrizték képességüket a fogadó gonádok kolonizálására transzplantáció után. 2+ év tenyésztés után gamétákat nyertünk két recipiensből és egy kontroll halból. Azonban a spermiumoknak a pontya ponty spermiumaira jellemző morfológiájuk volt, és csak a közönséges ponty dnd1-jét fejezték ki, nem az európai angolna dnd1-jét. Ez azt jelzi, hogy ezek az egyedek endogén ponty gamétákat állítottak elő, és nem donor-származékú európai angolna gamétákat. Ezért figyelembe kell venni, hogy nem minden dnd-MO-val sterilizált egyed sterilizált, és előfordulhatnak technikai problémák. Továbbá a ponty nem megfelelő recipiens az európai angolna gaméták és utódok szurrogát termeléséhez. Ez valószínűleg a két faj közötti nagy filogenetikai távolságnak tudható be. Ezért megfelelőbb

fogadó fajt, valószínűleg az anguillid családból kellene találni az európai angolna szurrogát termeléséhez. Mindazonáltal, ebben a disszertációban kifejlesztett protokollok jelentősen hozzájárulnak az európai angolna szurrogát szaporítási technológiájának fejlesztéséhez, és hozzájárulhatnak az európai angolna fontosabb genetikai erőforrásainak megőrzéséhez, amíg új technikák nem jelennek meg vagy az in vitro ivarsejt-kultúra a faj számára.

10 APPENDICES

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10.2 References

Aarts, B.G.W., Van Den Brink, F.W.B., Nienhuis, P.H., 2004. Habitat loss as the main cause of the slow recovery of fish faunas of regulated large rivers in Europe: the transversal floodplain gradient. River Res. Appl. 20, 3–23.

Albert, V., Jónsson, B., Bernatchez, L., 2006. Natural hybrids in Atlantic eels (*Anguilla anguilla*, *A. rostrata*): evidence for successful reproduction and fluctuating abundance in space and time. Mol. Ecol. 15, 1903–1916.

Allan, J.D., Abell, R., Hogan, Z., Revenga, C., Taylor, B.W., Welcomme, R.L., Winemiller, K., 2005. Overfishing of inland waters. Bioscience 55, 1041–1051.

Als, T.D., Hansen, M.M., Maes, G.E., Castonguay, M., Riemann, L., Aarestrup, K., Munk, P., Sparholt, H., Hanel, R., Bernatchez, L., 2011. All roads lead to home: panmixia of European eel in the Sargasso Sea. Mol. Ecol. 20, 1333–1346.

Altmayer, A., 2024. Addressing the critical state of European eel stocks. Brussels, Belgium.

Ananthanarayanan, V., Pins, M.R., Meyer, R.E., Gann, P.H., 2005. Immunohistochemical assays in prostatic biopsies processed in Bouin's fixative. J. Clin. Pathol. 58, 322–324.

Andrae, C.S., Oliveira, E.C.S., Ferraz, M.A.M.M., Nagashima, J.B., 2021. Cryopreservation of grey wolf (*Canis lupus*) testicular tissue. Cryobiology 100, 173–179.

Aroua, S., Schmitz, M., Baloche, S., Vidal, B., Rousseau, K., Dufour, S., 2006. Endocrine evidence that silvering, a secondary metamorphosis in the eel, is a pubertal rather than a metamorphic event. Neuroendocrinology 82, 221–232.

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.

Asturiano, J.F., Pérez, L., Garzón, D.L., Marco-Jiménez, F., Peñaranda, D.S., Vicente, J.S., Jover, M., 2004. Physio-chemical characteristics of seminal plasma and development of media and methods for the cryopreservation of European eel sperm. Fish Physiol. Biochem. 30, 283–293.

Avise, J.C., Nelson, W.S., Arnold, J., Koehn, R.K., Williams, G.C., Thorsteinsson, V., 1990. The evolutionary genetic status of Icelandic eels. Evolution (N. Y). 44, 1254–1262.

Baust, J.M., 2004. Molecular mechanisms of cellular demise associated with cryopreservation failure. Cell Preserv. Technol. 1, 17–31.

Begum, S., Gnanasree, S.M., Anusha, N., Senthilkumaran, B., 2022. Germ cell markers in fishes - A review. Aquac. Fish. 7, 540–552.

Belpaire, C.G.J., Goemans, G., Geeraerts, C., Quataert, P., Parmentier, K., Hagel, P., De Boer, J., 2009. Decreasing eel stocks: survival of the fattest? Ecol. Freshw. Fish 18, 197–214.

Bergersen, R., Klemetsen, A., 1988. Freshwater eel *Anguilla anguilla* (L.) from North Norway, with emphasis on occurrence, food, age and downstream migration. Nord. J. Freshw. Res. 64, 54–66.

Bernery, C., Bellard, C., Courchamp, F., Brosse, S., Gozlan, R.E., Jarić, I., Teletchea, F., Leroy, B., 2022. Freshwater fish invasions: A comprehensive review. Annu. Rev. Ecol. Evol. Syst. 53, 427–456.

Beullens, K., Eding, E.H., Gilson, P., Ollevier, F., Komen, J., Richter, C.J.J., 1997. Gonadal differentiation, intersexuality and sex ratios of European eel (*Anguilla anguilla* L.) maintained in captivity. Aquaculture 153, 135–150.

Bielanski, A., Bergeron, H., Lau, P.C.K., Devenish, J., 2003. Microbial contamination of embryos and semen during long term banking in liquid nitrogen. Cryobiology 46, 146–152.

Bielanski, A., Nadin-Davis, S., Sapp, T., Lutze-Wallace, C., 2000. Viral contamination of embryos cryopreserved in liquid nitrogen. Cryobiology 40, 110–116.

Blanes-García, M., Marinović, Z., Herranz-Jusdado, G., Xuan, X., Ferrão, L., Gallego, V., Pérez, L., Baloch, R.A., Horváth, Á., Pšenička, M., Asturiano, J.F., Morini, M., 2024. Characterization of potential spermatogonia biomarker genes in the European eel (*Anguilla anguilla*). Fish Physiol. Biochem. In Press.

Boëtius, I., Boëtius, J., 1967. Studies in the European Eel, Anguilla anguilla (L.). Experimental induction of the male sexual cycle, its relation to temperature and other factors. Meddelelser Fra Danmarks Fisk. Og Havundersøgelser 4, 339–405.

Borgsteede, F.H.M., Haenen, O.L.M., de Bree, J., Lisitsina, O.I., 1999. Parasitic infections of European eel (*Anguilla anguilla* L.) in the Netherlands. Helminthologia 36, 251–260.

Bos-Mikich, A., Marques, L., Rodrigues, J.L., Lothhammer, N., Frantz, N., 2012. The use of a metal container for vitrification of mouse ovaries, as a clinical grade model for human ovarian tissue cryopreservation, after different times and temperatures of transport. J. Assist. Reprod. Genet. 29, 1267–1271.

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. J. Appl. Ichthyol. 26, 623–635.

Callol, A., Pajuelo, D., Ebbesson, L., Teles, M., MacKenzie, S., Amaro, C., 2015. Early steps in the European eel (*Anguilla anguilla*) – *Vibrio vulnificus* interaction in the gills: Role of the RtxA13 toxin. Fish Shellfish Immunol. 43, 502–509.

Cao, Q., Gu, J., Wang, D., Liang, F., Zhang, H., Li, X., Yin, S., 2018. Physiological mechanism

of osmoregulatory adaptation in anguillid eels. Fish Physiol. Biochem. 44, 423–433.

Chaytor, J.L., Tokarew, J.M., Wu, L.K., Leclre, M., Tam, R.Y., Capicciotti, C.J., Guolla, L., Von Moos, E., Findlay, C.S., Allan, D.S., Ben, R.N., 2012. Inhibiting ice recrystallization and optimization of cell viability after cryopreservation. Glycobiology 22, 123–133.

Ciruna, B., Weidinger, G., Knaut, H., Thisse, B., Thisse, C., Raz, E., Schier, A.F., 2002. Production of maternal-zygotic mutant zebrafish by germ-line replacement. Proc. Natl. Acad. Sci. U. S. A. 99, 14919–14924.

Colombo, G., Grandi, G., 1996. Histological study of the development and sex differentiation of the gonad in the European eel. J. Fish Biol. 48, 493–512.

Cresci, A., 2020. A comprehensive hypothesis on the migration of European glass eels (*Anguilla anguilla*). Biol. Rev. 95, 1273–1286.

Dalsgaard, J., Lund, I., Thorarinsdottir, R., Drengstig, A., Arvonen, K., Pedersen, P.B., 2013. Farming different species in RAS in Nordic countries: Current status and future perspectives. Aquac. Eng. 53, 2–13.

Daverat, F., Limburg, K.E.., Thibault, I., Shiao, J.S., Dodson, J.J., Caron, F., Tzeng, W.N., Wickström, H., 2006. Phenotypic plasticity of habitat use by three temperate eel species, *Anguilla anguilla*, *A. japonica* and *A. rostrata*. Mar. Ecol. 308, 231–241.

Deelder, C.L., 1984. Synopsis of biological data on the eel *Anguilla anguilla* (Linnaeus, 1758). Food and Agriculture Organization of the United Nations (FAO).

Delrez, N., Zhang, H., Lieffrig, F., Mélard, C., Farnir, F., Boutier, M., Donohoe, O., Vanderplasschen, A., 2021. European eel restocking programs based on wild-caught glass eels: Feasibility of quarantine stage compatible with implementation of prophylactic measures prior to scheduled reintroduction to the wild. J. Nat. Conserv. 59, 125933.

Demirel, M.A., Acar, D.B., Ekim, B., Çelikkan, F.T., Alkan, K.K., Salar, S., Erdemli, E.A., Özkavukçu, S., Yar, S.S., Kanca, H., Baştan, A., 2018. The evaluation of xenotransplantation of feline ovarian tissue vitrified by needle immersed vitrification technique into male immunodeficient mice. Cell Tissue Bank. 19, 133–147.

Dollerup, J., Graver, C.M.C., 1985. Repeated induction of testicular maturation and spermiation, alternating with periods of feeding and growth in silver eels, Anguilla anguilla (L.). Dana 4, 19–39.

Dufour, S., Le Belle, N., Baloche, S., Fontaine, Y.A., 1989. Positive feedback control by the gonads on gonadotropin (GTH) and gonadoliberin (GnRH) levels in experimentally matured female silver eels, *Anguilla anguilla*. Fish Physiol. Biochem. 7, 157–162.

Dufour, S., Lopez, E., Le Menn, F., Le Belle, N., Baloche, S., Fontaine, Y.A., 1988.

Stimulation of gonadotropin release and of ovarian development, by the administration of a gonadoliberin agonist and of dopamine antagonists, in female silver eel pretreated with estradiol. Gen. Comp. Endocrinol. 70, 20–30.

Dufour, S., Montero, M., Le Belle, N., Bassompierre, M., King, J.A., Millar, R.P., Peter, R.E., Fontaine, Y.A., 1993. Differential distribution and response to experimental sexual maturation of two forms of brain gonadotropin-releasing hormone (GnRH) in the European eel, *Anguilla anguilla*. Fish Physiol. Biochem. 11, 99–106.

Durif, C.M.F., Elie, P., 2008. Predicting downstream migration of silver eels in a large river catchment based on commercial fishery data. Fish. Manag. Ecol. 15, 127–137.

Durif, C.M.F., van Ginneken, V., Dufour, Sylvie, Müller, T., Elie, P., 2009. Seasonal evolution and individual differences in silvering eels from different locations. In: Thillart, G., Dufour, S., Rankin, J.C. (Eds.), Spawning Migration of the European Eel. Springer, Dordrecht, pp. 13–38.

Elkouby, Y.M., Mullins, M.C., 2017. Methods for the analysis of early oogenesis in zebrafish. Dev. Biol. 430, 310–324.

Falahatkar, B., Poursaeid, S., Kitada, R., Yoshizaki, G., 2017. Hypothermic storage of isolated spermatogonia and oogonia from rainbow trout (*Oncorhynchus mykiss*). Cryobiology 76, 125–128.

Fernández-Díez, C., Pérez-Sanchiz, R., Sarasquete, C., Cabrita, E., Herráez, M.P., Fernandez-Diez, C., Perez-Sanchiz, R., Sarasquete, C., Cabrita, E., Herraez, M.P., 2012. New tools for genome preservation: grafting germinal cells in brown trout (*Salmo trutta*). J. Appl. Ichthyol. 28, 916–918.

Franěk, R., Cheng, Y., Fučíková, M., Kašpar, V., Xie, X., Shah, M.A., Linhart, O., Šauman, I., Pšenička, M., 2022. Who is the best surrogate for germ stem cell transplantation in fish? Aquaculture 549, 737759.

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.

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.

Froese, R., Puley, D., 2024. FishBase [WWW Document]. URL https://www.fishbase.de/ (accessed 4.25.24).

Geeraerts, C., Belpaire, C., 2010. The effects of contaminants in European eel: A review.

Ecotoxicology 19, 239–266.

Goto, R., Saito, T., 2019. A state-of-the-art review of surrogate propagation in fish. Theriogenology 133, 216–227.

Grandi, G., Colombo, G., 1997. Development and early differentiation of gonad in the European eel (*Anguilla anguilla* [L.], Anguilliformes , Teleostei): A cytological and ultrastructural study. J. Morphol. 216, 195–216.

Gravallese, E.M., Monach, P.A., 2015. The rheumatoid joint: Synovitis and tissue destruction. In: Hochberg, M.C., Silman, A.J., Smolen, J.S., Weinblatt, M.E., Weisman, M.H. (Eds.), Rheumatology. Mosby, pp. 768–784.

Grizzle, W.E., Stockard, C.R., Billings, P.E., 2001. The effects of tissue processing variables other than fixation on histochemical staining and immunohistochemical detection of antigens. J. Histotechnol. 24, 213–219.

Gross-Thebing, T., Yigit, S., Pfeiffer, J., Reichman-Fried, M., Bandemer, J., Ruckert, C., Rathmer, C., Goudarzi, M., Stehling, M., Tarbashevich, K., Seggewiss, J., Raz, E., 2017. The vertebrate protein *dead end* maintains primordial germ cell fate by inhibiting somatic differentiation. Dev. Cell 43, 704–715.

Guan, M., Rawson, D.M., Zhang, T., 2008. Cryopreservation of zebrafish (Danio rerio) oocytes using improved controlled slow cooling protocols. Cryobiology 56, 204–208.

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

Haenen, O., van Ginneken, V., Engelsma, M., van den Thillart, Guido, 2009. Impact of eel viruses on recruitment of European eel. In: Thillart, G., Dufour, S., Rankin, J.C. (Eds.), Spawning Migration of the European Eel. Springer, Dordrecht, pp. 387–400.

Haenen, O.L.M., Davidse, A., 2001. First isolation and pathogenicity studies with *Pseudomonas anguilliseptica* from diseased European eel *Anguilla anguilla* (L.) in The Netherlands. Aquaculture 196, 27–36.

Hanel, R., Briand, C., Diaz, E., Döring, R., Sapounidis, A., Warmerdam, W., Andrés, M., Freese, M., Marcelis, A., Marohn, L., Pohlmann, J.D., van Scharrenburg, M., Waidmann, N., Walstra, J., Werkman, M., de Wilde, J., Wysujack, K., 2019. Research for PECH Committee - Environmental , social and economic sustainability of European eel management. Brussels (Belgium).

Herranz-Jusdado, J.G., Gallego, V., Morini, M., Rozenfeld, C., Pérez, L., Müller, T., Horváth, Á., Ohta, H., Asturiano, J.F., 2019. Eel sperm cryopreservation: An overview. Theriogenology 133, 210–215.

Horváth, L., Szabó, T., Burke, J., 1997. Hatchery testing of GnRH analogue-containing pellets on ovulation in four cyprinid species. Polish Arch. Hydrobiol. 44, 221–226.

Howroyd, P., Hoyle-Thacker, R., Lyght, O., Williams, D., Kleymenova, E., 2005. Morphology of the fetal rat testis preserved in different fixatives. Toxicol. Pathol. 33, 300–304.

Huang, Y.S., Rousseau, K., Le Belle, N., Vidal, B., Burzawa-Gérard, E., Marchelidon, J., Dufour, S., 1999. Opposite effects of insulin-like growth factors (IGFs) on gonadotropin (GtH-II) and growth hormone (GH) production by primary culture of European eel (*Anguilla anguilla*) pituitary cells. Aquaculture 177, 73–83.

Inoue, J.G., Miya, M., Miller, M.J., Sado, T., Hanel, R., Hatooka, K., Aoyama, J., Minegishi, Y., Nishida, M., Tsukamoto, K., 2010. Deep-ocean origin of the freshwater eels. Biol. Lett. 6, 363–366.

Isachenko, E., Isachenko, V., Katkov, I.I., Rahimi, G., Schöndorf, T., Mallmann, P., Dessole, S., Nawroth, F., 2004. DNA integrity and motility of human spermatozoa after standard slow freezing versus cryoprotectant-free vitrification. Hum. Reprod. 19, 932–939.

Isachenko, V., Isachenko, E., Reinsberg, J., Montag, M., van der Ven, K., Dorn, C., Roesing, B., van der Ven, H., 2007. Cryopreservation of human ovarian tissue: Comparison of rapid and conventional freezing. Cryobiology 55, 261–268.

Isachenko, V., Maettner, R., Petrunkina, A.M., Sterzik, K., Mallmann, P., Rahimi, G., Sanchez, R., Risopatron, J., Damjanoski, I., Isachenko, E., 2012. Vitrification of human ICSI/IVF spermatozoa without cryoprotectants: New capillary technology. J. Androl. 33, 462–468.

Isayeva, A., Zhang, T., Rawson, D.M., 2004. Studies on chilling sensitivity of zebrafish (*Danio rerio*) oocytes. Cryobiology 49, 114–122.

Jéhannet, P., Palstra, A.P., Giménez Nebot, I., Schipper, H., Swinkels, W., Heinsbroek, L.T.N., Komen, H., 2023. Recombinant gonadotropins to induce oocyte development *in vitro* and *in vivo* in the European eel *Anguilla anguilla*. Fishes 8, 123.

Jung, H., 2020. Hyaluronidase: An overview of its properties, applications, and side effects. Arch. Plast. Surg. 47, 297–300.

Kagawa, H., 2003. Artificial induction of oocyte maturation and ovulation. In: Aida, K., Tsukamoto, K., Yamauchi, K. (Eds.), Eel Biology. Springer, Tokyo, pp. 401–414.

Kása, E., Bernáth, G., Kollár, T., Żarski, D., Lujić, J., Marinović, Z., Bokor, Z., Hegyi, Á., Urbányi, B., Vílchez, M.C., Morini, M., Peñaranda, D.S., Pérez, L., Asturiano, J.F., Horváth, Á., 2017. Development of sperm vitrification protocols for freshwater fish (Eurasian perch, *Perca fluviatilis*) and marine fish (European eel, *Anguilla anguilla*). Gen. Comp. Endocrinol. 245, 102–107.

Kása, E., Lujić, J., Marinović, Z., Kollár, T., Bernáth, G., Bokor, Z., Urbányi, B., Lefler, K.K., Jesenšek, D., Horváth, Á., 2018. Development of sperm vitrification protocols for two endangered salmonid species: the Adriatic grayling, *Thymallus thymallus*, and the marble trout, *Salmo marmoratus*. Fish Physiol. Biochem.

Khan, I.A., Lopez, E., Leloup-Hâtey, J., 1987. Induction of spermatogenesis and spermiation by a single injection of human chorionic gonadotropin in intact and hypophysectomized immature European eel (*Anguilla anguilla* L.). Gen. Comp. Endocrinol. 68, 91–103.

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.

Kirkegaard, E., Aarestrup, K., Pedersen, M.I., Jepsen, N., Koed, A., Larsen, E., Lund, I., Tomkiewicz, J., 2010. European eel and aquaculture, DTU Aqua Report. Kongens Lyngby.

Kottelat, M., Freyhof, J., 2007. Handbook of European freshwater fishes. Imprimerie du Democrate, SA, Delemond, Switzerland.

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, 1–9.

Lacerda, S.M.S.N., Batlouni, S.R., Silva, S.B.G., Homem, C.S.P., França, L.R., 2006. Germ cells transplantation in fish: the Nile-tilapia model. Anim. Reprod. 3, 146–159.

Lane, M., Schoolcraft, W.B., Gardner, D.K., Phil, D., 1999. Vitrification of mouse and human blastocysts using a novel cryoloop container-less technique. Fertil. Steril. 72, 1073–1078.

Latendresse, J.R., Warbrittion, A.R., Jonassen, H., Creasy, D.M., 2002. Fixation of testes and eyes using a modified Davidson's fluid: Comparison with Bouin's fluid and conventional Davidson's fluid. Toxicol. Pathol. 30, 524–533.

Lawrence, A.J., Elliott, M., 2003. Introduction and conceptual model. In: Lawrence, A., Hemingway, K. (Eds.), Effects of Pollution on Fish. John Wiley & Sons, Ltd, pp. 1–13.

Le Gac, F., Blaise, O., Fostier, A., Le Bail, P.Y., Loir, M., Mourot, B., Weil, C., 1993. Growth hormone (GH) and reproduction: a review. Fish Physiol. Biochem. 11, 219–232.

Lee, S., Iwasaki, Y., Shikina, S., Yoshizaki, G., 2013. Generation of functional eggs and sperm from cryopreserved whole testes. Proc. Natl. Acad. Sci. U. S. A. 110, 1640–1645.

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.

Lee, S., Katayama, N., Yoshizaki, G., 2016b. Generation of juvenile rainbow trout derived from cryopreserved whole ovaries by intraperitoneal transplantation of ovarian germ cells. Biochem. Biophys. Res. Commun. 478, 1478–1483.

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

Leloup-Hâtey, J., Oudinet, J.P., Lopez, E., 1985. Testicular steroidogenesis during gonadotropin-induced spermatogenesis in male European eel (Anguilla anguilla L.). In: Lofts, B., Holmes, W.N. (Eds.), Current Trends in Comparative Endocrinology. Hong Kong University Press, Hong Kong, pp. 229–232.

Lenz, J., Macháčová, D., Konečná, P., Fiala, L., Kyllar, M., Tichý, F., 2022. Effects of different fixatives over different fixation times, including Antigenfix, on immunohistochemical studies. Acta Vet. Brno 91, 179–188.

Lin, T.C., Yen, J.M., Kuo, T.C., Gong, K.B., Hsu, K.H., Hsu, T.T., 2008. Comparison of the developmental potential of 2-week-old preantral follicles derived from vitrified ovarian tissue slices, vitrified whole ovaries and vitrified/transplanted newborn mouse ovaries using the metal surface method. BMC Biotechnol. 8, 1–13.

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 J. Anim. Sci. 59, 381–390.

Liu, J., Cheng, K.M., Silversides, F.G., 2012. Novel needle-in-straw vitrification can effectively preserve the follicle morphology, viability, and vascularization of ovarian tissue in Japanese quail (*Coturnix japonica*). Anim. Reprod. Sci. 134, 197–202.

Liu, J., Cheng, K.M., Silversides, F.G., 2013. Production of live offspring from testicular tissue cryopreserved by vitrification procedures in Japanese quail (*Coturnix japonica*). Biol. Reprod. 88, 124.

Lokman, P.M., Young, G., 1998. An intersexual migratory (silver) longfinned New Zealand eel and its gonadal response to treatment with salmon pituitary homogenate. J. Fish Biol. 52, 547–555.

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.

Lujić, J., Marinović, Z., Kása, E., Šćekić, I., Urbányi, B., Horváth, Á., 2018a. Preservation of common carp germ cells under hypothermic conditions: Whole tissue vs isolated cells. Reprod. Domest. Anim. 1–6.

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.

Lujić, J., Marinović, Z., Sušnik Bajec, S., Djurdjevič, I., Urbányi, B., Horváth, Á., 2018b. Interspecific germ cell transplantation: a new light in the conservation of valuable Balkan trout genetic resources? Fish Physiol. Biochem. 44, 1487–1498.

Ma, X., Liu, Y., Fan, W., Cui, Z., 2011. Cryopreservation: Organ preservation. In: Moo-Young, M. (Ed.), Comprehensive Biotechnology. Academic Press, pp. 83–98.

Macente, B.I., Fonseca-Alves, C.E., Magalhães, G.M., Tavares, M.R., Mansano, C.F.M., Mouttham, L., Apparício, M., Toniollo, G.H., Comizzoli, P., 2022. Influence of vitrification device, warming protocol, and subsequent *in vitro* culture on structural integrity of testicular fragments from adult domestic cats. Biopreserving and Biobanking 20, 392–400.

Maitland, P., 2003. Ecology of the river brook and sea lamprey - Conserving Natura 2000 Rivers. EN, CCW, EA, SEPA, SNH & SNIFFER.

Majhi, S.K., Hattori, R.S., Yokota, M., Watanabe, S., Strüssmann, C.A., 2009. Germ cell transplantation using sexually competent fish: An approach for rapid propagation of endangered and valuable germlines. PLoS One 4, e6132.

Marco-Jiménez, F., Pérez, L., Castro, M.P.V. De, Garzón, D.L., Peñaranda, D.S., Vicente, J.S., Jover, M., Asturiano, J.F., 2006. Morphometry characterisation of European eel spermatozoa with computer-assisted spermatozoa analysis and scanning electron microscopy. Theriogenology 65, 1302–1310.

Marinović, Z., 2021. Intra- and inter-specific transplantation of spermatogonial stem cells in freshwater fish. Hungarian University of Agriculture and Life Sciences. PhD Thesis.

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.

Marinović, Z., Lujić, J., Kása, E., Bernáth, G., Urbányi, B., Horváth, Á., 2017. Cryosurvival of isolated testicular cells and testicular tissue of tench *Tinca tinca* and goldfish *Carassius auratus* following slow-reezing. Gen. Comp. Endocrinol. 245, 77–83.

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. J. Vis. Exp. e56118.

Marques, L.S., Bos-Mikich, A., Godoy, L.C., Silva, L.A., Maschio, D., Zhang, T., Streit, D.P., 2015. Viability of zebrafish (*Danio rerio*) ovarian follicles after vitrification in a metal

container. Cryobiology 71, 367–373.

Martinez-Paramo, 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.

Mazur, P., Leibo, S.P.P., Seidel, G.E.G.R.J., 2008. Cryopreservation of the germplasm of animals used in biological and medical research: importance, impact, status, and future directions. Biol. Reprod. 78, 2–12.

Mazur, P., Seki, S., 2011. Survival of mouse oocytes after being cooled in a vitrification solution to -196 °C and 95° to 70000 °C/min and warmed at 610° to 118000 °C/min: A new paradigm for cryopreservation by vitrification. Cryobiology 62, 1–7.

McCleave, J.D., 1993. Physical and behavioural controls on the oceanic distribution and migration of leptocephali. J. Fish Biol. 43, 243–273.

McCleave, J.D., Kleckner, R.C., 1987. Distribution of leptocephali of the catadromous *Anguilla* species in the western Sargasso Sea in relation to water circulation and migration. Bull. Mar. Sci. 41, 789–806.

Meske, C., 1973. Experimentally induced sexual maturity in artificially reared male eels (*Anguilla anguilla*). In: Schröder, J.H. (Ed.), Genetics and Mutagenesis of Fish. Springer, Berlin, Heidelberg, pp. 161–170.

Miller, M., 2009. Ecology of Anguilliform leptocephali: Remarkable transparent fish larvae of the ocean surface layer. Aqua-BioScience Monogr. 2, 1–94.

Molnár, K., Székely, C., Baska, F., 1991. Mass mortality of eel in Lake Balaton due to *Anguillicola crassus* infection. Bull. Eur. Assoc. Fish Pathol. 11, 211–212.

Moriarty, C., 2003. The yellow eel. In: Aida, K., Tsukamoto, K., Yamauchi, K. (Eds.), Eel Biology. Springer, Tokyo, pp. 89–105.

Müller, T., Urbányi, B., Váradi, B., Binder, T., Horn, P., Bercsényi, M., Horváth, Á., 2004. Cryopreservation of sperm of farmed European eel *Anguilla anguilla*. J. World Aquac. Soc. 35, 225–231.

Ottolenghi, F., Silvestri, C., Giordano, P., Lovatelli, A., New, M.B., 2004. Capture-based aquaculture. The fattening of eels, groupers, tunas and yellowtails. FAO (Food and Agriculture Organization of the United Nations), Rome (Italy).

Palstra, A., Curiel, D., Fekkes, M., de Bakker, M., Székely, C., van Ginneken, V., van den Thillart, G., 2007. Swimming stimulates oocyte development in European eel. Aquaculture 270, 321–332.

Palstra, A., van Ginneken, V., van den Thillart, Guido, 2009. Effects of swimming on silvering

and maturation of the European eel, *Anguilla anguilla* L. In: Thillart, G., Dufour, S., Rankin, J.C. (Eds.), Spawning Migration of the European Eel. Springer, Dordrecht, pp. 229–251.

Palstra, A.P., van den Thillart, G.E.E.J.M., 2010. Swimming physiology of European silver eels (*Anguilla anguilla* L.): Energetic costs and effects on sexual maturation and reproduction. Fish Physiol. Biochem. 36, 297–322.

Panagiotidis, Y., Vanderzwalmen, P., Prapas, Y., Kasapi, E., Goudakou, M., Papatheodorou, A., Passadaki, T., Petousis, S., Nikolettos, N., Veletza, S., Prapas, N., Maroulis, G., 2013. Open versus closed vitrification of blastocysts from an oocyte-donation programme: a prospective randomized study. Reprod. Biomed. Online 26, 470–476.

Pankhurst, N.W., Sorensen, P.W., 1984. Degeneration of the alimentary tract in sexually maturing European *Anguilla anguilla* (L.) and American eels *Anguilla rostrata* (LeSueur). Can. J. Zool. 62, 1143–1149.

Pedersen, M.I., Jepsen, N., Aarestrup, K., Koed, A., Pedersen, S., Økland, F., 2012. Loss of European silver eel passing a hydropower station. J. Appl. Ichthyol. 28, 189–193.

Peñaranda, D.S., Gallego, V., Rozenfeld, C., Herranz-Jusdado, J.G., Pérez, L., Gómez, A., Giménez, I., Asturiano, J.F., 2018. Using specific recombinant gonadotropins to induce spermatogenesis and spermiation in the European eel (*Anguilla anguilla*). Theriogenology 107, 6–20.

Peñaranda, D.S., Pérez, L., Gallego, V., Jover, M., Asturiano, J.F., 2009. Improvement of European eel sperm cryopreservation method by preventing spermatozoa movement activation caused by cryoprotectants. Cryobiology 59, 119–126.

Pérez, L., Aturiano, J.F., Tomás, A., Zegrari, S., Barrera, R., Espinós, F.J., Navarro, J.C., Jover, M., 2000. Induction of maturation and spermiation in the male European eel: assessment of sperm quality throughout treatment. J. Fish Biol. 57, 1488–1504.

Pike, C., Crook, V., Gollock, M., 2020. *Anguilla anguilla*, European eel. IUCN Red List Threat. Species^{TM} 2020, e.T60344A152845178.

Pirollo, T., Perolo, A., Mantegari, S., Barbieri, I., Scali, F., Alborali, G.L., Salogni, C., 2023. Mortality in farmed European eel (*Anguilla anguilla*) in Italy due to *Streptococcus iniae*. Acta Vet. Scand. 65, 1–8.

Pšenička, M., Saito, T., 2020. Specificity of germ cell technologies in sturgeons. In: Yoshida, M., Asturiano, J.F. (Eds.), Reproduction in Aquatic Animals: From Basic Biology to Aquaculture Technology. Springer Singapore, Singapore, pp. 335–356.

Pšenička, M., Saito, T., Linhartová, Z., Gazo, I., 2015. Isolation and transplantation of sturgeon

early-stage germ cells. Theriogenology 83, 1085–1092.

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, 119–122.

Querat, B., Hardy, A., Fontaine, Y.A., 1991. Regulation of the type-II gonadotrophin α and β subunit mRNAs by œstradiol and testosterone in the European eel. J. Mol. Endocrinol. 7, 81–86.

Rahimi, G., Isachenko, E., Isachenko, V., Sauer, H., Wartenberg, M., Tawadros, S., Hescheler, J., Mallmann, P., Nawroth, F., 2004. Comparison of necrosis in human ovarian tissue after conventional slow freezing or vitrification and transplantation in ovariectomized SCID mice. Reprod. Biomed. Online 9, 187–193.

Rawlings, N.D., Barrett, A.J., 1993. Evolutionary families of peptidases. Biochem. J. 290, 205–218.

Rezazadeh Valojerdi, M., Eftekhari-Yazdi, P., Karimian, L., Hassani, F., Movaghar, B., 2009. Vitrification versus slow freezing gives excellent survival, post warming embryo morphology and pregnancy outcomes for human cleaved embryos. J. Assist. Reprod. Genet. 26, 347–354.

Rodrigues, R.B., Uczay, M., Brito, V.B., Godoy, A.C., Moura, D.J., Vogel, C., Vasconcelos, A.C.N., Streit, D.P., 2021. Oxidative stress and DNA damage of zebrafish sperm at different stages of the cryopreservation process. Zebrafish 18, 97–109.

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. Biol. Reprod. 78, 159–166.

Saito, T., Goto-Kazeto, R., Kawakami, Y., Nomura, K., Tanaka, H., Adachi, S., Arai, K., Yamaha, E., 2011. The mechanism for primordial germ-cell migration is conserved between Japanese eel and zebrafish. PLoS One 6, e24460.

Schulz, R.W., de França, L.R., Lareyre, J.J., LeGac, F., Chiarini-Garcia, H., Nobrega, R.H., Miura, T., 2010. Spermatogenesis in fish. Gen. Comp. Endocrinol. 165, 390–411.

Schulz, R.W., Nobrega, R.H., 2011a. Regulation of Spermatogenesis. In: Encyclopedia of Fish Physiology. pp. 627–634.

Schulz, R.W., Nobrega, R.H., 2011b. The reproductive organs and processes. In: Encyclopedia of Fish Physiology. pp. 616–626.

Sébert, M.E., Amérand, A., Vettier, A., Weltzien, F.A., Pasqualini, C., Sébert, P., Dufour, S., 2007. Effects of high hydrostatic pressure on the pituitary–gonad axis in the European eel,

Anguilla anguilla (L.). Gen. Comp. Endocrinol. 153, 289–298.

Sébert, M.E., Weltzien, F.A., Moisan, C., Pasqualini, C., Dufour, S., 2008. Dopaminergic systems in the European eel: characterization, brain distribution, and potential role in migration and reproduction. Hydrobiologia 602, 27–46.

Seki, S., Jin, B., Mazur, P., 2014. Extreme rapid warming yields high functional survivals of 8-cell mouse embryos even when suspended in a half-strength vitrification solution and cooled at moderate rates to -196 °C. Cryobiology 68, 71–78.

Seki, S., Mazur, P., 2008. Effect of Warming Rate on the Survival of Vitrified Mouse Oocytes and on the Recrystallization of Intracellular Ice. Biol. Reprod. 79, 727–737.

Seki, S., Mazur, P., 2012. Ultra-rapid warming yields high survival of mouse oocytes cooled to -196 ℃ in dilutions of a standard vitrification solution. PLoS One 7.

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*). Mol. Reprod. Dev. 80, 763–773.

Sinha, V.R.P., Jones, J.W., Pritchett, R.S., 1975. The European freshwater eel. Liverpool University Press.

Slabbert, M., du Plessis, S.S., Huyser, C., 2015. Large volume cryoprotectant-free vitrification: an alternative to conventional cryopreservation for human spermatozoa. Andrologia 47, 594–599.

Sorensen, P.W., Pankhurst, N.W., 1988. Histological changes in the gonad, skin, intestine and olfactory epithelium of artificially-matured male American eels, *Anguilla rostrata* (LeSueur). J. Fish Biol. 32, 297–307.

Succu, S., Leoni, G.G., Bebbere, D., Berlinguer, F., Mossa, F., Bogliolo, L., Madeddu, M., Ledda, S., Naitana, S., 2007. Vitrification devices affect structural and molecular status of in vitro matured ovine oocytes. Mol. Reprod. Dev. 74, 1337–1344.

Suvarna, S.K., Layton, C., Bancroft, J.D., 2019. Bancroft's theory and practice of histological techniques, Eighth edi. ed, Techniques in Histopathology and Cytopathology. Elsevier.

Szabó, G., Müller, T., Bercsényi, M., Urbányi, B., Kucska, B., Horváth, Á., 2005. Cryopreservation of European eel (*Anguilla anguilla*) sperm using different extenders and cryoprotectants. Acta Biol. Hung. 56, 173–175.

Tamburrino, L., Traini, G., Marcellini, A., Vignozzi, L., Baldi, E., Marchiani, S., 2023. Cryopreservation of human spermatozoa: Functional, molecular and clinical aspects. Int. J. Mol. Sci. 24, 4656.

Tang, Q., Abdelfattah, N.S., Blackburn, J.S., Moore, J.C., Martinez, S.A., Moore, F.E.,

Lobbardi, R., Tenente, I.M., Ignatius, M.S., Berman, J.N., Liwski, R.S., Houvras, Y., Langenau, D.M., 2014. Optimized cell transplantation using adult *rag2* mutant zebrafsh. Nat. Methods 11, 821–824.

Tesch, F.W., Wegner, G., 1990. The distribution of small larvae of *Anguilla* sp. related to hydrographic conditions 1981 between Bermuda and Puerto Rico. Int. Rev. der gesamten Hydrobiol. und Hydrogr. 75, 845–858.

Thomson, L.K., Fleming, S.D., Aitken, R.J., De Iuliis, G.N., Zieschang, J.A., Clark, A.M., 2009. Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. Hum. Reprod. 24, 2061–2070.

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

Tsukamoto, K., Kuroki, M., Watanabe, S., 2020. Common names for all species and subspecies of the genus *Anguilla*. Environ. Biol. Fishes 103, 985–991.

U.S. Fish and Wildlife Service, 2019. European eel (Anguilla anguilla) - Ecological risk screening summary. Washington DC, USA.

Vajta, G., Holm, P., Kuwayama, M., Booth, P.J., Jacobsen, H., Greve, T., Callesen, H., 1998. Open pulled straw (OPS) vitrification: A new way to reduce cryoinjuries of bovine ova and embryos. Mol. Reprod. Dev. 51, 53–58.

Van Beurden, S.J., Engelsma, M.Y., Roozenburg, I., Voorbergen-Laarman, M.A., Van Tulden, P.W., Kerkhoff, S., Van Nieuwstadt, A.P., Davidse, A., Haenen, O.L.M., 2012. Viral diseases of wild and farmed European eel *Anguilla anguilla* with particular reference to the Netherlands. Dis. Aquat. Organ. 101, 69–86.

van der Oost, R., Beyer, J., Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: a review. Environ. Toxicol. Pharmacol. 13, 57–149.

van Ginneken, V., Bruijs, M., Murk, T., Palstra, A., van den Thillart, Guido, 2009. The effect of PCBs on the spawning migration of European silver eel (*Anguilla anguilla* L.). In: Thillart, G., Dufour, S., Rankin, J.C. (Eds.), Spawning Migration of the European Eel. Springer, Dordrecht, pp. 365–386.

van Ginneken, V.J.T., Maes, G.E., 2005. The European eel (*Anguilla anguilla*, Linnaeus), its lifecycle, evolution and reproduction: A literature review. Rev. Fish Biol. Fish. 15, 367–398.

Verreault, G., Mingelbier, M., Dumont, P., 2012. Spawning migration of American eel *Anguilla rostrata* from pristine (1843–1872) to contemporary (1963–1990) periods in the St Lawrence Estuary, Canada. J. Fish Biol. 81, 387–407.

Vollestad, L.A., 1992. Geographic variation in age and length at metamorphosis of maturing European eel: Environmental effects and phenotypic plasticity. J. Anim. Ecol. 61, 48.

Wang, H., Yang, L.L., Ji, Y.L., Chen, Y.H., Hu, J., Zhang, C., Zhang, J., Xu, D.X., 2016. Different fixative methods influence histological morphology and TUNEL staining in mouse testes. Reprod. Toxicol. 60, 53–61.

Wang, W.Y., Meng, L.J., Xu, Y.J., Gong, T., Yang, Y., 2020. Effects of 4% paraformaldehyde and modified Davidson's fluid on the morphology and immunohistochemistry of Xiang pig testes. J. Toxicol. Pathol. 33, 97–104.

Wang, Y., Xiao, Z., Li, L., Fan, W., Li, S.W., 2008. Novel needle immersed vitrification: A practical and convenient method with potential advantages in mouse and human ovarian tissue cryopreservation. Hum. Reprod. 23, 2256–2265.

Xing, W., Zhou, C., Bian, J., Montag, M., Xu, Y., Li, Y., Li, T., 2010. Solid-surface vitrification is an appropriate and convenient method for cryopreservation of isolated rat follicles. Reprod. Biol. Endocrinol. 8, 1–9.

Yamada, Y., Zhang, H., Okamura, A., Tanaka, S., Horie, N., Mikawa, N., Utoh, T., Oka, H.P., 2001. Morphological and histological changes in the swim bladder during maturation of the Japanese eel. J. Fish Biol. 58, 804–814.

Yang, Y., Honaramooz, A., 2010. Effects of medium and hypothermic temperatures on preservation of isolated porcine testis cells. Reprod. Fertil. Dev. 22, 523–532.

Yang, Y., Steeg, J., Honaramooz, A., 2010. The effects of tissue sample size and media on short-term hypothermic preservation of porcine testis tissue. Cell Tissue Res. 340, 397–406.

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. Physiol. Part D Genomics Proteomics 6, 55–61.

Yoshizaki, G., Takashiba, K., Shimamori, S., Fujinuma, K., Shikina, S., Okutsu, T., Kume, S., Hayashi, M., 2016. Production of germ cell-deficient salmonids by *dead end* gene knockdown, and their use as recipients for germ cell transplantation. Mol. Reprod. Dev. 83, 298–311.

Yoshizaki, G., Yazawa, R., 2019. Application of surrogate broodstock technology in aquaculture. Fish. Sci. 83, 429–437.

Zacchei, A.M., Tavolaro, P., 1988. Lateral line system during the life cycle of *Anguilla anguilla* (L.). Ital. J. Zool. 55, 145–153.

Zhou, L., Wang, X., Liu, Q., Yang, J., Xu, S., Wu, Z., Wang, Y., You, F., Song, Z., Li, J., 2021.

Successful spermatogonial stem cells transplantation within Pleuronectiformes: First breakthrough at inter-family level in marine fish. Int. J. Biol. Sci. 17, 4426–4441.

10.3 Supplement 1

Reagents

- 2-phenoxyethanol - VWR, cat. no. 26244.290
- Benzocaine - Sigma-Aldrich, cat. no. E1501
- Phosphate buffered saline (PBS) tablets - Sigma-Aldrich, cat. no. P4417
- Leibovitz (L-15) medium - Sigma-Aldrich, cat. no. L1518
- Collagenase - Gibco, cat. no. 9001-12-1
- Trypsin - Sigma-Aldrich, cat. no. T8003
- DNase I - Pan-Reac AppliChem, cat. no. A3778
- Fetal bovine serum (FBS) - Sigma-Aldrich, cat. no. F9665
- Trypan blue - Sigma-Aldrich, cat. no. T6146
- Formalin - Reanal, cat. no. 10492-1-01-65
- Bouin's solution - Sigma-Aldrich, cat. no. HT10132
- Ethanol - Lachner, cat. no. 20025-A99
- Xylol - Molar Chemicals, cat. no. 09690-507-410
- Paraffin - Molar Chemicals, cat. no. 08010-469-230
- Hematoxylin - Merck, cat. no. 75290
- Eosin - Merck, cat. no. 45380
- PKH-26 fluorescent linker dye - Sigma-Aldrich, cat. no. MIDI26
- Agar - Reanal, cat. no. 03340-1-99-33
- TRI reagent - Molecular Research Center, cat. no. TR118
- Dimethyl sulfoxide - Reanal, cat. no. 08860-1-08-65
- Propylene glycol - Reanal, cat. no. 00190-1-01-65
- Ethylene glycol - Reanal, cat. no. 01250-0-99-65
- Glycerol - Sigma-Aldrich, cat. no. G5516
- Methanol - Reanal, cat. no. 20740-0-01-65
- Glucose - Reanal, cat. no. 07072-1-08-38
- Trehalose - Arcos Organics, cat. no. 309870250
- Sucrose - Reanal, cat. no. 07140-1-08-38
- Fructose - Reanal, cat. no. 07190-1-08-38
- Bovine serum albumin (BSA) - Fisher Scientific, cat. no. BF9701-100
- HEPES - Sigma-Aldrich, cat. no. H3375

- RevertAid first strand cDNA synthesis kit - ThermoFisher Scientific, cat. no. K1622
- HOT FIREPol EvaGreen qPCR supermix - Solis BioDyne, cat. no. 08-36-00008
- Transcriptor High Fidelity cDNA Synthesis Kit - Sigma-Aldrich, cat. no. 5091284001
- Penicillin/Streptomycin mix - Sigma-Aldrich, cat. no. P4333
- RNAlater - Sigma-Aldrich, cat. no. R0901
- First-DNA all tissue kit - Genial, cat. no. D1002000