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Revised

Testing cryopreserved European eel sperm for hybridization (*A. japonica* × *A. anguilla*)

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Abstract

The objective of this study was to assess impact of cryopreserved European eel sperm and Japanese eel native sperm on early fertilization, hatch, survival, and malformation rates of larvae, as well as develop molecular techniques to distinguish different eel species. Eggs from Japanese eel females (*Anguilla japonica*) were artificially fertilized with sperm of Japanese eel males and cryopreserved sperm from European eel (*A. anguilla*, extender was modified Tanaka solution and methanol as cryoprotectant). There were no statistical differences ($p>0.05$) among the measured parameters such as fertilization, hatch and survival after 10 days post-hatch rates due to large individual differences. The malformation rate of larvae compared to the hatching rate was higher in cryopreserved groups than in the control indicating that the methodology needs further refinement. Genetic analyses (PCR-RFLP, PCR-HRM) proved a clear result in the detection of paternal contribution in hybridization between the Japanese and the European eel and applied PCR-HRM method is a quick and cost effective tool to identify illegally imported *A. anguilla* at the glass eel stage, which can be transported from Europe to Asia.

Keywords: propagation, eel larvae, SNP, High Resolution Melting analyses

1. Introduction

Several studies have been published on the drastic reduction of European eel (*Anguilla anguilla*) stocks from the 1980's due to overfishing of glass eels and adults, climatic changes, persistent pollutants, nematode infection, etc. [1, 2, 3]. European eel population originate from natural spawning. Eel farms only grow glass eels collected from natural sources. For eel, a management framework aimed at the recovery of the global stock is currently ongoing under a specific European Regulation [4]. There are several successful propagation experiments resulting viable living European eel larvae in early stages [5, 6, 7, 8, 9, 10, 11,12, 13] and initial steps of culture of larvae rearing has been developed [10, 11, 12, 13] but no one has managed to rear larvae till glass eel stage yet. The lack of larval rearing methods makes the development of stocks even more difficult. The economic and environmental importance of eel production by artificial propagation is evident.

Eel sperm cryopreservation would offer enormous comparative advantages in the development of induced spawning methods. In the initial phases of the work cryopreserved sperm could be used for propagation practices by simplifying broodstock management in order to concentrate exclusively on induction of sexual maturation of females. Sperm cryopreservation would allow to preserve the best-quality sperm samples. Cryopreserved sperm is obviously free of parasitic infections, thus, it facilitates the formation of parasite-free stocks even by bringing in sperm from infected ones. Apart from this, it has been found suitable for the preservation of genetic resources of endangered species (*A. anguilla* is listed by the International Union for Conservation of Nature (IUCN) as a critically endangered fish species) as well for general breeding purposes in cultured species (sperm bank).

There are several reports on the sperm cryopreservation of European eel [7, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24] however, most publications evaluated only post-thaw sperm motility and very few reported fertilization tests. There are two published results from two

fertilization tests using European eel cryopreserved sperm in different crosses (*A. japonica* eggs × *A. anguilla* cryopreserved sperm [24] and *A. anguilla* × *A. anguilla* [7]). In both cases, the hatching rates were low; <1% [24], n=1 female) or "some free swimming larvae" [7] n=1 female) indicating the need for more studies to reveal the real effect of cryopreserved sperm samples on fertilization. According to literature data, there is no phylogenetic barrier factor that limits hybridization between the Japanese and European eel. Okamura et al [25] reported on the hybridization between the Japanese eel and the European eel and there were no significant differences between the fertilization results (Japanese eel control fertilization rate was 75±7.1%, larvae survived for up to 34 days, Japanese eel × European eel hybrid fertilization rate was 78±11.3% and larvae survived for up to 30 days). Matsubara et al [26] managed to rear hybrids from leptocephali to glass eels in captivity. Thus, cryopreserved European eel sperm can be used for fertilization tests with the eggs of Japanese eel. In contrast to the results of the European eel, Japanese eel life cycle has been closed in laboratory conditions [27]. Thanks to the programmed propagation of Japanese eel we had a chance to carry out fertilisation test by cryopreserved sperm samples from the European eel.

The objective of this study was to conduct fertilization trials with cryopreserved sperm from European eels on an increased scale in order to collect more information on the fertilizing capacity of sperm and the survival of embryos and larvae. An additional objective was to develop a new genetic analysis protocol to distinguish the two species and detect maternally and paternally derived DNA fragments in order to verify the hybrid nature of the offspring, which would be a quicker and more cost effective tool than previous one [15, 28] and it can be used for identification of Japanese and European eel as well.

2. Materials and methods

2.1. Male maturation and cryopreservation of sperm

Ten males were kept in 400 L aquaria with an external filtration system in 2005. Water temperature was maintained at 20-21 C, and the photoperiod was close to natural, about 9-10 h of light a day. Fish were not fed during the experiment. The fishes were anesthetized by clove oil, *Syzygium aromaticum* (10 drops in 10-L water) before treatments and milt collections. All fish were given 250 IU human chorion gonadotropin (hCG) / body weight kg / week. The calculated dosage of hormone was injected intrapentoneally each week. Cryopreservation of sperm was performed following the ninth injection. Sperm was collected 24 h following the injection. The genital area was dried with a soft towel, and sperm was collected with a gentle pressure on the abdomen into 2-mL sterile dry syringes having 0.1-mL marks. The volume of sperm collected into the syringes was recorded, and the sperm was subjected to motility analysis. 19 µL of artificial seawater (3.5% NaCl solution) was dropped onto a glass slide and 0.1 µL of diluted sperm was mixed with the seawater. The motility of the sperm sample was estimated at 400× magnification using a Zeiss Laboval microscope. Sperm samples showing motility higher than 70% were chosen for cryopreservation (n=7). The sperm samples were pooled. A modified Tanaka solution [29] was prepared (137 mM NaCl, 76.2 mM $NaHCO_3$) as an extender. Ten percent methanol in v/v final concentration was used as cryoprotectant. Sperm was diluted at a ratio of 1:9 with the diluent containing the extender and the cryoprotectant. Diluted sperm was loaded into 0.5 mL straws. Samples were frozen in the vapor of liquid nitrogen in an insulated polystyrene box (rectangular form; length, width, height: 360×260×210 mm). Liquid nitrogen was poured into the box and a polystyrene frame was placed onto the surface of liquid nitrogen. The height of frame was 3 cm. Straws were placed onto the frame

for 3 min, and then they were plunged directly into liquid nitrogen. After freezing samples had been transferred into storage Dewar bottles and kept for 5 years before being transported to Japan by plane. There was possibility to check the post-thawned sperm samples by Computer-assisted sperm analysis (CASA) in 2010. The main parameters, progressive motility of samples 12.3±10.87% (min-max: 0.23-37%), curvilinear velocity (VCL): 57.4±39.73% (min-max: 22.7 -204.9%).

The maturation of Japanese eel was performed in the Tokyo University of Agriculture, Abashiri, Japan. Male eels (200–300 g) were injected repeatedly with human chorionic gonadotropin [30]. 1 ml sperm (sperm mortality over 70%) were diluted with 99 ml eel Ringer solution as described [31]. A diluent was prepared in a test tube containing Artificial Seminal Plasma (ASP - 149.3 mM NaCl + 15.2 mM KCl + 1.3 mM $CaCl_2$ + 1.6 mM $MgCl_2$ + 20 mM $NaHCO_3$ buffered with 20 mM TAPS-NaOH at pH 8.1, [32]. The milt was mixed with ASP in 1:100 ratios. The process of the cryopreservation of Japanese eel samples was the same as the one described in European eel. The samples were stored in liquid Nitrogen for 2-5 days until fertilization tests. Straws were thawed for 13 second in a water bath at 40 ºC before the fertilization tests.

2.2. Female maturation and fertilization

Cultured Japanese eels were acclimated to seawater at the Tokyo University of Agriculture, Abashiri, Japan. After acclimation, eels were induced to mature by hormonal treatment in circulation water tanks holding 2500 L of seawater at 20 °C. After anesthesia in 0.1% 2-phenoxy ethanol (Wako Co. Ltd., Tokyo, Japan), feminized eels (500–700 g: received weekly intramuscular injections of salmon pituitary homogenates at 40 µg/g body weight suspended in eel Ringer solution to induce maturation [33], then a single intramuscular injection of 17α, 20β-

dihydroxy-4-pregnene-3-one at 1 µg/kg body weight suspended in eel Ringer solution to induce ovulation [31]. Eggs, which originated from four females (initial body weight was 578±129.4 g), were collected into dry plastic bowls by gentle abdominal pressure. Samples of ovulated eggs weighing 1 gram (1700-1800 eggs) were distributed into 5 cm Ø 50 ml beakers and controls were inseminated with 500 µl of pre-diluted milt (sperm mortality over 70%) as described earlier [31]. Treated samples were inseminated with thawed milt (500 µl). Forty-eight-well tissue culture plates (Iwaki Glass Co. Ltd., Tokyo, Japan) were filled with 1 mL/well of filtered (pore size, 0.2 mm) natural seawater containing antibiotics (Penicillin G potassium, 5000 IU/L; Banyu Pharmaceutical Co. Ltd., Tokyo, Japan, and streptomycin sulfate, 0.05 g/L; Meiji Seika Kaisha Ltd., Tokyo, Japan) and 1 mg/L bovine serum albumin fraction V (Nacalai Tesque, Inc., Kyoto, Japan). Ninety-six buoyant eggs were transferred randomly from each dish to two 48-well plates (1 egg/well) within 1 h after insemination. The two 48-well plates (96 eggs each) were maintained for the determination of hatch and survival percentages as well as abnormality. Egg samples were incubated in a thermostat (23 °C). Live larvae were observed under a stereoscopic microscope every day until the stage at which larvae had completely resorbed the yolk. Digital images were taken of the developing embryos.

The following parameters were recorded:

Fertilization rate = number of fertilized eggs / total number of eggs × 100

Hatching rate = number of hatched larvae / total number of eggs × 100

Malformation rate 1. = number of hatched malformed larvae / total number of eggs × 100

Malformation rate 2. = number of hatched malformed larvae / total number of hatched larvae × 100

Spinal malformations in tail fin and pericardium oedemas were assessed at hatching by observation with a dissecting microscope. Statistical analyses were carried out with SPSS for

Windows. Independent Samples Test was used to test the main effects of the treatments using alpha = 0.05 for significance.

2.3. Genetic analyses

Randomly selected 6 days old *A. japonica*, *A. anguilla* and hybrid larvae were preserved in RNALater to be used for genetic analyses. In order to ensure the hybrid character of the embryos concerned, PCR RFLP analyses of Follicle Stimulating Hormone - beta subunit (FSH) were used as described earlier [24]. The marker can distinguish the nuclear genomes of the species (*A. anguilla*, *A. japonica*) and their F1 hybrids.

For cost effective genotyping of the species-discriminative SNP, High Resolution Melting (HRM) analysis was optimized. The amplification of the 100bp long fragment of the FSH gene a Rotor-Gene Q 5plex HRM Platform and Type-it HRM PCR Kit (Qiagen, Hilden, Germany) were used with EvaGreen intercalating dye. The PCR reactions were performed using 17 ng total DNA as template and the 2× HRM PCR Master mix, according to the instruction of the manufacturer in 10 μl final reaction volumes. The primers for the SNP were FSH_Angolna_RsaI_F" (5′-CAACAGGCCTGCAACTTCA) and "FSH_Angolna_RsaI_R" (5′-CTCAGAGCCACAGGGTAGGT), respectively. Reactions were carried out with an initial denaturation step at 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 30 sec and then HRM curves were generated by acquiring florescence data between 65 and 90°C (Fig. 1.).

The protocols of fish sperm cryopreservation and fish propagation and the template informed consent forms contained in Appendix (Scientific Ethics Council for Animal Experimentation; XIV-001-2299-4/2012, XIV-001-2306-4/2012) were reviewed and approved and applicable by

National Food Chain Safety Office, Animal Health and Animal Welfare Directorate of Government Office of Pest Country with respect to scientific content and compliance with applicable research subjects regulations.

3. Results

Successful fertilization tests were carried out using cryopreserved sperm from European eel males and four Japanese eel females. Hybrid and native Japanese eel larvae were successfully hatched 34 hours after fertilization. There were no statistical differences ($p>0.05$) among the measured parameters such as fertilization, hatch and survival after 10 days post-hatch rates due to the large individual fluctuations (Table 1., Fig. 2.). The malformation rate of larvae compared to the hatching rate was higher in the cryopreserved group (min-max: 42.8-100%) but statistical differences ($p=0.069$) were not observed from Japanese eel group (min-max: 16-49.9%). The genetic results clearly revealed that each of the samples were hybrid. The negative control had a band corresponding the dimerised primers with absence of template and presence of all other PCR components. Incidentally, it had about the same size as the lower (40 bp) restricted band for hybrid/European eel. Visualized with an overlapping digestion pattern, the hybrid clearly showed the two parental alleles, revealing that artificial crossing of *A. anguilla* to *A. japonica* can lead to successful fertilization (Fig. 2.). The homozygous and heterozygous genotypes were confirmed with the newly developed HRM based SNP analyses. The results were evaluated using the Rotor-Gene software and visual inspection (Figure 1.).

4. Discussion

Cryopreserved sperm could be used for fertilization of eggs when synchronization of gamete maturation in both fish sexes is problematic, thus good quality sperm may not be available for fertilization. In case of the eel, sperm cryopreservation would offer enormous comparative advantages in the development of induced spawning methods. Tanaka et al [29] were the first to publish and apply a practical protocol of cryopreservation of Japanese eel sperm. Cryopreserved sperm containing 76.2 mM NaHCO3, 137mM NaCl, 1.4% Soya lecithin and 10% dimethyl sulphoxide was used for fertilizing eggs, which hatched successfully afterwards (2.4–22.5%, n=3 females). In our previous study we used ASP as extender and methanol as cryoprotectant of Japanese eel yielded 6.2 – 32.6% hatch comparing to fresh sperm (4.2 – 29.2% [35]. In the present study a similar hatching rate (6.17 – 32.63 %, n=4 females) as that described by Tanaka et al [29] was observed. Although this could prove that the cryopreserved and thawed European eel sperm samples were able to fertilize the eggs of Japanese eel, accurate analyses of these preliminary results were not possible due to poor egg quality. Also, in this experimental period a high rate of malformation was observed following the use of cryopreserved sperm samples. The exact causes of this high percentage of malformations are still unclear and explanations can only be speculated. An increased rate of larval deformities following fertilization with cryopreserved sperm has been observed in some cases [34], however this is not a general observation and typically, larvae hatching from eggs fertilized with cryopreserved sperm are normal [35, 36]. Spermatozoa are exposed to several damaging factors during cryopreservation, including cell membrane disruption and genotoxic effects [37, 38]. A significant effect of cryopreservation on the motility, viability and head morphology of European eel spermatozoa was observed by Asturiano et al [21]. As the effects of these damaging factors on the fertilizing capacity of cryopreserved sperm and subsequent

embryogenesis are not known, their effect on larval deformities cannot be excluded. The malformation was higher in larvae originating from cryopreserved fertilization [39], but there were no significant differences ($p>0.05$) 19.9 – 62.5 % using cryopreserved sperm samples and 16 – 49.9 % fertilized with ASP-diluted native sperm.

The genetic analysis, which has already been used previously [24, 40] proved that the malformation was not caused by haploidy, as each investigated hybrid larva developed and the investigated random hybrid samples clearly showed two parental alleles. The cause of morphological deformities cannot be attributed to the fact of hybridization because it occurred in pure *A. japonica* control fertilization as well. The rearing water temperature and salinity effects on the rate of larval malformation in Japanese eel have been observed previously, too [41, 42]. Hybrid and pure Japanese eel controls were kept in the same condition in this experiment. Based on these data, the European eel cryopreservation methodology needs further refinement in the near future. Similarly to the observations of Asturiano et al [7], who carried out a successful fertilization with cryopreserved sperm using the cryopreservation protocol described by Peñaranda et al [43] and European eel eggs, the control fertilization rate was between 69 and 94%, while in contrast the cryopreserved sperm fertilized 0-33% of the eggs and “a few larvae” hatched. They suggested to check if possible DNA damages or differences in the ambient environment, i.e.: osmolality conditions, during activation in fertilization medium, causes the reduction of fertilization rate as well as the high rate of larvae malformation. Sperm motility of Japanese eel K30 artificial seminal plasma (K30 ASP) before experiments was investigated using CASA [44]. Similarly to our earlier observation in European eel [15, 17, 24] and Japanese eel [39] 10% and 15% MeOH as cryoprotectant was the most successful cryoprotectant with percentage of the initial motility of 59.7 ± 12.1% but in this case there was no fertilization test. These experimental results showed that MeOH is more suitable cryoprotectant for cryopreservation of eel sperm than dimethyl sulphoxide (DMSO [17, 44]).

Developmental differences in the larval ontogeny of two genotypes was not investigated. Matsubara et al. [26] reported that hybrids needed 322 days for the metamorphosis from leptocephalus to glass eel stage, while *A. japonica* undergone this processes earlier (179 days). In the future it will be necessary to follow the differences in embryonic and larval development of the hybrids and *A. japonica* or *A. anguilla*, which results led to successful larvae rearing in the European eel as well.

The presented new HRM based discrimination of the *A. japonica, A. anguilla* and their hybrids is a useful and more efficient method than the previously developed PCR-RFLP based detection of the species specific SNP-s of the FSH gene. The HRM is also used for identification of oyster or tuna species [45, 46] as well as other animals [47] or carp linages [48], but this is the first HRM based method for eel species identification. The method applied is significantly (nearly 3 times) quicker and cheaper (about half the price per reaction) than our PCR-RFLP based detection method described earlier [24]. This method is suitable for wide screening of different stocks of larvae as well as fresh and processed fish products. The quicker molecular method to identify the parental species in juvenile form (in glass eel stage) is advantageous, because of the problems of illegal trade of *A. anguilla* from Europe to Asia [28, 40].

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Conflict of interest

None of the authors have any conflict of interest to declare

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438 **Table**

439 **Table 1.** Fertilization, hatch, 10-day survival as well as larval malformation of Japanese eel
440 (*Anguilla japonica*) eggs fertilized with either fresh Japanese eel or cryopreserved European
441 eel (*A. anguilla*) sperm. (Malformation rate 1. = number of hatched malformed larvae / number
442 of total eggs × 100, Malformation rate 2. = number of hatched malformed larvae / number
443 hatched larvae × 100).

444

445 **Figure captions**

446

447 **Figure 1.** The normalised HRM profile of Japanese, European and hybrid eels. The HRM
448 analysis produced robust results confirming the usability of the method for distinguish the
449 Japanese and European eels and their hybrids. The amplified PCR products using FSH Angolna
450 F and FSH Angolna R primers were amplified and then HRM curves were generated by
451 acquiring florescence data between 65°C and 90°C. Arrows link with corresponding curves of
452 genotypes.

453

454 **Figure 2.** PCR-RFLP analyses of Japanese, European and hybrid eels. Gel electrophoresis (3%
455 agarose gel). The amplified PCR products using FSH Angolna F and FSH Angolna R primers
456 were digested by using restriction enzyme RsaI. Marker =50 bp molecule weight marker
457 (Fermentas, EU) at the left side, and 100 bp molecule weight marker at the right side.

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