Deliverable Report

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Deliverable Title	Cryopreservation method for wreckfish			
WP No:	6		**WP Lead beneficiary:**	P8. IEO
WP Title:	Reproduction and Genetics – Wreckfish			
Task No:	6.4		**Task Lead beneficiary:**	P14. IFREMER
Task Title:	Evaluation of sperm characteristics and cryopreservation protocols			
Other beneficiaries:	P1. HCMR	P3. IRTA	P14. IFREMER	P15. ULL
P19. CMRM	P32. MC2			
Status:	Delivered		**Expected month:**	24
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Objective: The goal of this deliverable was to provide a reliable method to store live wreckfish sperm after having tested and adapted on this species, different media and protocols developed for other marine fish species.

Background

In preliminary experiments, wild caught wreckfish exhibited a high growth rate and a good behavior in captivity (Papandroulakis et al, 2004 Peleteiro et al 2010), which indicated that this species was an interesting candidate for aquaculture. However, these preliminary experiments were based on a few individuals and the catches have remained very low and scattered so that few organisms are available to research the potential of wreckfish culture. Therefore, the control of wreckfish reproduction in captivity is a prerequisite to provide individuals for the research of the species biology and aquaculture potential.

In captivity, wreckfish have been observed to spawn spontaneously in very large aquaria and both fertilized eggs and larvae were collected, but in an unreliable way (observed in DIVERSIFY). The first trials using hormonal stimulation to induce spawning provided some eggs either by spontaneous egg release or by hand-stripping the females.

Fertilization failures may not be due to semen fertility since wreckfish males produce a high volume of easily expressible milt with a concentration considered as medium range for marine fish and of course much higher than that of flatfish. On the top of those general features, the setup of a CASA protocol adapted to

wreckfish sperm allowed showing that this sperm exhibits a high percentage of motile cells at activation, one of the highest initial speed associated with a long swimming duration among the studied marine fish (***Deliverable 6.1 Computer Assisted Sperm Analysis (CASA) method for wreckfish)***. Moreover, artificial fertilization of hand-stripped eggs produced viable embryos (Papandroulakis et al 2004; Fauvel et al. 2008). These observations in captivity and preliminary trials indicated that hand stripping and artificial fertilization may be an alternative method for a reliable production of embryos for the wreckfish propagation in aquaculture.

To secure artificial fertilization methods, the availability of live and efficient wreckfish sperm at the right time is mandatory. In order to anticipate this requirement, it was proposed to test the ability of wreckfish sperm to cope with cryopreservation and to develop methods providing efficient sperm, available for artificial fertilization protocols. The present deliverable is a report of the applied method for the cryopreservation of wreckfish sperm.

Description

The experiments of storage of wreckfish sperm were performed in the laboratories of P8 (IEO) during the data collection campaigns of April 2014 and January 2015 using only sperm from the captive males of Vigo center in Galicia (NW Spain), and taking profit of their characterization described in ***Deliverable 6.1 Computer Assisted Sperm Analysis (CASA) method for wreckfish*** . Moreover, sperm was collected from 6 males from P32 (Acuarium Finisterrae, MC2) facilities, 2 males of Luso Hispana de Aquicultura (LHA) and transported to Vigo. These samples were analyzed the day after collection. The laboratory methods of analysis according to Fauvel et al, 2010, were shared between researchers of the different partners and a researcher from P14 (IFREMER) was in charge of this task in the laboratory of P8.

Short term storage without any medium

The semen was collected by hand stripping in the different locations mentioned above. The collected semen was divided into various aliquots in Eppendorf 1.5 ml microtubes. Each aliquot was of a small volume of either 10 µl or 1 ml of semen. The aliquots were stored undiluted between 0 and 4°C until the day after (24 h) or for several days.

The motility of sperm was evaluated with the following steps: 1) a small volume of around 1 µl of sperm was collected by plunging a syringe needle in the semen, 2) the 1 µl drop of sperm was deposited on a microscope slide, 3) a drop of seawater (50 µl) was deposited beside the sperm without making contact with the sperm, 4) the contact between sperm and seawater was triggered by the depositing of a coverslip over both drops and 5) after a rapid focus at 200X magnification, the initial motility was subjectively assessed at-a-glance, on a scale of 5 classes covering 20% mobile spermatozoa in each class.

After this first evaluation, the samples were assayed again by a Computer Assisted Sperm Anaylsis (CASA) method as described in ***Deliverable 6.1 Computer Assisted Sperm Analysis (CASA) method for wreckfish***. Briefly, sperm motion was video recorded, then it was analyzed by an image sequence analysis after image binarization. This study used the free software ImageJ (NIH, USA), and the dedicated CASA plugin from Wilson-Leedy and Alderman (2007).

Cryopreservation

Direct freezing of cells generally causes the complete mortality of all cells and it is necessary that the cryopreservation of live cells is made with a specific preparation that prevents membrane wounds and subcellular organelle degradation. The freezing process involves the solidification of both the whole cell and the surrounding environment. During cell life, the cytosol contains ions, which are exchanged with the external saline environment through the cell membrane. The ionic status of both internal and external media decreases the freezing point to below 0°C. The solidification of a saline solution involves an increase of ionic

concentration around the ice crystals and the change of state from liquid to solid is exothermic. The challenge to maintain the cells alive is to overcome physical problems such as the differential freezing between the cell and the environment, the deleterious effects of hyperosmotic shock linked to peri-ice increase of salt concentration and of the freezing/thawing vibration due to the exothermic reaction at the "seeding point" when ice crystal formation is initiated (Cloud and Patton, 2008).

As a consequence, among the parameters to be controlled for successful cryopreservation, the main issues are the slope of temperature decrease and the composition of the environment of the cells including the environment of organelles inside the cells. The temperature decrease depends on the container used (temperature gradient linked to the volume of sample) and the temperature applied, while the environment of cells is adjusted by the use of cryoprotectants.

The cryoprotectants must respect the ionic equilibrium between the cells environment and the external environment before freezing. In addition to this, the cryoprotectants must limit the deleterious effects of the increase of external osmotic pressure at the time of freezing. To fulfill these requirements, the media are constituted of isotonic or lightly hypotonic saline, complemented with large proteins such as egg yolk or bovine serum albumin which protect cell membranes, and finally with products such as methanol or dimethyl sulfoxide (DMSO), which penetrate the cells without membrane lesion and allow a solidification of the whole solution of diluted semen including the external environment, cell structure and cytosol.

Freezing protocol

The freezing protocol tested in the frame of this task was a simple transfer from the protocols already successfully used in different species like seabass and turbot with the following main characteristics:

Cryoprotectants

In the current task, two different cryoprotectants were tested. The first one, Cryofish is a commercial product from the company IMV (http://www.imv-technologies.com/), which has been adapted for several species of both marine and freshwater fish, such as salmonids, turbot, gilthead seabream (*Sparus aurata*) and tilapia (*Oreochromis spp*). This product has also been used for European seabass (*Dicentrarchus labrax*) sperm cryopreservation to establish a large cryobank with the purpose to conserve genetic strains.

Since the precise composition of the Cryofish is patented, hence not precisely described, an experimental diluent especially designed for either short term chilled sperm conservation or cryopreservation of European seabass sperm was also tested. This diluent is composed of a cell culture medium - Leibovitz L15 (the formula is publically available and the medium is available from sigma Aldrich, ref L4386,) and 2 amino acids - pyruvic acid sodium salt and glutamine (Table 1). The amino acids are involved in the respiration chain which sustains the ATP content of sperm for 3 days in European seabass (Fauvel et al., 2012), hence permitting a high recovery of sperm after thawing.

Table 1. Modified Leibovitz composition and instructions for use

	Storage diluent (250mOsm, pH: 8)	Cryoprotectant added to diluted sperm
Leibovitz L15 350mOsm, pH:7.5	100ml	
Sodium pyruvate	600mg	
Glutamine	30mg	
NaOH (1N)	250µl	

Distilled water	80ml	
BSA		2% (then shake)
DMSO		10% (then shake and freeze)

Sperm was either diluted in a ratio 1/3 (vol. sperm / vol. cryoprotectant) in modified Leibovitz, then complemented at time of freezing by 10%vol of DMSO, or diluted in the same ratio in Cryofish prepared according to producer's recommendation

Temperature

In order to get an acceptable decrease of temperature in field conditions without sophisticated devices, plastic straws from IMV of 250 µl and 1.8 ml cryotubes (Nalgene) filled with 1 ml diluted sperm (**Fig 1**) were deposited at either 2 cm or 6 cm into the cold vapors above liquid nitrogen (LN) on styrofoam vessels floating on the LN surface (**Fig 2**). Temperature decrease was monitored by a microprobe located in the sperm samples.

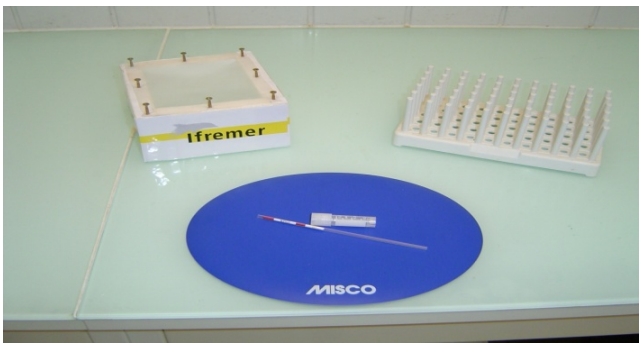

Figure 1. Material for cryopreservation of fish sperm: 250 µl (straw) and 1.8 ml (Cryotube) as containers and a handcrafted Styrofoam vessel of 6 cm height

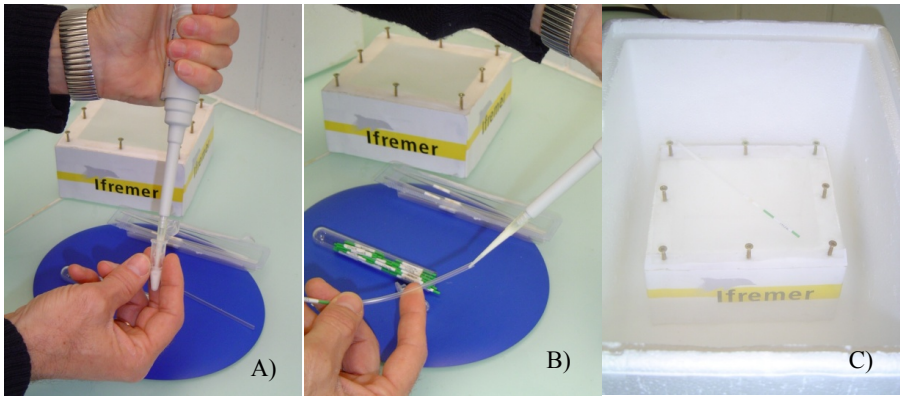

Figure 2. Freezing protocol for wreckfish sperm with 3 steps: A) Sperm dilution into adapted diluents, B) Straw filling and C) Sperm cooling in a straw in cold vapors at 6cm over liquid nitrogen surface

At thawing, straws or microtubes were plunged into a waterbath at 38°C until the total liquefaction of the diluted sperm and they were immediately tested for motility.

Results

The temperature decrease recorded in the diluted sperm that was inside straws held at 6 cm and cryotubes held at 2 cm above liquid nitrogen (LN) actually revealed the phenomenon of exothermic reaction at seeding point. The cooling rate imparted by LN vapors at the different heights were sufficient to prevent temperature vibrations, hence avoiding successive deleterious freezing/thawing of spermatozoa. (**Fig. 3**).

Sperm recovery after storage was evaluated from the analyses of 3 best records of each of 3 male sperm samples at thawing, after cryopreservation using either the patented Cryofish or the modified Leibovitz.

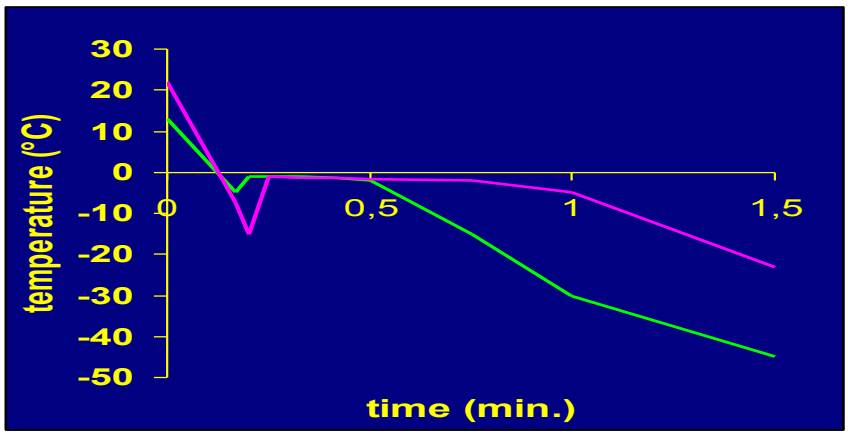

Figure 3. *In situ* variations of temperature during cooling of straws at 6 cm above liquid nitrogen (green line) and cryotubes at 2 cm only above liquid nitrogen (pink line).

Chilled storage without dilution

The storage of tiny volumes (10 µl) of undiluted sperm in Eppendorf tubes maintained at 4°C did not reveal deleterious effects on the semen, since significant motility of sperm at activation was recorded for up to 12 days. On the contrary the storage of larger volumes of sperm (1 ml) in 5 ml tube either with or without oxygen supplementation was unsuccessful and no sperm could be activated after a 24 h storage period. Such a result indicates that a dilution for chilled storage or cryopreservation are necessary to manage larger volumes for sperm storage.

Chilled storage with dilution using modified L15 and Cryofish

At 24 h after collection, wreckfish sperm diluted in both media in a ratio 1/3 (v/v) and stored as large volumes (1 ml) at around 4°C showed a high variability activation that ranged from no activation at all to 50% initial motility whatever the medium and the rank of trial. Moreover the duration of spermatozoa movement was impaired compared to that of fresh sperm. As a matter of fact, this chilled storage method that has been efficient for other species such as European seabass may cannot be used as such for wreckfish, but only after a long process of adjustment of settings.

Cryopreservation

Wreckfish sperm showed a good ability for cryopreservation under usual protocols developed for other species so that it was possible to describe the motility after thawing in the different media and compare with fresh sperm. There were no significant global differences (integrated motility over time of movement) between the tested media particularly for the first part of the movement while similar performances were obtained with sperm from cryotubes cooled at 2 cm from LN surface and from 250 µl straws cooled in the vapors at 6 cm above LN (**Fig 4**).

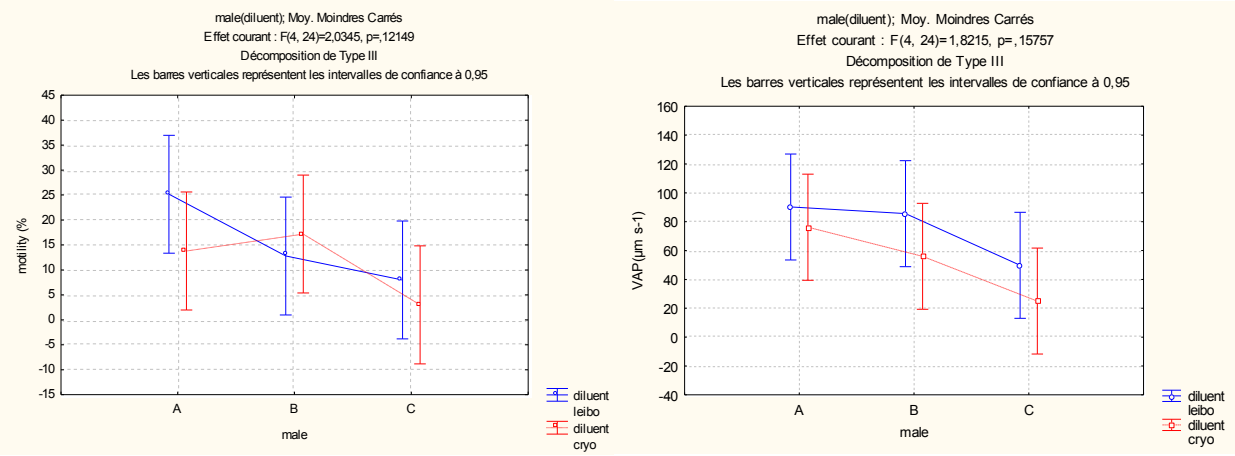

Figure 4. Analysis of variance of motility and velocity of sperm after cryopreservation using the general linear model (Statistica, Statsoft, Tulsa, USA) with individuals and diluents as predictive factors.

The higher initial motility at activation was 42% in modified Leibovitz while it was only 20% of mobile sperm in the commercial product Cryofish. In both cases, the initial motility was reduced when compared to the activated fresh sperm in which motility of 60% was recorded (Deliverable 6.1). Moreover in both diluents, thawed sperm showed a quicker decrease of velocity and of the number of mobile spermatozoa which finally resulted in a shorter duration of motility reduced to 2 minutes instead of 4 minutes for fresh sperm (Deliverable 6.1). The dilution in Cryofish seemed less efficient since in a subjective way, we observed noticeable aggregations of cells after thawing in this medium (**Fig. 5B**). **Figure 5** summarizes the main features of sperm quality after thawing in the different media illustrated by one particular individual from IEO.

Conclusion and perspectives

The present work shows the feasibility of wreckfish sperm cryopreservation while chilled storage does not seem to be a good solution for the management of sperm for artificial fertilization. The performances of frozen/thawed wreckfish sperm are globally half those of fresh sperm in terms of percentage of motile sperm and duration of swimming while the velocity in modified Leibovitz was similar to that of fresh sperm. Since wreckfish produce large volumes of high quality sperm in terms of concentration, velocity and duration of motility (Deliverable 6.1), the losses of sperm quality due to freezing may be compensated by increasing the number of spermatozoa per egg as is usually practiced in other species. The short duration of rapid movement may not be harmful since generally fertilization occurs in the first seconds of contact between

gametes of both sexes. However, the current results only describe the movement of spermatozoa. They are a good index of cryopreservation coping capacity of sperm. Nevertheless, these results have to be confirmed in the future by a test of cryopreserved sperm ability to fertilize.

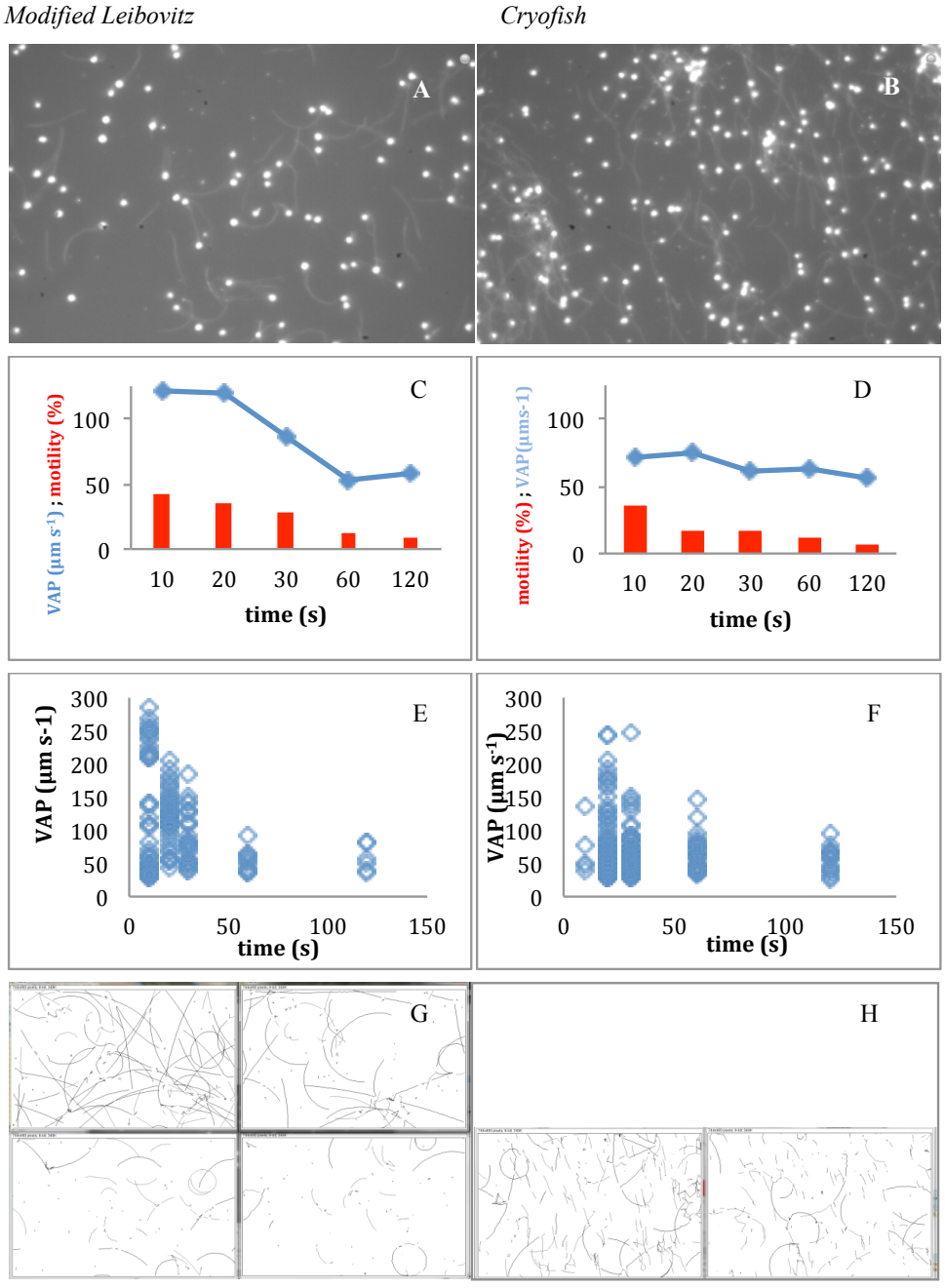

Figure 5. Wreckfish sperm status after cryopreservation in modified Leibovitz and Cryofish. A and B: Pictures of the sperm diluted in the different media (extracted from video records), Cryofish samples (B) show aggregations of sperm unlike modified Leibovitz (A). C and D: Mean velocity decrease and variations of the percentage of motile sperm with time in the different media. E and F: individual velocities of spermatozoa recorded in the different media showing that modified Leibovitz (E) allows a high recovery of a larger number of spz compared to Cryofish (F). G and H: illustration of tracks generated by CASA for the spz stored in the two media: Leibovitz (G) and Cryofish (H).

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Deviations: No deviations


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