EEL SPERM CRYOPRESERVATION: AN OVERVIEW

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ABSTRACT

The eels are teleost fishes from the order Anguilliformes that includes several species with high commercial value. Due to the high interest for aquaculture production of some eel species and for the need to restore eel species that are endangered, several research groups have directed their research toward developing protocols to cryopreserve the spermatozoa of Japanese eel (*Anguilla japonica*) and European eel (*Anguilla anguilla*). In this review, we provide an overview on the different protocols that have been developed so far. The first developed protocols used DMSO as cryoprotectant in both species with good success, obtaining sperm motilities of over 45% in Japanese eel and over 35% in European eel. Moreover, sperm cryopreserved using DMSO was successfully used in fertilization trials, although with low fertilization rates. However, recent studies show that DMSO produce epigenetic changes in eel sperm and therefore, the last developed protocols used methanol as cryoprotectant instead. Cryopreservation protocols using methanol as cryoprotectant, showed improved motility values in both Japanese and European eel. In addition, the latest protocols have been adapted to cryopreserve larger volumes of sperm of up to 5 mL, which is useful for larger scale fertilization trials.

The present study introduces the state of the art and future perspectives of the eel sperm cryopreservation to be applied in aquaculture and biological conservation programs.

Keywords: Fish; *Anguilla*; DMSO; Methanol; Spermatozoa motility; Vitrification

1. Introduction

Freshwater eels of the genus *Anguilla* include 19 species [1], all of which display a complex catadromous life cycle, with oceanic migrations ranging from few hundreds to thousands of kilometers depending on the species [2]. Recent studies have indicated that the genus *Anguilla* is originated in the deep ocean of tropical areas and freshwater eels radiated out from the tropics to colonize the temperate regions [3,4].

Since the early 80´s, the population of temperate eels has continuously declined. Particularly, the populations of European eel (*A. anguilla*) and Japanese eel (*A. japonica*) became reduced by 90% in the last 30 years, however the decline of the American eel (*A. rostrata*) population is less dramatic [5]. In the case of tropical eels, the actual situation is uncertain, since data of fisheries are unavailable. The causes behind the decline of the temperate eel populations are most likely due to a combination of global climate change, habitat degradation, pollution, parasite infection and overfishing. These eels are common in the traditional diets of many countries, especially in Europe and Asia. The country where eels are consume the most is Japan. There, eels are smoked and processed into a dish named “kabayaki”. This delicatessen is made using Japanese eel, but imported eels from the United States (American eel) and Europe (European eel) were also used due to the low availability of Japanese eels. However, since 2010, due to the decline of European eel catches, the EU imposed export restrictions, making illegal to sell European-caught eel to markets outside the EU [6]. In Europe, eels are consumed smoked principally in northern European countries or consumed at elver stage in places like northern Spain. In the United States, although eels were consumed by the pilgrims from Europe, the nowadays catches are mostly used for export of elvers to Europe or Asia [7]. However, all three temperate eel species have been included in the Red List of the International Union for Conservation of Nature (IUCN) as threatened due to population decline, with

A. japonica and *A. rostrata* categorized as “Endangered” [8,9], and *A. anguilla* included as “Critically Endangered” [10], which is the highest category before extinction rating. The conservation status of the eel species justifies the needs for taking actions such as development of reproduction in captivity and control of fisheries based on life cycle. The complexity of their life cycle include a metamorphosis. First, the larvae hatched as leptocephalus, which has a laterally compressed body and looks like a leaf with a small head. These first larvae are transported by the oceanic currents to the continental coasts, where they metamorphose into glass eels. At this stage, they display the anguilliform shape but they are thin, small and unpigmented. Thereafter, the glass eels migrate into coastal waters and turn into pigmented elver eels, that later migrate into continental waters and become yellow eels. At this stage, eels undergo a sedentary and feeding phase in freshwater prior to enter the silver eel stage (called silvering). Silvering is a puberty related event, which marks the beginning of sexual maturation, migration and the reproductive phase. Silver eels are still sexually immature when they start their reproductive migration, with sexual maturation occurring during the migration period towards the reproduction site in the ocean. However, in captivity, dopaminergic inhibitions in addition to a deficient stimulation of gonadotropin-releasing hormone (GnRH) block the eel sexual maturation as long as the reproductive oceanic migration is not performed [11-13]. Therefore, eels are blocked in a pre-pubertal stage and do not mature spontaneously in captivity. To induce an artificial full maturation in eels, costly hormonal treatments are required that last for several weeks in males and even months in females [14-17]. Moreover, there is frequently a maturation asynchrony between sexes. In females, the period of time after ovulation during which oocytes are viable for fertilization is very short [18,19]. Therefore, preservation of sperm would be essential to facilitate successful artificial fertilization.

Interestingly, eel spermatozoon present an unusual structure (Figure 1). It possesses a crescent-shaped nucleus with a flagellum consisting of a 9+0 pattern, whereas the typical axonemal structure of the flagellum is 9+2. Moreover, it has a pseudoflagelum and a single large spherical mitochondrion on the anterior surface at the superior end of the nucleus [20,21].

Cryopreservation is the conservation of biological material in liquid nitrogen (LN) at very low temperatures (-196 ºC) that may potentially preserve its viability indefinitely [22]. In addition to long-term conservation of sperm, cryopreservation of sperm presents several additional advantages; for instance providing research scholars with biological materials to perform comparative experiments, to promote exchange of genetic material for use in breeding and genetic studies [23,24].

Cryopreservation of eel sperm was first achieved by Tanaka et al [25] in the early 2000´s for Japanese eel. Many advances have been achieved since, including the development of cryopreservation protocols for European eel. In this review, we performed an overview of the historical development of different sperm cryopreservation protocols of two freshwater eels including the European and the Japanese eels.

2. Eels artificial maturation

Good gamete quality is crucial for successful sperm cryopreservation [24]. Eels (*Anguilla* spp.) do not mature spontaneously in captivity, so to obtain good quality sperm in the lab, male eels need to be treated with long-term hormonal treatments, i.e. gonadotropins, to induce maturation [26-28]. These treatments produce a boost in the plasma levels of 11-ketotestosterone (11-KT), which is the effective androgen in most fish species, including eels [29]. Leydig cells are considered as the major source of androgens, while androgen receptors are mainly expressed in Sertoli cells and in interstitial cells. However, androgen

receptors are also expressed in Leydig cells, where androgens modulate the expression of steroidogenic genes [30], suggesting that androgens develop biological activity via testicular somatic cells [31]. Sertoli cells produce different growth factors during spermatogenesis, and their expression or repression seems to regulate spermatogonial mitosis and germ cell differentiation [31]. Consequently, the hormonal treatment with gonadotropins promotes spermatogenesis and spermiation.

The traditional hormonal treatment with gonadotropins to induce maturation in Japanese eel and European eel males typically consists of weekly injections of human chorionic gonadotropins (hCG) [26,32] and has been used as the preferred method to obtain high quality sperm for cryopreservation trials in the eels [25,33-41]. However, application of heterologous hormonal treatments with hCG have been observed to produce low rates of fertilization and hatching due to low gamete quality [25], and a new line of studies focuses on the development of homologous gonadotropic hormones to induce eel maturation.

In European eel, Peñaranda et al. [42] used homologous recombinant LH and FSH, which were obtained by transfection of mammalian cells of Chinese hamster ovary. They treated immature European eels with weekly injections of recombinant LH and FSH and successfully induced full spermatogenesis and spermiation *in vivo*. Nonetheless, there were high variations in sperm quality among treated males [42], and thus sperm obtained from this protocol has not yet been used for any cryopreservation trial. In parallel, in the Japanese eel, Kazeto et al. [43] succeeded in producing homologous recombinant gonadotropins of Japanese eel synthetized as well from cell lines of Chinese hamster ovary. Soon after, Ohta et al. [44] developed a protocol for Japanese eel maturation consisting of weekly injections of recombinant LH at a dose of 500 μg/kg fish, that induced a high volume of spermiation and fast stimulation of spermatogenesis. This maturation method has been successfully used in cryopreservation and fertilization trials

151 with positive results [45].

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153 **3. Cryopreservation protocols**

154 **3.1 Japanese eel sperm cryopreservation**

155 A cryopreservation protocol for Japanese eel sperm was first developed by Tanaka et al.
156 [25]. In this work, the researchers first designed a cryopreservation diluent or extender,
157 to prevent cryoinjury of the spermatozoa and to avoid the spermatozoa activation. This is
158 crucial, since when spermatozoa start their motility, the stored ATP required for the
159 movement of the flagellum will last for only a few minutes [46]. The cryoprotectant used
160 was dimethyl sulphoxide (DMSO) at 10% v/v, which is the most common compound
161 used as cryoprotectant in sperm of marine fish species [47], and the extender diluent
162 included NaCl, $NaHCO_3$ and soya lecithin (Table 1). The use of DMSO caused a
163 hypertonicity in the medium that activated sperm motility [48], but due to the inclusion
164 of $NaHCO_3$ in the extender, the spermatozoa motility was prevented and the protective
165 capacity of the freezing medium was improved [49]. Furthermore, the cryopreservation
166 protocol used 2 mL cryovials that were cooled in LN vapor for 5 min, 2 mm above the
167 LN surface, before immersion and storage, and the thawing consisted in immersion in a
168 water bath at 40 ºC for 70 s.

169 Using this protocol, Tanaka et al. [25] obtained good post-thaw sperm motility values
170 (37-46%), and therefore, they used it for fertilization trials. In these trials, they
171 successfully fertilized Japanese eel oocytes using cryopreserved sperm, however the
172 hatchability of the fertilized eggs was lower than eggs fertilized with fresh sperm.

173 For long time, this was the only published Japanese eel sperm cryopreservation protocol,
174 until Müller et al. [50] published a new cryopreservation protocol in which an artificial
175 seminal plasma (ASP) and methanol were used as extender and cryoprotectant,

176 respectively. The composition of ASP was based on the Ohta et al.'s study [26], and was
177 prepared with (in mM) 149.3 NaCl, 15.2 KCl, 1.3 $CaCl_2$, 1.6 $MgCl_2$ and 20 $NaHCO_3$,
178 buffered with 20 mM TAPS-NaOH at pH 8.1, and possess iso-ionic osmolality to the
179 seminal plasma of artificially maturated Japanese eel. In contrast to DMSO, methanol is
180 osmotically inert avoiding the spermatozoa motility activation that has been caused by
181 DMSO. Moreover, the protocol used 0.5 mL straws and the freezing was conducted in
182 LN vapor for 3 min, 3 cm over the LN surface, before immersion and storage, and the
183 thawing consisted of immersion for 13 s in water at 40 ºC. Although Müller et al. [50]
184 successfully cryopreserved Japanese eel sperm, the embryos hatched using cryopreserved
185 samples showed lower survival and higher malformation rate than those of fresh sperm,
186 indicating that the protocol was still sub-optimal.
187 In parallel, Koh et al. [51] conducted a series of experiments focused on the use of K30
188 ASP [52] as extender in an alternative cryopreservation protocol for Japanese eel sperm.
189 The K30 ASP consisted of (in mM) 134.3 NaCl, 30 KCl, 20 $NaHCO_3$, 1.6 $MgCl_2$, 1.3
190 $CaCl_2$, and buffered at pH 8.1 with 20 mM TAPS-NaOH. Moreover, they tested different
191 cryoprotectants in various concentrations, addition of fetal bovine serum (FBS), different
192 storage temperatures before cooling, sperm dilution ratios and cooling rates. The
193 cryoprotectants tested were methanol, DMSO, N-N,dimethyl formamide (DMF), N-
194 N,dimethyl-acetamide (DMA) and a combination of methanol and DMA. Their results
195 showed that the optimal protocol with the tested parameters consisted of 10 or 15%
196 methanol as cryoprotectant, with 22.5% FBS and 67.5% K30 ASP as extender solutions,
197 obtaining results close to 60% of comparative post thaw motility (CPM), which is a
198 parameter calculated from [sperm motility (%) after cryopreservation × sperm motility
199 (%) before preservation^{-1}] × 100. Furthermore, the cooling rates used were 6.3 – 28.6
200 ºC/min, corresponding to cooling at 10-16 cm above the LN surface when 0.25 mL straws

were used, and the temperature at which the samples were immersed in LN was -40 to -70 ºC. Interestingly, the use of DMSO as cryoprotectant was incompatible with the use of K30 ASP as extender for Japanese eel sperm.

Following the work of Koh et al. [51], Nomura et al. [45] established a large-scale cryopreservation protocol for Japanese eel sperm to be used for fertilization programs. They used 5 mL straws and adapted the cooling rate to that volume, and the cryoprotectants and extenders were as described in the latest protocol [51]. Further, the fertilization trials did not show any difference in egg hatching or survival rates between cryopreserved sperm and fresh sperm. Moreover, the morphology of larvae produced from cryopreserved sperm was similar to that of larvae from fresh sperm, and the larvae were further grown into normal glass eels, representing a great refinement of the Japanese eel sperm cryopreservation protocol.

3.2 European eel sperm cryopreservation

Cryopreservation of the European eel sperm was developed shortly after the first cryopreservation protocols for Japanese eel. Two independent research groups established their own cryopreservation protocols in Spain and Hungary. These protocols differed in most aspects, from the rearing conditions of the eels to the type of cryoprotectants applied into their experiments (Table 2).

The group from Spain developed a primary protocol [33,53] mimicking the protocol previously developed for Japanese eel [25] using DMSO as cryoprotectant (Table 1). Different extenders were tested, including two developed for Japanese eel (Tanaka´s and K30) and two developed for the European eel (P1 and P2) designed to be iso-ionic to the seminal plasma of European eel [54]. The Tanaka medium, developed for Japanese eel had (in mM) 137 NaCl, 76.2 $NaHCO_3$ and 20 TAPS at pH 8.2, and the K30 medium with (in mM) 134.5 NaCl, 20 $NaHCO_3$, 30 KCl, 1.6 $MgCl_2$, 1.3 $CaCl_2$, and at pH 8.1. The P1

medium developed for European eel, was composed by (in mM) 125 NaCl, 20 $NaHCO_3$, 30 KCl, 2.5 $MgCl_2$, 1 $CaCl_2$, and the pH was adjusted to 8.5, and the P2 medium was prepared with (in mM) 70 NaCl, 75 $NaHCO_3$, 30 KCl, 2.5 $MgCl_2$, 1 $CaCl_2$, and pH 8.5. All media were supplemented with 10% DMSO as cryoprotectant and different sperm dilution ratios were also examined. The freezing process was carried out in 0.25 mL straws placed for 10 min, 5 cm above LN surface before plunging them into LN, and the thawing was conducted by submerging the straws in a water bath at 20 ºC during 45 s. In this first approach, Asturiano el al. [33,53] reported that sperm samples diluted 1:5 in Tanaka extender or P1 extender with 10% DMSO showed the highest spermatozoa motility post thawing.

In parallel, the group from Hungary [34] developed a cryopreservation protocol using a modified Kurokura solution as extender (in mM: 61.6 NaCI, 134.1 KCI, 1.98 $CaCl_2$, 0.84 $MgCl_2$, 2.4 $NaHCO_3$) and 10% methanol as cryoprotectant (Table 1). The dilution rate used was 1 sperm: 8 extender: 1 methanol using 0.25 mL straws, and cooling them 4 cm over the LN for 3 min before plunging them into the LN. A water bath at 40 ºC was used to thaw cryopreserved sperm for 5 s. Under application of this protocol, Müller et al. [34] obtained similar results to those of the Spanish protocol, although protocols show differences. Following this study, Szabó el al. [35] conducted a series of experiments to test different extenders and cryoprotectants (DMSO and methanol), aiming at improvement of the protocol described by Müller et al. [34]. They observed that application of DMSO (10%) with Tanaka extender, and application of methanol (10%) with Tanaka extender resulted in the highest success compared to other treatments. The samples cryopreserved using the protocol with methanol could be further diluted 1:9 in Tanaka's medium to reduce the toxicity of the cryoprotectant, which may be important for further short-term storage of frozen-thawed sperm. This was not a possibility when

using DMSO as cryoprotectant, probably explained by the change in osmolality of sperm following dilution [48].

Similarly, the Spanish group conducted a study where they tested the effect of DMSO, methanol and other cryoprotectants, with different dilution ratios and freezing medium supplementation with FBS, on European eel spermatozoa motility, viability and on spermatozoa head size [36,37]. Here, they found that viability for frozen-thawed eel spermatozoa with DMSO and methanol was similar, but the spermatozoa heads when cryopreserved in methanol medium were smaller than with DMSO. Furthermore, the researchers found a positive effect when the freezing medium was supplemented with FBS (25%). In a different study, a similar effect as spermatozoa membrane protector was found when adding L-α-phosphatidylcholine [55]. However, this compound also increased the osmolality and density of the media, being therefore impractical to use.

Although valuable results were obtained following the protocol using DMSO in terms of percentage of spermatozoa motility, viability and spermatozoa head size, the use of this cryoprotectant still increased the medium osmolality resulting in inducing spermatozoa motility activation and premature ATP consumption. To avoid this drawback, Peñaranda et al. [38] tested different combinations of pH and $NaHCO_3$ concentrations. The use of $NaHCO_3$ was previously included in the Japanese eel sperm cryopreservation protocols, developed by Tanaka et al. [25] because of its inhibitory role on spermatozoa motility. Based on this feature, Peñaranda et al. [38] developed an improved medium based on the P1 medium, but containing 100 mM $NaHCO_3$ and pH 6.5 that partially prevented the activation effect of DMSO. Furthermore, the researchers refined the protocol and used a 1:2 (sperm:freezing medium) dilution, and the 0.25 mL straws were cooled 1.6 cm above LN surface for 5 min before being immersed into LN. With this protocol, they obtained post-thaw spermatozoa motility values close to 40%, which is well sufficient for

fertilization trials.

Following this last protocol, Asturiano et al. [40] successfully used cryopreserved sperm in fertilization trials, to produce viable offspring. Despite the low percentage of fertilized eggs, lower than that observed with fresh sperm, embryos developed and a few larvae from cryopreserved sperm were obtained at 55 h after fertilization. Similarly, Müller et al. [39,56] successfully used cryopreserved European eel sperm in fertilization trials, but in this case, they used Japanese eel eggs and successfully obtained hybrid larvae of *A. japonica x A. anguilla*. The sperm used in this fertilization trial was cryopreserved following a protocol based on those described by Müller et al. [34] and Szabó et al. [35], using a modified Tanaka solution [25] as extender and methanol 10% as cryoprotectant (Table 1), obtaining progressive motility results in the thawed sperm samples of 12.3 ± 10.87%. Although the hatching rate was low, they demonstrated that the cryopreservation protocol worked successfully on the European eel sperm.

Despite the fact that cryopreservation protocols developed by the groups from Spain and Hungary were proved to succeed in fertilization trials, they differed in many aspects (Table 2) and a need of standardization of the protocol was evident. With this aim, both groups conducted together a joined study [41], where both protocols were tested using the same sperm samples. In this study, in addition to analysis of viability and motility in frozen-thawed samples, epigenetic effects of cryopreservation on spermatozoa DNA was also tested. Several studies suggested that the drastic changes occurring during freezing and thawing may affect the DNA of cryopreserved spermatozoa [57,58]. Furthermore, the use of methylated cryoprotectants is known to induce the production of reactive oxygen species (ROS) that can cause several damages, such as cytosine methylation in fish spermatozoa DNA [59], which is one of the principal epigenetic mechanisms [60], and have been suggested to be a good indicator for sperm quality [61], affecting

consequently the success of a cryopreservation protocol. In this comparative study, Herranz-Jusdado et al. [41] showed that the protocol using methanol, initially developed by the Hungarian group [35,39], was better in terms of higher spermatozoa viability and motility than the protocol with DMSO developed by the Spanish group [38,40]. Furthermore, the protocol with DMSO induced a hypo-methylation of them spermatozoa DNA, whereas no changes in DNA methylation were observed when sperm was cryopreserved with the protocol with methanol.

The most recent work on the European eel sperm cryopreservation aimed at the improvement of the protocol by using sperm membrane protection additives and to adapt the protocol to larger volumes [62] as done with Japanese eels [45]. In this latest work, using the protocol described by Herranz-Jusdado et al. [41], the researchers successfully scaled up the volume of sperm cryopreserved using 2 and 5 mL cryotubes, by adapting the cooling rate. Furthermore, adding egg yolk to the extender solution, they improved the frozen-thawed sperm quality, reaching motility values over 50%, which are the highest motility ever reported in cryopreserved European eel sperm. These improvements in the protocol represent a great advance for future large-scale reproduction programs in European eel.

4. Vitrification

Vitrification is a cryopreservation technique that leads to a glass like-solidification while preventing intracellular and extracellular ice crystallization, that has been proposed as an alternative to traditional cryopreservation [63]. Although exists several methods for vitrification [64], its application with fish sperm is typically based on the combined use of high concentrations of cryoprotectants and fast cooling rates [65]. The use of vitrification of fish spermatozoa is a relatively new application, however, it has been

already tested on sperm of several fish species [66]. The success of sperm vitrification depends on several factors, including initial sperm quality, type and concentration of cryoprotectants, equilibration time and cooling and warming rates [67]. Normally, the concentration of cryoprotectants used must be very high to prevent the formation of ice crystals during the fast cooling process, but can be toxic to the cells. Therefore, finding a proper cryoprotectant and its concentration is critical to develop new sperm vitrification protocols [65].

Recently, a new vitrification protocol has been developed for European eel sperm [68]. The protocol consisted in a sperm:diluent ratio of 1:1, with 40% cryoprotectant (20% methanol and 20% propylene glycol), and 10% FBS using Cryotops of 2 µL as cooling device. The percentage of spermatozoa motility obtained from this vitrification protocol was low compared to conventional sperm cryopreservation. However, this was the first protocol described for European eel, proving the feasibility of this technique with European eel sperm.

5. Conclusion and future remark

Since first was developed an eel sperm cryopreservation protocol in the early 2000´s, lots have been changed and improved. The latest protocols for sperm cryopreservation of European and Japanese eel use methanol as cryoprotectant and they have been adapted to large volumes. In the case of the protocol for Japanese eel sperm, successful fertilization have been achieved and with similar survival rates as with fresh sperm. Moreover, the morphology of the larvae produced with cryopreserved sperm was similar as larvae produced from fresh sperm. In the case of the protocol for European eel sperm, the latest protocol has not been tested for fertilization trials yet, but the motility of frozen-thawed sperm obtained was over 50%, which is the highest ever obtained in this species and

351 future studies should aim to test whether is suitable for large-scale fertilizations.
352 Moreover, future work should aim to investigate the effect of large periods of cryogenic
353 storage (over 2 years) on eel sperm.
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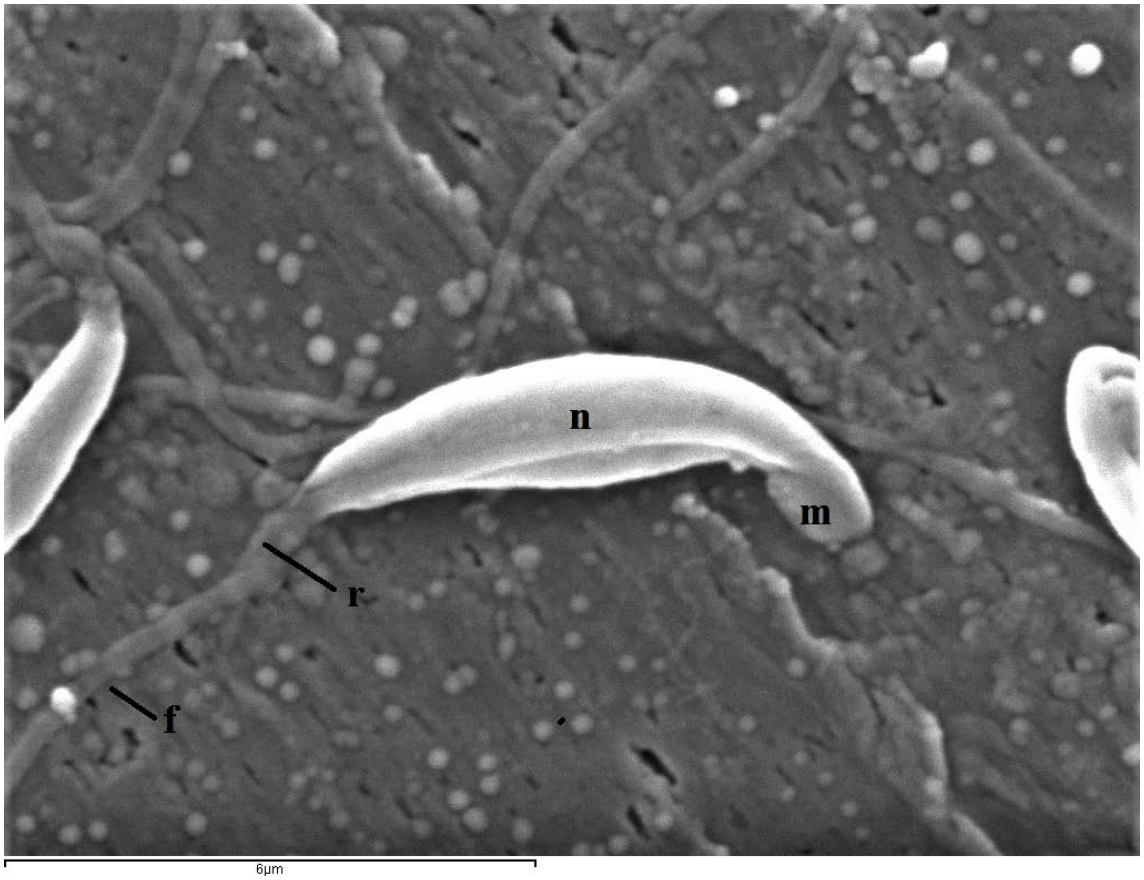
610 **Tables and figure**



611
612 **Figure 1.** Electron microscope picture of eel spermatozoa (European eel). m,
613 mitochondria; n, nucleus; r, rootlet (pseudoflagellum); f, flagellum.
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Table 1. Extender composition, cryoprotectant concentration and pH used for sperm cryopreservation in Japanese eel (*Anguilla japonica*) and European eel (*Anguilla anguilla*).

Species	Extender	Extender composition (in mM)	Cryoprotectant	pH	Volume (mL)	Thawing (t/T[a])	Motility (%)	References
Japanese eel	Tanaka	137 NaCl, 76.2 $NaHCO_3$, 24 Soya lecithin.	DMSO (10%)	8.2	1	70 s/40 ºC	46.6	[25]
	ASP	149.3 NaCl, 15.2 KCl, 1.3 $CaCl_2$, 1.6 $MgCl_2$, 20 $NaHCO_3$, 20 TAPS-NaOH.	MeOH (10%)	8.1	0.5	13 s/40 ºC	-	[50]
	K30 ASP	134.3 NaCl, 30 KCl, 1.3 $CaCl_2$, 20 $NaHCO_3$, 1.6 $MgCl_2$, 20 TAPS- NaOH, 22.5% FBS.	MeOH (10-15%)	8.1	0.25 0.25, 2.5 & 5	10 s/20 ºC	59.7 CPM 54.8 CPM	[51] [45]
European eel	TNK	137 NaCl, 76.2 $NaHCO_3$, 20 TAPS.	DMSO (10%)	8.1	0.25	45 s/20 ºC	32.2	[53]
	P1	125 NaCl, 20 $NaHCO_3$, 30 KCl, 2.5 $MgCl_2$, 1 $CaCl_2$, 1.4% L-α-phosphatidylcholine.	DMSO (10%)	8.5	0.25	45-60 s/20 ºC	36.6	[33,55]
	Kurokura	61.6 NaCI, 134.1 KCI, 1.98 $CaCl_2$, 0.84 $MgCl_2$, 2.4 $NaHCO_3$.	MeOH (10%)	8.0	0.25	5 s/40 ºC	36	[34]
	Tanaka	137 NaCl, 76.2 $NaHCO_3$.	MeOH (10%)	8.2	0.25 0.5	5 s/40 ºC 13 s/40 ºC	40-47 31	[35] [39,56,69]
	P1+FBS	125 NaCl,, 20 $NaHCO_3$, 30 KCl, 2.5 $MgCl_2$, 1 $CaCl_2$, 25% FBS.	DMSO (10%)	8.5	0.25	60 s/20 ºC	-	[36]
	Modified P1	125 NaCl, 75 $NaHCO_3$, 30 KCl, 2.5 $MgCl_2$, 1 $CaCl_2$, 25% FBS.	DMSO (10%)	8.5	0.25	60 s/20 ºC	22.2	[37]
	Modified P1 (M5)	50 NaCl, 100 $NaHCO_3$, 30 KCl, 2.5 $MgCl_2$, 1 $CaCl_2$, 25% FBS.	DMSO (10%)	6.5	0.25	15 s/20 ºC 10 s/40 ºC	38	[38] [40]
	P1+egg yolk	125 NaCl,, 20 $NaHCO_3$, 30 KCl, 2.5 $MgCl_2$, 1 $CaCl_2$, 5% egg yolk	MeOH (10%)	8.5	2 & 5	75 s/70 ºC 105 s/70 ºC	51.6	[62]

Table 2. Comparison of the main technical aspects of European eel sperm cryopreservation protocols developed by the Spanish and Hungarian research groups previous to standardization by Herranz-Jusdado et al. [41]

Protocols	**Spanish [33,38]**	**Hungarian [34,35]**
Fish origin	Farmed fish	Farmed fish
Rearing water	Seawater	Freshwater
Hormonal treatment	hCG recombinant	Natural hCG
Extender solution	P1	Tanaka
Dilution ratio	1:2	1:9
Cryoprotectants	10% DMSO & 25% FBS	10% Methanol
Straws (in mL)	0.25	0.5