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Additional Information

Successful cryopreservation in biodegradable containers of sperm from aquaculture Mediterranean fishes

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Highlights

- Hard-capsules maintain the cryopreserved sperm motility, as well as plastic straws.
- Sperm thawing in an extender did not compromise the cell membrane integrity.
- Capsules' thawing process can damage the gilthead seabream sperm DNA.
- Alkaline comet assay protocol for European eel sperm worked well.

ABSTRACT

We aimed to evaluate the efficiency of hard-gelatin and hard-hydroxypropyl methylcellulose (HPMC) capsules as biodegradable alternative containers to plastic straws in European eel (*Anguilla anguilla*), gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) sperm cryopreservation. Sperm samples from each European eel (n=12) were diluted 1:8:1 (sperm: extender P1+5% egg yolk: methanol). Gilthead seabream (n=12) samples were individually diluted in a cryoprotectant solution of 5% Me_2SO + NaCl 1% plus BSA (10 mg mL^{-1}) at a ratio of 1:6 (sperm: cryoprotectant solution). European sea bass (n=10) sperm from each male was diluted in non-activating medium (NAM) at a ratio of 1:5.7 (sperm: NAM), and 5% of Me_2SO was added. The diluted European eel and sea bass sperm aliquots (0.5 mL) were individually filled in plastic straws (0.5 mL), hard-gelatin, and HPMC capsules (0.68 mL). Gilthead seabream diluted sperm (0.25 mL) were filled in plastic straws (0.25 mL) and identical capsules described. All samples were frozen in liquid nitrogen vapor and stored in a liquid nitrogen tank. Sperm kinetic parameters were evaluated by CASA-Mot software. Sperm membrane integrity was performed using a Live and Dead KIT and an epifluorescence microscope. To quantify DNA damage, the alkaline comet assay was performed and TailDNA (TD-%) and Olive Tail Moment (OTM) were evaluated by CaspLab software. Sperm cryopreservation of the three Mediterranean species in straws, gelatin, or HPMC capsules reduced the kinetic parameters and cell membrane integrity. Generally, the post-thawing samples cryopreserved in straws and capsules did not differ for the kinetic parameters and cell membrane integrity, except for European sea bass sperm, where the samples stored in gelatin capsules showed higher velocities (VCL - 100; VSL - 76; VAP - 90 μm s^{-1}) than the sperm stored in HPMC capsules (VCL - 87; VSL - 59; VAP - 73 μm s^{-1}). The cryopreservation process did not damage the sperm DNA of European eel and European sea bass, regardless of the containers used. On the other hand, gilthead seabream sperm cryopreserved in gelatin (TD - 9.8%; OTM - 9.7) and HPMC (TD - 11.1%; OTM - 11.2) capsules showed higher DNA damage than fresh samples (TD - 3.6%; OTM - 2.7) and the sperm stored in straws (TD - 4.4%; OTM - 5.2). The hard-gelatin and HPMC biodegradable capsules can be used as an alternative to straws for European eel, gilthead seabream, and European sea bass sperm cryopreservation.

Keywords: Capsule; HPMC; Gelatin; Cryobiology; Fish

1. Introduction

In 2020, global aquaculture achieved a remarkable milestone, producing 122.6 million tons of aquatic organisms, representing a noteworthy 2.7% increase compared to the previous year's output [1]. This notable growth in aquaculture production can be attributed to the ever-increasing demand for food and the economic development, spurred by the relentless growth of the world's population [2]. As fish capture encounters stagnation or decline in certain regions, the spotlight has turned to aquaculture as a crucial solution to meet the surging food demands. Looking ahead to 2030, some regions and countries are targeting a substantial increase of 35 to 40% in the production of aquatic organisms through aquaculture. The surge in aquaculture production contributes to a proportional increase in the amount of waste released into the environment. Therefore, measures that make production more sustainable from an environmental point of view and reduce waste should be practiced [3]. One of them is the use of the circular economy, which aims to prevent resource depletion, close energy and materials loop uses, and facilitate sustainable development [4]. This entails optimizing resource utilization to enhance efficiency and minimize waste generation and emissions by aquaculture [5]. This ambitious effort requires innovating and advancing sustainable techniques within the industry [1].

Cryopreservation is a biotechnique that maintains cells and tissues at extremely low temperatures, ensuring their functionality, development, and growth can be preserved even after thawing, with minimal loss of efficiency. Among its various applications, fish sperm cryopreservation is a valuable tool for enhancing aquaculture practices, particularly in optimizing broodstock management at hatcheries while safeguarding natural fish stocks [6,7]. Nowadays, several protocols are established for the cryopreservation of fish sperm [7]. Most protocols use plastic straws (0.25, 0.5, 1, 2 and 5 mL) or cryovials (1, 2, 3 and 5 mL) as sperm storage containers. These containers are made of Polyvinyl chloride (PVC) or polypropylene, both plastic and recyclable. However, it is a common practice not to recycle these containers after use due to their contact with biological samples. Consequently, this leads to waste with significant pollution potential, given that these containers are made from durable plastics. Furthermore, the limited number of industries manufacturing plastic containers results in higher market prices and hinders accessibility in certain countries. To find a biodegradable alternative to plastic containers for fish sperm cryopreservation, a groundbreaking methodology has been reported by [8]. This innovative approach employs hard-gelatin capsules and hard-hydroxypropyl methylcellulose (HPMC) capsules as the storage containers for freshwater fish sperm. These capsules are crafted from biodegradable and biocompatible byproducts from animal and plant production, making them environmentally friendly and compatible with biological samples. Moreover, their widespread availability on the

market and cost-effectiveness makes them a practical and accessible solution. Using these capsules represents a significant step towards reducing environmental harm compared to plastic containers.

The European eel *Anguilla anguilla* is a catadromous fish species that performs a long-spawning migration, reaching thousands of kilometers along the Atlantic Ocean [9]. Since the 1980s, natural eel stocks have been decreasing due to anthropic actions. Today the species is on the Red List of the International Union for Conservation of Nature (IUCN), categorized as "Critically Endangered" [10] the highest classification level before extinction rating. Moreover, the European eel is a highly prized delicacy in European and Asian cuisine, boasting great economic value. This places a considerable strain on the wild eel population due to the high demand, emphasizing the urgency for implementing a captive breeding program. Such a program aims to alleviate the fishing pressure on natural eel populations, ensuring their sustainable preservation for the future [6]. In last decades, protocols for cryopreserving the sperm of this species have undergone continuous development and enhancement [11]. Our research group has established the latest and most efficient protocol in this regard [12].

Gilthead seabream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) hold prominent positions as two of the top ten most produced marine fishes globally [1]. Concentrated mainly in the Mediterranean Sea, their combined production in 2021 reached 619,000 tons [13,14]. As the aquaculture sector continues to expand in the Mediterranean Sea with a positive growth trajectory [15], adopting new strategies to mitigate potential environmental impacts becomes crucial. In this context, sperm cryopreservation can be an important tool in producing these species, assisting in genetic improvement programs (genetic backup), and genetic editing for the development of infertile animals, since fish escape from cages are recurrent and cause serious environmental problems [16]. Gilthead seabream and European seabass sperm cryopreservation protocols have been developed and improved for decades [17-19]. Nowadays, protocols for sperm cryopreservation of gilthead seabream [20] and European sea bass [21] in plastic straws are already established. However, new protocol adjustments are always welcome to make it cheaper and environment-friendly.

In several areas, the use of biodegradable polymers is an alternative to minimize the pollution caused by plastic [22]. By adopting these sustainable alternatives, the fish sperm cryopreservation process becomes more ecologically responsible, positively contributing to environmental conservation and sustainability efforts. Thus, we aimed to evaluate the efficiency of hard-gelatin and hard-hydroxypropyl methylcellulose (HPMC) capsules as biodegradable alternative containers in European eel, gilthead seabream and European sea bass sperm cryopreservation.

2. Materials and Methods

Reagents were purchased from Sigma Aldrich (Madrid, Spain), unless otherwise stated.

2.1. Ethics statement

This study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of the Spanish Royal Decree 53/2013 (BOE, 2013). The protocol used with European eel was approved by the Experimental Animal Ethics Committee from the Universitat Politècnica de València (UPV). Protocols used with gilthead seabream (1295/2022) and European sea bass (1273/2022) were approved by the Institute of Aquaculture Torre de la Sal (IATS) Experimental Animal Ethics Committee and CSIC Ethics Committee. Final permissions (European eel: 2023-VSC-PEA-0039; gilthead seabream: 2022-VSC-PEA-0230; European sea bass: 2022-VSC-PEA-0213) were given by the local government (Generalitat Valenciana).

2.2. Experimental design

The experimental design is summarized in Fig. 1. The study was performed using European eel, gilthead seabream, and European sea bass sperm samples. The motility was compared in fresh and post-thawing sperm samples after being cryopreserved in three containers: plastic straw (0.25 or 0.5 mL - IMV Technologies, l'Aigle, France), hard-gelatin capsule (Nadiprana SL, Els Pallaresos, Spain) manufactured with collagen, size 0 – locked length 21.7 mm, and with a 0.68 mL volume, and hard-HPMC capsule (Nadiprana SL, Els Pallaresos, Spain) manufactured with hydroxypropyl methylcellulose and having the same size. After thawing, samples were evaluated testing in triplicate their kinetic parameters, membrane integrity, and grade of DNA damage.

2.3. Fish handling and sperm collection

2.3.1. European eel

All experimental procedures were performed at the Fish Reproduction Laboratory of the Universitat Politècnica de València (UPV, Spain). European eel males (n = 36; 127 ± 21 g) from commercial fish farm (Valenciana de Acuicultura S.A.; Puzol, Spain) were transferred to UPV. The animals were accommodated in three 96-L freshwater aquaria in the laboratory, 12 fish in each aquarium, and gradually acclimatized to seawater (salinity = 38 ± 0.5 g L^{-1}) during a week. Aquaria water was kept at 20 °C and covered to decrease the light, reducing the eels stress. After 10 days of acclimatization, the hormonal protocol to induce sexual maturation started. Eels were weekly anesthetized using a benzocaine solution (60 ppm – Thermo Fisher, Kandel, Germany) and received an intraperitoneally injection of 1.5 IU g^{-1} fish of recombinant human chorionic

gonadotropin (rhCG; Ovitrelle, Merck S. L., Madrid) [23]. From the seventh week of hormone treatment, the animals began to produce sperm. After 24 hours of hormone administration, the sperm samples were collected weekly by abdominal massage and collected in a plastic tube (15 mL) [24]. The samples were maintained at 4 °C until experimental procedures.

2.3.2. Gilthead seabream and *European sea bass*

The sperm collection was performed at the Institute of Aquaculture of Torre la Sal (IATS, Ribera de Cabanes, Castellón, Spain) during the species spawning season (gilthead seabream: Dec/2022; European sea bass Feb-Mar/2023). Gilthead seabream (n = 32; 997 ± 114 g) and European sea bass (n = 12; 3.4 ± 0.5 kg) males were maintained in 5000 L tanks in an open seawater system with salinity (39 ± 0.5 g L^{-1}), natural temperature (10.2 ± 2 °C), and photoperiod (11 h light: 13 h dark). Animals of both species were fed by hand using commercial fish feed once a day to apparent satiation.

Before the sperm collection, the animals were not hormonally induced following the IATS reproduction protocol. Both breeder species produce a sperm volume of at least 2 mL, which does not justify the use of hormone. Each animal was anesthetized with Ethyl 3-aminobenzoate methanesulfonate (Tricaine methanesulfonate, MS-222 - 60 ppm). The genital area was cleaned with distilled water and dried to avoid contamination of samples by seawater, urine, and feces. Sperm from each male was individually collected by a gentle abdominal massage using a syringe and placed in a plastic tube (15 mL). In a previous test, it was observed that undiluted gilthead seabream fresh samples could be transported to the UPV without loss of quality. On the other hand, undiluted European sea bass sperm samples showed decreased quality after transport. Thus, individualized gilthead seabream fresh sperm samples were transported undiluted, while individualized European seabass samples were diluted in two tubes containing non-activation medium (NAM seabass - in mM: NaCl 59.83, KCL 1.47, $MgCl_2$ 12.91, $CaCl_2$ 3.51, $NaHCO_3$ 20, glucose 0.44; BSA 1% (w:v); 310 mOsm kg^{-1} and pH adjusted to 7.7) described by [19]. In the first plastic tube (15 mL), 1430 µL of fresh sperm were diluted in 8145 µL NAM seabass reaching a ratio of 1:5.7 (sperm: extender). In the second plastic tube (2 mL), 80 µL of fresh sperm were diluted in 1920 µL of NAM seabass, reaching a ratio of 1:25 (sperm:extender) (Fig. 1). All samples were kept in a cooled box at 4 °C and transported (approximately 50 min) to the Fish Reproduction Laboratory at UPV.

2.4. Sperm samples selection

Once at the Fish Reproduction Laboratory at UPV, fresh sperm samples from the three species

were selected for the experiments using the Computer-Assisted Sperm Analyzer (CASA ISASv1; Proiser R+D, S.L., Spain), following the method described by [25]. After CASA evaluation, 12 European eel sperm samples demonstrating a minimum of 65% MOT, 12 gilthead seabream samples exhibiting at least 55% MOT, and 10 European sea bass samples with a minimum of 55% MOT were selected for the experiment.

2.5. Cryopreservation and thawing

The European eel sperm samples (n = 12) were individually diluted at a proportion of 1:8:1 (sperm: P1 extender plus 5% egg yolk (v:v): methanol) in plastic tubes (1.5 mL). The egg yolk (from commercial hen eggs) was diluted in P1 extender, then the methanol was added, followed by the sperm. Diluted samples were incubated for 1 h at 4 °C, permitting the cryoprotectant penetration into the cells [12]. Further, the 8 plastic straws, 8 hard-gelatin, and 8 hard-HPMC capsules per male were filled with 0.5 mL of diluted sperm. Immediately, the samples were frozen (both in straws and capsules) for 3 min, 3 cm over the liquid nitrogen vapor, and then thrown into the liquid nitrogen [12]. Afterward, all the samples were stored in a liquid nitrogen tank (Minitube, Tiefenbach, Germany) at -196 °C for one month. For thawing, the plastic straws were individually submerged in water at 40 °C for 13 s [12]. The capsules were individually removed from the nitrogen tank. The upper part of each capsule was broken with a clamp pression and placed inside plastic tubes with a capacity of 15 mL. These tubes contained 5 mL of P1 extender previously warmed at 40 °C in a water bath. As soon as the capsule was placed in the extender, the tube was shaken in a vortex (VWR Test tube shaker – model 4441378, Leuven, Belgium) at 2000 rpm for 25 seconds to dissolve the capsule [8]. The post-thawing samples were maintained at 4 °C until analyzed.

Gilthead seabream sperm samples (n = 12) were cryopreserved using a solution of 5% of the permeable cryoprotectant dimethyl sulfoxide (Me_2SO) in extender NaCl 1% plus BSA (10 mg mL^{-1}). The cells were diluted in the cryoprotectant solution at a ratio of 1:6 (sperm: cryoprotectant solution) [18,26]. Immediately, 8 plastic straws, 8 hard-gelatin, and 8 hard-HPMC capsules per male were filled with 0.25 mL of diluted sperm. Without equilibrium time, the samples were frozen (both in straws and capsules) for 10 min, 1 cm over the liquid nitrogen vapor [20]. Later, the containers were stored in a nitrogen liquid tank at -196 °C for two weeks. Plastic straws were thawed in a water bath at 60 °C for 5 s [20], and capsules were thawed as described above, but using the extender NAM seabream previously warmed at 40 °C in a water bath. All post-thawing samples were maintained at 4 °C until analyzed.

In the European sea bass sperm samples (n = 10) previously individually diluted in NAM sea bass

(200 mOsm kg^{-1}; pH 7.7) were added 5% Me_2SO (v:v), reaching a final concentration of 4.29% Me_2SO and a final ratio of 1:6 (sperm: cryoprotectant solution). Then, 8 plastic straws, 8 hard-gelatin, and 8 hard-HPMC capsules per male were filled with 0.5 mL of diluted sperm. Immediately, the samples were frozen 6.5 cm over the liquid nitrogen vapor for 15 min [21]. After, the containers were stored in a liquid nitrogen tank at -196 °C for one month. Plastic straws were thawed in a water bath at 35 °C for 15 s [21]. Capsules were thawed using the previously described method, except they were placed in extender NAM sea bass pre-warmed at 35 °C in a water bath. The post-thawing samples were maintained at 4 °C until analyzed.

2.6. Sperm kinetic parameters

The evaluation of European eel (n = 12), gilthead seabream (n = 12), and European sea bass (n = 10) fresh samples and post-thawing sperm samples cryopreserved in plastic straws and biodegradable capsules were carried out in triplicate using CASA-Mot system. Before the analysis, European eel samples were diluted in P1 medium (in mM: NaCl 125, $NaHCO_3$ 20, $MgCl_2$ 2.5, $CaCl_2$ 1, KCl 30; and pH adjusted to 8.5; [27]. Gilthead seabream sperm was diluted using as extender a non-activating medium (NAM seabream - in mM: NaCl 75, $NaHCO_3$ 20, $MgCl_2$ 12.9, $CaCl_2$ 2.65, KCl 1.5, glucose 4.4, bovine serum albumin (BSA) 0.015; 280 mOsm kg^{-1} and pH adjusted to 7.7) described by [28]. To evaluated European sea bass samples, fresh sperm previously diluted in a 1:25 ratio (sperm: NAM sea bass) was used (Fig. 1).

All samples were activated using artificial seawater (ASW - in mM: NaCl 354.7, $MgCl_2$ 52.4, $CaCl_2$ 9.9, Na_2SO_4 28.2, KCl 9.4, in distilled water) with 2% BSA (w:v), pH adjusted to 8.2 and osmolality of 1100 mOsm kg^{-1}. Briefly, a fresh sperm diluted aliquot of 0.5 µL was activated with 4.5 µL ASW. The post-thawing samples cryopreserved in plastic straws were evaluated by mixing 0.2 µL of sperm in 10 µL of ASW, and in the case of the samples stored in capsules, an aliquot of 1 µL was mixed with 5 µL of ASW. The samples activation was performed in a counting chamber ISAS Spermtrack 10 (Proiser R+D, S.L., Spain) under a microscope in negative phase with a 10× magnification (Nikon Eclipse 80i, Tokyo, Japan) with a camera (ISAS 782M, Proiser R+D, S.L., Spain) attached for video recording connected to a computer. Videos of spermatozoa displacements were captured at 60 frames per second for 1 s. For all samples, the analyses were performed in triplicate 10 s after activation using CASA software. The kinetic parameters analyzed were the percentage of total motile spermatozoa (MOT - %), the percentage of spermatozoa swimming forward (MOTp - %), curvilinear velocity (VCL - µm s^{-1}), straight line velocity (VSL - µm s^{-1}), and average path velocity (VAP - µm s^{-1}).

2.7. Sperm membrane integrity

The percentage of viable spermatozoa was analyzed in every fresh and thawed sample from European eel (n = 12), gilthead seabream (n = 12), and European sea bass (n = 10). The evaluation was performed using a fluorescence kit (LIVE/DEAD Sperm Viability Kit, Thermo Fisher Scientific, MA, USA) composed of SYBR-14 at a final concentration of 2 μM and propidium iodide (PI) at 240 μM. Firstly, European eel fresh sperm previously diluted (1:25) was diluted again in P1 at a ratio of 1:350 (diluted sperm: extender). Post-thawing sperm frozen in plastic straws was diluted in P1 at a ratio of 1:15 (post-thawing sperm: extender), and those samples cryopreserved in biodegradable capsules were not diluted to perform this evaluation. In all the cases 50 μL aliquots were used, 1.5 μL of SYBR-14 were added and incubated for 10 min. Then 3 μL of PI were added and incubated 5 min more [20].

Gilthead seabream fresh sperm samples were diluted with NAM seabream at a ratio of 1:1000 (fresh sperm: extender). Post-thawing sperm from plastic straws was diluted in the same extender at a ratio of 1:20 (post-thawing sperm: extender), and the samples cryopreserved in capsules were not diluted. Similarly, in all the cases 50 μL aliquots were used, 1.5 μL of SYBR-14 were added and incubated for 10 min, and 3 μL of PI were added and incubated 5 min more [20].

For European sea bass, the fresh sperm samples previously diluted (1:25) was diluted again in NAM sea bass at a ratio of 1:1000 (fresh sperm:extender). Post-thawing sperm from plastic straws was diluted in NAM sea bass at 1:20 (post-thawing sperm:extender), and the samples cryopreserved in capsules were not diluted. In this case, 2 μL of SYBR-14 were added to 50 μL of samples, and after 5 min, 0.2 μL of PI were added and incubated 5 min more [20]. All the samples from the three species were incubated at 25 °C in the dark and immediately visualized.

For observation, two sperm-stained aliquots of 20 μL were pipetted onto each corner of the histological slide and covered with a coverslip under a fluorescence microscope (Nikon Eclipse 80i, Tokyo, Japan). With the aid of a camera (Moticam 1080, Xiamen, China) attached to the microscope and the Motic Image Plus software (Version 3.1.1, Motic, Xiamen, China), photos were taken in the same field using 450-490 nm filter to visualize viable gametes stained by SYBR-14, and 510–560 nm fluorescence filters to visualize non-viable spermatozoa stained by PI. The percentage of viable cells in relation to non-viable cells was evaluated using the FIJI software (Version 1.53t, Image J, National Institutes of Health, Bethesda, USA) with the cell counter plug-in, where the images were superimposed and at least 400 cells on each slide were counted.

2.8. DNA fragmentation quantification

The alkaline comet assay was performed to determine the DNA fragmentation of fresh and post-

thawing sperm samples using the protocol described by [18] with adaptations. Before the analyses, the histological slides were prepared with normal melting point agarose (0.5% diluted in PBS). After removing the excess of agarose, the slides were stored in the dark at 4 °C.

To prepare the samples, 1 µL of fresh sperm, 10 µL of sperm cryopreserved in straws, or 200 µL of sperm cryopreserved in capsules were diluted in 5 mL of extender (European eel - P1; gilthead seabream - NAM seabream; European sea bass - NAM sea bass). A positive control, aimed to cause severe damage to DNA sperm using hydrogen peroxide (H_2O_2 30%), also was prepared to certify the accuracy of the analysis. A work solution was prepared, diluting H_2O_2 in PBS at a ratio of 1:10. In each fresh and post-thawing sample of European eel was added 2 µL, gilthead seabream 250 µL, and European sea bass 350 µL. Later, the positive control samples were incubated for 15 min at 4 °C. Then, all the samples were centrifuged at 4000 x g for 5 min at 4 °C and resuspended in 200 µL of the extender used for sperm dilution in each species. After this procedure, 10 µL of the samples were pipetted into a plastic tube (1.5 mL). In these same tubes, 180 µL of low melting point agarose (0.5% diluted in PBS) were added. The slides received two 75 µL aliquots (semen + agarose), one from each end of the slide, which were covered with coverslips. The slides were stored for 30 min at 4 °C for the agarose to let the agarose solidify, and then the coverslips were gently removed. The European eel samples slides were exposed to a lysis solution (Na_2 EDTA 100 mM; NaCl 2.5 M; Tris pH 10 10.0 mM; 1% lauryl sarcosine; Triton X-100 1% - diluted in distilled water and adjusted to pH 10) for 30 min at 4 °C. Gilthead seabream and European sea bass samples slides were exposed to a lysis solution for 60 min at 4 °C. Then, they were exposed to denaturizing solution (lysis solution containing dithiothreitol 10 mM) for 30 min at 4 °C, and finally, a denaturizing solution with lithium diiodosalicylate 4 mM for 90 min at room temperature. Once the cell lysis phase was concluded, the slides were subjected to electrophoresis (Biorad PowerPac, Basic Sub-Cell GT Horizontal Electrophoresis System, Hercules, USA) at 15 v, 300 mA for 10 min submerged in an electrophoresis solution (Na_2 EDTA 1 mM; NaOH 0.3 M – diluted in distilled water and adjusted to pH 13). Once finished the electrophoresis step, the slides were removed from the cube and washed three times with the neutralization solution (Tris pH 10 0.4 M - diluted in distilled water and adjusted to pH 7.5). After this step, the slides were fixed using methanol for 3 min and stored in the dark at 4 °C.

The cells were stained with PI (0.5 mM) to evaluate the comets. Aliquots of PI (10 µL) were pipetted on each side of the slide and covered by coverslips. The samples were observed under an epifluorescence microscope (Nikon Eclipse 80i, Tokyo, Japan) at 400 x magnification and using an excitation filter of 450-490 nm. One slide per male and treatment was observed, evaluating at least 50 cells per slide. The images were acquired with a camera (Moticam 1080, Xiamen, China)

attached to the microscope and connected to a computer with Motic Image Plus software (Version 3.1.1, Motic, Xiamen, China). The images of comets were individually analyzed using the CASP Lab software (version 1.2.3). From the several parameters analyzed by the software, the percentage of tail DNA (TailDNA - %) and Olive tail moment (OTM) were used to characterize DNA damage of sperm samples (fresh and cryopreserved at different containers) from the three species. The TailDNA % refers to the total amount of DNA migrated from the cell nucleus. Moreover, the OTM is the product of the TailDNA % and the median migration distance that occurs due to the distance between the comet's center head and tail gravity center (Fig. 2). Greater values of these parameters indicate higher cellular DNA fragmentation. Fresh and post-thawing samples of European eel (n = 8), gilthead seabream (n = 10), and European sea bass (n = 7) cryopreserved in plastic straw and hard biodegradable capsules were evaluated in triplicate.

2.9. Statistical analyses

The data are presented as means ± SD. Normality (Shapiro-Wilk test) and homogeneity (O'Neill & Mathews test) were verified. When necessary, data were transformed using LOG. After verifying compliance with the statistical assumptions, data were analyzed using a one-way ANOVA followed by Tukey's test. All analyses were performed with a 95% confidence level. The graphs and analyses were performed using R (RStudio version 2022.07.01) and GraphPad Prism software (Version 9.3.0).

3. Results

European eel, gilthead seabream, and European sea bass sperm kinetic parameters results of fresh and post-thawing sperm cryopreserved in plastic straw, hard gelatin, and hard HPMC capsules are shown in the Figure 3. The cryopreservation process, independently of the used container, decreased the post-thawing sperm MOT and MOTp of the three species. In addition, no differences were observed for these parameters between the samples cryopreserved in plastic straws and hard biodegradable capsules (Fig. 3A).

European eel fresh samples showed higher VCL values (179 ± 14 μm s^{-1}) than those found in samples cryopreserved in capsules (gelatin – 139 ± 9 μm s^{-1}; HPMC – 143 ± 18 μm s^{-1}), and higher VAP values (133 ± 18 μm s^{-1}) than sperm stored in hard HPMC capsules (109 ± 11 μm s^{-1}). In gilthead seabream samples, the cryopreservation process caused a reduction of the post-thawing sperm velocities independently of the used container. A similar result was observed in European sea bass samples, but the sperm cryopreserved in hard gelatin capsules showed higher velocities (VCL 101 ± 15 μm s^{-1}; VSL 76 ± 18 μm s^{-1}; VAP 90 ± 18 μm s^{-1}) than those stored in hard HPMC

capsules (VCL 87 ± 7 μm s^{-1}; VSL 59 ± 11 μm s^{-1}; VAP 73 ± 10 μm s^{-1}) (Fig. 3B).

The fresh and post-thawing sperm membrane integrity percentage of the three studied species is shown in the Figure 4. European eel sperm membrane integrity of samples cryopreserved in hard HPMC capsules (65 ± 12%) did not differ from the value observed in fresh sperm (76 ± 11%). However, samples cryopreserved in plastic straws (61 ± 12%) and hard gelatin capsules (62 ± 14%) showed a significantly smaller percentage of live spermatozoa. In the gilthead seabream and European sea bass samples, a decrease of viable cells was observed after the cryopreservation process, independently on the used container.

The comet assay showed that European eel and European sea bass sperm samples did not show differences of DNA fragmentation after the cryopreservation in different containers (Fig. 5). However, in gilthead seabream, the sperm samples cryopreserved in capsules showed higher Tail DNA (gelatin – 10 ± 2%; HPMC – 11 ± 6%) and OTM (gelatin – 10 ± 3; HPMC – 11 ± 6) than fresh (Tail DNA – 4 ± 3%; OTM – 3 ± 2) and cryopreserved in plastic straws samples (Tail DNA – 4 ± 1%; OTM – 5 ± 2), evidencing a higher DNA damage through the freezing-thawing process.

4. Discussion

The present study is the first one testing biodegradable capsules as an alternative to plastic straws in the cryopreservation of sperm from three marine fish species with high environmental and commercial interest. We observed that biodegradable capsules were as efficient as plastic straws in preserving sperm kinetic parameters, membrane integrity, and DNA integrity after European eel, gilthead seabream, and European sea bass sperm cryopreservation.

During cryopreservation, cells are exposed to high thermal and osmotic stress, mainly during freezing and thawing, causing damage to the cells [29]. This damage caused during cryopreservation is called cryodamage, which can fully or partially compromise cellular functionality. Thus, cryopreserved sperm generally showed lower kinetic parameters than fresh sperm due to the damage caused during the cryopreservation process [30]. Sperm motility is the main biomarker of sperm quality [31]. In the present study, we observed in the three species that samples cryopreserved in plastic straws, hard-gelatin, or hard-HPMC capsules showed lower MOT and MOTp than fresh sperm samples. The same was observed in other studies by our group in European eel [12,32,33] and by other authors in gilthead seabream [34] and European sea bass [21,35]. On the other hand, the kinetic parameters of sperm samples cryopreserved in straws and capsules did not differ. These results support the efficiency of the sperm cryopreservation protocols for the three species in biodegradable capsules developed in this study. Sperm velocities are kinetic parameters only observed in motile spermatozoon and are related to the potential

fertilization capacity [36,37]. In the present study, we observed that the sperm velocities of European eel and gilthead seabream samples cryopreserved in the three containers did not differ. European sea bass thawed sperm cryopreserved in HPMC capsules showed a difference of VCL -13%, VSL -23%, and VAP -19% for sperm cryopreserved in gelatin capsules. We observed that after the thawing procedure, the HPMC capsules did not dissolve as well as the gelatin capsules. Small fragments left in the middle may have made it difficult for the sperm to swim, reducing the velocities. However, cryopreserved sperm in plastic straws showed similar velocities to samples stored in HPMC capsules. This may have happened due to the presence of the extender at the time and after defrosting because sperm dilution after thawing can increase sperm velocities [38]. The present study results show that the capsules maintained sperm kinetic parameters after the cryopreservation process, as well as straws of three crucial species of environmental and commercial fish from the Mediterranean.

The evaluation of cell viability by checking the membrane integrity and functionality is a widely employed method in research to assess the effectiveness of a sperm cryopreservation procedure [39]. The plasma membrane consists of a dual layer of lipids with distinct hydrophobic and hydrophilic regions. This membrane shields and isolates the cell from the external environment while also regulates the movement of substances into and out of the cell, primarily through transmembrane proteins. Maintaining the integrity and functionality of the plasma membrane is crucial for the survival of cells, including cryopreserved spermatozoa [7]. As expected, we observed that cryopreservation of European eel, gilthead seabream, and European sea bass sperm induced a decrease of the number of viable spermatozoa. A similar decrease of membrane integrity was observed by [12] when they tested the cryopreservation of European eel sperm in 2 and 5 mL containers, and the use of different cryoprotectant solutions. In addition, decreases in sperm membrane integrity in samples from both gilthead seabream [34] and European sea bass [21] has also been reported. However, when sperm samples from the three species cryopreserved in different containers were compared, we did not observe any difference between them in terms of post-thawing sperm membrane integrity. These results support the idea that the use of biodegradable capsules as containers could be successfully applied to preserve sperm membrane integrity, and it can be used in aquaculture and conservation projects of Mediterranean fish species.

The ultimate objective of spermatozoa is to transmit the male genetic information to the offspring, making imperative to prioritize the preservation of spermatozoa's genomic information during the design of a cryopreservation protocol [7]. Over the years, several studies have reported DNA damage in post-thawing fish sperm evaluated by alkaline comet assay [35,40-43]. Our study is the first to report results about DNA fragmentation in European eel fresh and post-thawed sperm using

comet assay analysis.

We did not observe an increase in sperm DNA damage in European eel and sea bass after freezing and thawing. In addition, we did not find any difference in the TailDNA and OTM between sperm samples of both species cryopreserved in plastic straws and biodegradable capsules. We also observed no difference in DNA damage parameters between gilthead seabream fresh and cryopreserved sperm in straws, coinciding with the reported by [18]. On the other hand, our results showed greater DNA damage in post-thawing sperm cryopreserved in biodegradable capsules compared to fresh and cryopreserved sperm in straws. The permeable cryoprotectant Me_2SO is toxic to the cells, and the toxicity is accentuated by the increase in temperature during thawing and the time of exposure to the cryoprotectant solution post-thawing [44]. When we were fixing our protocol, we first thawed gilthead seabream sperm samples frozen in capsules at 60 °C. However, this resulted in the absence of sperm motility. Thus, we decided to reduce the thawing temperature to 40 °C, and observed that the sperm kinetic parameters were similar to those of sperm samples cryopreserved in straws. In this way, decreasing the thawing temperature of capsules could minimize DNA damage to spermatozoa cryopreserved in these containers. Anyhow, we observed a Tail DNA of 10 and 11% in sperm cryopreserved in gelatin and HPMC capsules, respectively. In our opinion, and considering previous studies [18,45] these results indicate low sperm DNA damage and it is probable that the oocyte could repair this level of damage during early embryogenesis, without jeopardizing a right embryonic development and, consequently, a regular larvae production. Our results showed that the use of biodegradable capsules as containers for the cryopreservation of sperm from Mediterranean fishes of commercial interest preserves the sperm DNA integrity at a level than can be considered good enough to maintain normal embryonic development.

In this study, our data showed that it is possible to cryopreserve European eel, gilthead seabream, and European sea bass sperm using hard-gelatin and hard-HPMC capsules. The sperm quality parameters evaluated in the present study are widely used in fish reproduction studies [7,46]. In addition, they have a high correlation with reproductive success for several fish species [36,37,47]. Even though we did not evaluate the fertilization capacity of the cryopreserved sperm samples, we observed that they presented similar sperm quality to those stored in plastic straws. Thus, the cryopreservation methodology of the three Mediterranean species sperm in gelatin or HPMC capsules can be apparently used without losing sperm reproductive potential. Consequently, using capsules as a container for sperm cryopreservation from marine fish species of high commercial interest can make larvae production more sustainable from an environmental and economic point of view.

5. Conclusion

We observed that biodegradable hard-gelatin and hard-HPMC capsules could maintain the sperm quality of European eel (*Anguilla anguilla*), gilthead seabream (*Sparus aurata*), and European sea bass (*Dicentrarchus labrax*) after cryopreservation. Thus, the capsules can be considered as an alternative container to plastic straws for storing sperm of marine fish species at ultra-low temperatures. Our study describes the methodology for using these containers in three species, which could be adapted to other ones. In addition, it paves the way for developing this research area intending to reduce the cost and the amount of plastic waste generated in the sperm cryopreservation process.

Declaration of interest

TSF and DPSJ filed (September/2022) a patent (pending) application on the methodology described in the study. The remaining authors have no conflicts of interest that could be perceived as prejudicing the impartiality of the research reported.

Author contribution statement

TSF: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing - original draft; WAG-L: Formal analysis, Investigation, Methodology; MPS: Formal analysis, Investigation; LF: Formal analysis, Investigation, Methodology; FF-G: Formal analysis, Investigation; LPB: Formal analysis, Investigation; AB: Investigation; PGH: Investigation; JCCG: Investigation; AF: Investigation, Project administration, Funding acquisition; AG: Investigation, Project administration, Funding acquisition; JPS: Investigation, Project administration, Funding acquisition; DPSJ: Conceptualization, Supervision; JFA: Investigation, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

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Figure captions

Figure 1. Fresh sperm dilutions and experimental design to compare European eel (*Anguilla anguilla*; n = 12), gilthead seabream (*Sparus aurata*; n = 12), and European sea bass (*Dicentrarchus labrax*; n = 10) post-thawing sperm quality after cryopreservation in plastic straws, hard-gelatin capsules, and hard-HPMC capsules. Plastic straws were thawed in water-bath and capsules in tubes with extender under vortex shake. The sperm kinetic parameters, membrane integrity, and DNA damage of fresh and post-thawing sperm were performed in triplicate.

Figure 2. Schematic image demonstrating TailDNA (%) and Olive Tail Moment formulas used by CaspLab software after the alkaline comet assay. Both parameters show DNA fragmentation that indicates cellular DNA integrity. The median DNA migration is the distance between the center head comet and tail center gravity; numbers indicate both points.

Figure 3. Sperm kinetic results of European eel (n = 12), gilthead seabream (n = 12), and European sea bass (n = 10) fresh and post-thawing sperm. The samples were cryopreserved in plastic straws, hard-gelatin, and hard-HPMC capsules. Graphs show motility (MOT – A), progressive motility (MOTp - A), and velocities (VCL, VSL, VAP – B) evaluated by CASA-Mot. Values are shown individually, and horizontal lines indicate the means ± SD. Different letters indicate differences ($P < 0.05$; Tukey's test) between means.

Figure 4. Sperm membrane integrity of European eel (n = 12), gilthead seabream (n = 12), and European sea bass (n = 10) fresh and post-thawing sperm. The samples were cryopreserved in plastic straws, hard-gelatin, and hard-HPMC capsules. Graphs show viable cells percentage observed by epifluorescence microscope using SYBR-14 (2 μM) and propidium iodide (PI - 2.4 mM) stains. Photos were taken, and the results were calculated after counting cells using the FIJI software. Values are shown individually, and horizontal lines indicate the means ± SD. Different letters indicate differences ($P < 0.05$; Tukey's test) between means.

Figure 5. Sperm DNA fragmentation of European eel (n = 8), gilthead seabream (n = 10), and European sea bass (n = 7) fresh and post-thawing sperm. The samples were cryopreserved in plastic straws, hard-gelatin, and hard-HPMC capsules. Graphs show DNA in the comet tail percentage and the Olive tail moment index calculated observed by epifluorescence microscope using PI stain and evaluated by CaspLab software. Values are shown individually, and horizontal

lines indicate the means ± SD. Different letters indicate differences ($P < 0.05$; Tukey's test) between means.

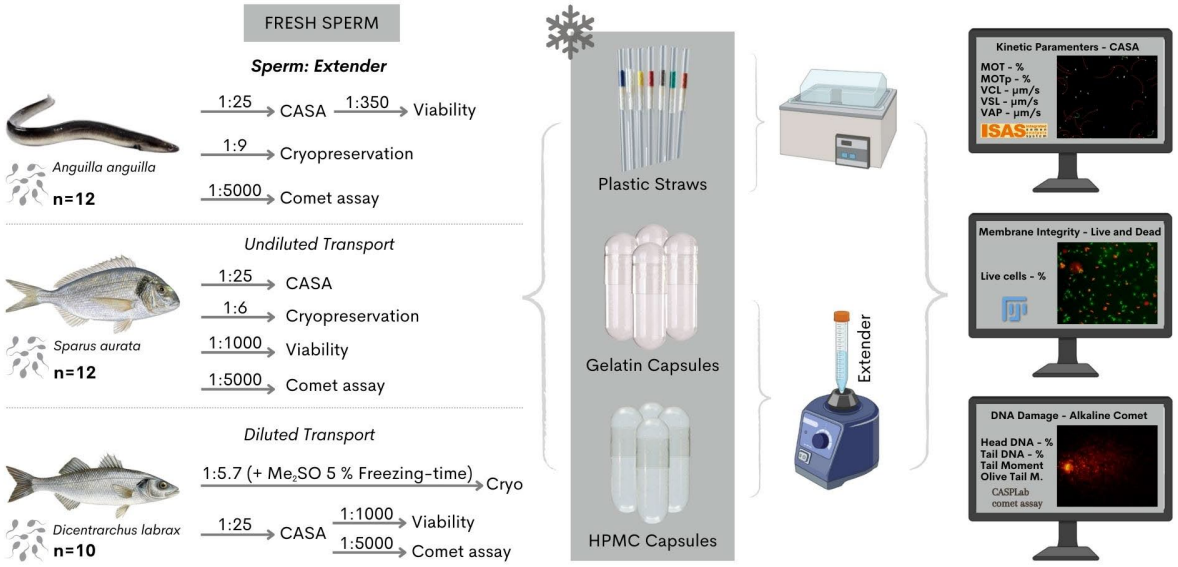
FRESH SPERM
Sperm: Extender
Anguilla anguilla
n=12
1:25
CASA
1:350
Viability
1:9
Cryopreservation
1:5000
Comet assay
Undiluted Transport
Sparus aurata
n=12
1:25
CASA
1:6
Cryopreservation
1:1000
Viability
1:5000
Comet assay
Diluted Transport
Dicentrarchus labrax
n=10
1:5.7 (+ Me2SO 5 % Freezing-time)
Cryo
1:25
CASA
1:1000
Viability
1:5000
Comet assay
Plastic Straws
Gelatin Capsules
HPMC Capsules
Extender
Kinetic Paramenters - CASA
MOT - %
MOTp - %
VCL - µm/s
VSL - µm/s
VAP - µm/s
ISAS
Membrane Integrity - Live and Dead
Live cells - %
DNA Damage - Alkaline Comet
Head DNA - %
Tail DNA - %
Tail Moment
Olive Tail M.
CASPLab
comet assay

Figure 1

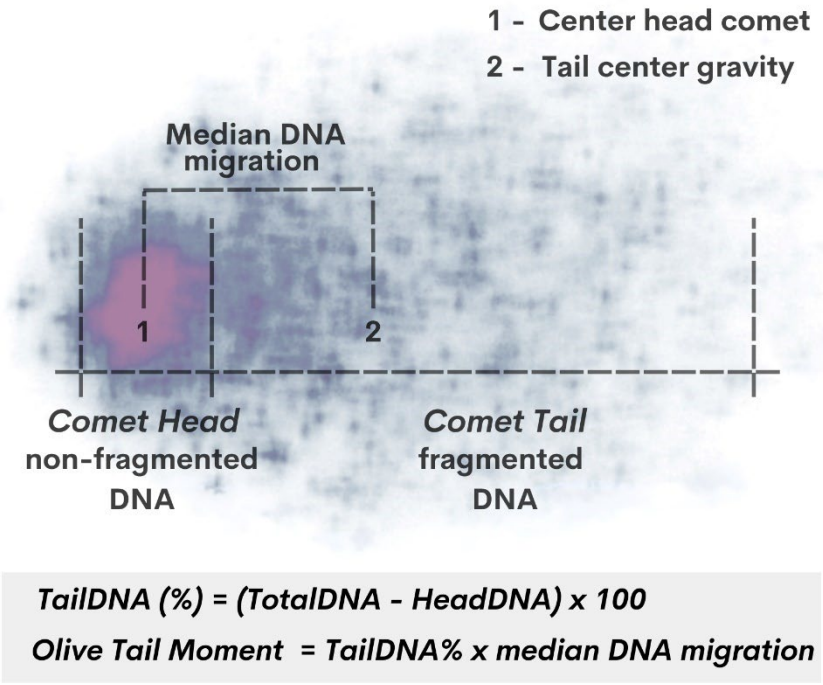
1 - Center head comet
2 - Tail center gravity
Median DNA migration
1
2
Comet Head
non-fragmented DNA
Comet Tail
fragmented DNA
TailDNA (%) = (TotalDNA - HeadDNA) x 100
Olive Tail Moment = TailDNA% x median DNA migration

Figure 2.

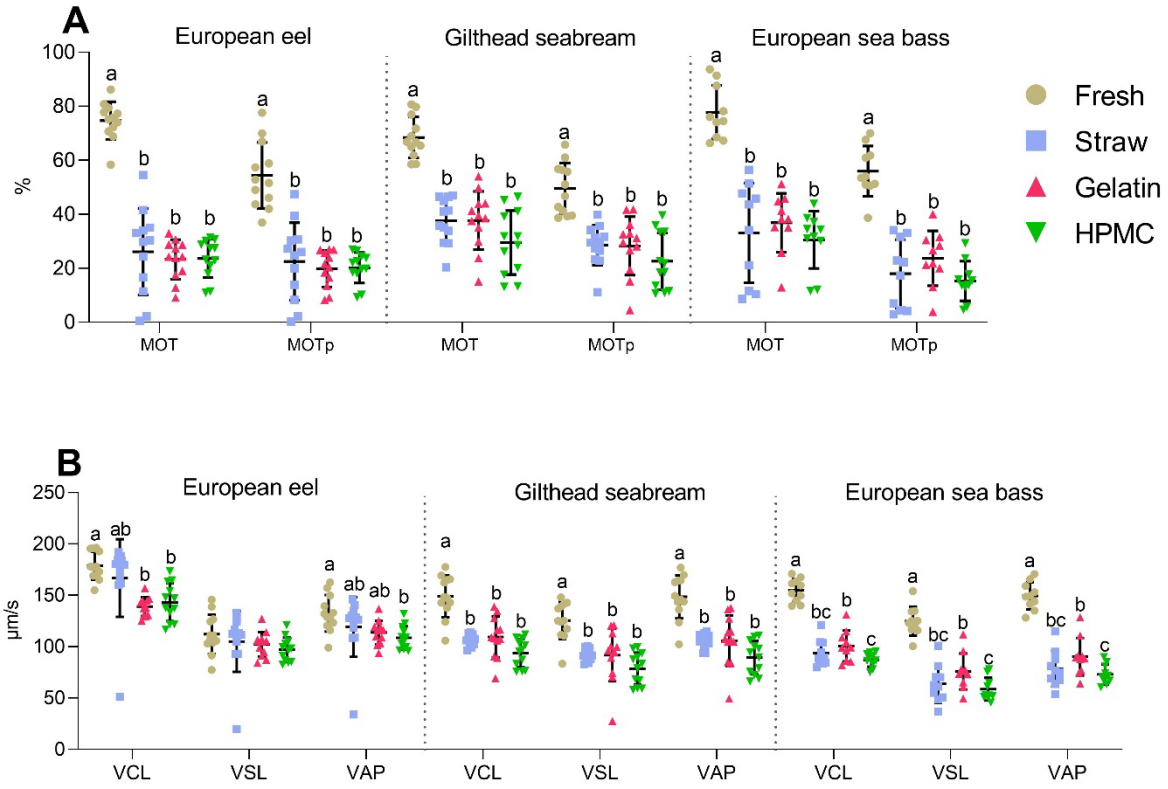
A
European eel
Gilthead seabream
European sea bass
%
MOT
MOTp
Fresh
Straw
Gelatin
HPMC
B
µm/s
VCL
VSL
VAP

Figure 3

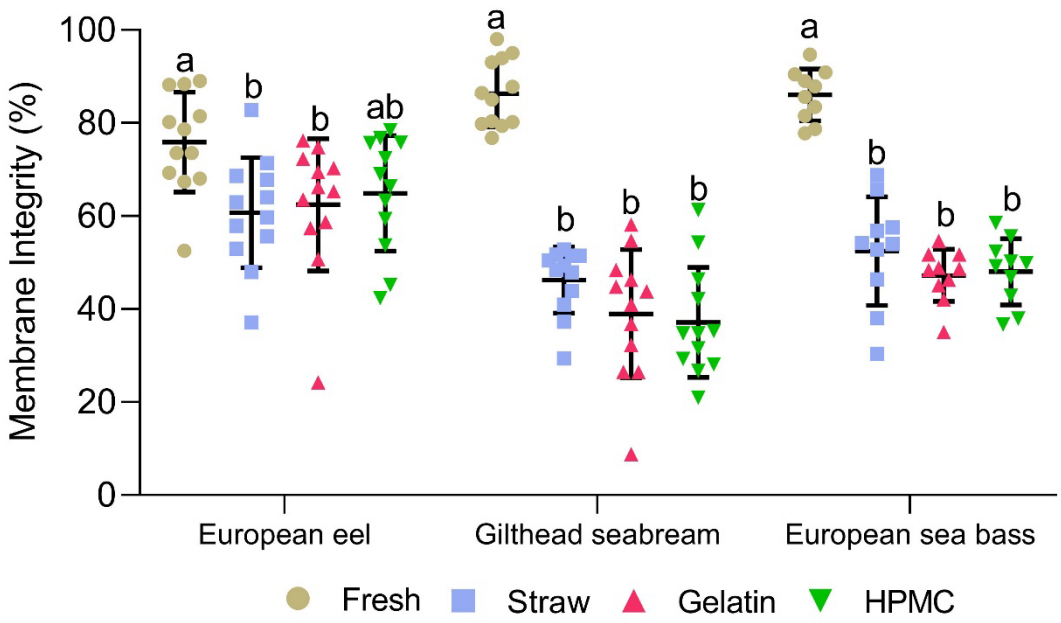
Membrane Integrity (%)
100
80
60
40
20
0
a
b
b
ab
a
b
b
b
a
b
b
b
European eel
Gilthead seabream
European sea bass
Fresh
Straw
Gelatin
HPMC

Figure 4

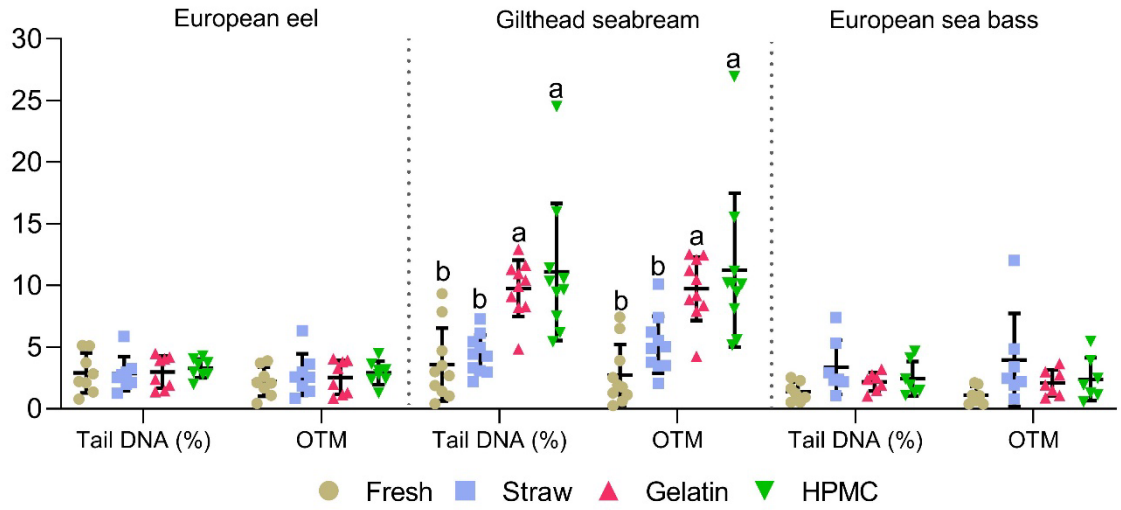
European eel
Gilthead seabream
European sea bass
30
25
20
15
10
5
0
a
a
a
b
b
b
b
b
Tail DNA (%)
OTM
Tail DNA (%)
OTM
Tail DNA (%)
OTM
Fresh
Straw
Gelatin
HPMC

Figure 5