STSM Scientific Report

COST STSM Reference Number: ECOST-STSM-FA1205-280414-043034

COST Action: FA1205 (Aquagamete) **Period:** 12-05-2014 to 16-05-2014

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STSM title: Effect of shifted reproductive season on seabass sperm quality and motility features

Fish sperm objective qualification: IFREMER-IOLR technology exchanges

Short Term Scientific Mission May 12-16, 2014

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In the STSM carried out on May 12-16th 2014 we aimed to study the effects of shifted reproductive season on European seabass sperm quality and become acquainted with the relevant evaluation techniques, particularly the Computer Assisted Sperm Analysis (CASA).

The CASA facilitates a simple and rapid quantitative assessment of the quality of fish sperm and may thus predict its ability to fertilize eggs. In the frame of a mutual EC project (DIVERSIFY), we intend to put into practice the CASA in the IOLR-NCM facilities in Eilat, in order to assess the effect of heterologous hormonal stimulation on grey mullet (*Mugil cephalus*) sperm quality.

General context

The success of reproduction is a prerequisite for the development of a self sustained aquaculture. A successful reproduction in captivity has been achieved for most of the current aquacultured species, by adopting different strategies such as spontaneous ovulation, spawning and mating in tanks (seabream, seabass, meager, tuna), induced ovulation followed by spontaneous spawning and species-specific mating, induced ovulation followed by stripping and artificial fertilization (salmonids, carp in genetics programs) or spontaneous ovulation followed by stripping and artificial fertilization (flatfishes).

In order to increase the variety for the market without depleting wild stocks, it is necessary to control the entire life cycle in captivity of new species with economic potential, such as amberjack or mullet, of whose aquaculture depends on juvenile catches in the wild. A better knowledge of these species' gamete characteristics both in wild and captive fish may allow a significant progress in broodstock management.

In the case of the mullet, a protocol for gonadal development and spawning induction in captivity has been developed at NCM and is being optimized. However, for a better understanding of the developing process for this and other fish species, there is a need for an objective assessment of the gamete characteristics.

For our studies of the effect of heterologous stimulation of sperm production in mullet, a plan to assess sperm quality through accessible techniques was initiated using the seabass as a model. However, due to time schedule constraints, the choice of the species was modified in order to study gametes from the broodstock during their full reproductive season.

The samples of meager sperm were provided by the private company "Les Poissons du Soleil", whose help we acknowledge and whose spirit of collaboration in the organization and performance of this project has been greatly appreciated.

General features of male fish reproduction in temperate seas.

Gametogenesis

- Discontinuous (seasonal) spermatogenesis and possible disconnection between spermatogenesis and spermiation (according to species)
- Ageing of sperm (variation of quality along the reproductive season)
- Possibility to collect sperm by simple pressure of fish abdomen (more or less easy according to species)
- Gametogenesis and spermiation may be altered by heterologous hormonal stimulations

Concentration/Volume of expressible milt

- Species specific: volume from 20 ml plus to only 10µl ; concentration from 10^9 to 6^{10} spz/ml
- Both values may vary along the reproductive season

Motility

- No motility in the genital tract
- Motility triggered by external medium at ejaculation.
- In marine fish motility triggered by hyperosmotic shock (seawater as well as glucose)
- Motility duration variable
    - species specific, e.g. from 30 sec in trout to 20 min in bluefin tuna
    - varies along the season (seabass, turbot)
    - linked to energy stored, since respiration does not compensate ATP expenses due to motility

Fertility

- Depends on species (optimized artificial fertilization for 6.10^3spz/egg in turbot and 7.10^4 spz/egg in seabass, for instance)
- Depends on time in the season
- More or less sensitive to storage, e.g. from several days in sturgeon or trout to several hours in seabass

Intended program

- Sperm quality
    - Sperm concentration: will be assessed by the use of counting cell (Thoma), which is a classic method, however improved by a subsequent image analysis. It requires taking a picture (x20 magnification) and count by image analysis using image J.
      A dilution to 1/500 is generally made to count concentrations of up to 10^{10} spz/ml.

    - Motility: generally estimated by the percentage of motile spz by class of around 20% (Class 5: 100-80%, class 4: 60-80% and so on). Since motility is highest at the time of activation by seawater, it is necessary to follow it as soon as possible after activation. It is also required for the sperm to be collected in a "clean-dry" way, without being exposed to seawater. A dilution between 1/1000 to 1/2500 of fresh sperm in seawater is adequate to categorize the motility classes.
      A two-step dilution (first, 1/10 in an isotonic medium, which does not activate sperm followed by a second step:1/100 to 1/250 in seawater) allows a better triggering of motility.
      Since motility decreases after activation, it is interesting to characterize the slope of decrease and to evaluate the motility class at activation, then record the time when progressive movement ceases (less than 5% motility).
      Recording the movement as soon as possible allows for the use of CASA. Movements of sperm will be recorded using a video camera (x20 magnification). It will provide original data of spermatozoa velocity and the trajectory variations with time will be highlighted.
      In order to limit the possibility for sperm to go out of focus during the observation, and to work under similar conditions, special cells - LEJA (with depth of 10µ) were tested.
      The activating medium will be seawater, to which different doses of bovine serum albumin or other protein such as yolk albumin will be added. This will considerably reduce the sticking of spermatozoa to the glass surface.
    - Sperm "shelf" life: analysing fresh sperm (in close proximity to collection time) is always preferable. However, when this is not feasible, sperm must be stored at low temperature (~5°C) either as is or diluted (1/3 to 1/5) in a

non activating medium (either saline or tissue culture media). Samples of sperm will be collected, maintained in different media and checked for motility in the lab.

- The fertilizing ability of fresh as well as stored sperm will be tested if ovulation of females can concomitantly be programmed

Schedule

Monday morning visit to "Les Poissons du Soleil "(LPDS), Balaruc (25 km from Palavas)

1) Participation in gamete collection in the framework of a specific control of fish crosses in the company

- Broodstock anesthesia and female selection for hormonal stimulation
- Assessment of male fluency and sperm sampling (considering the need of using these males as brooders)

2) Training: Sample 2ml from 5 males and prepare 2 sperm conditions:

Chilled storage

1ml	Storefish (v/v)	L15 modified	cryofish	Fresh (control)
1/ 2	100µl	100µl	100µl	250
1/3	100µl	100µl	100µl	
1/5	50µl	50µl	50µl	

Cryopreservation*

1ml	Storefish (v/v)	L15 modified	cryofish	Fresh (control)
1/ 2	100µl	100µl	100µl	250
1/3	100µl	100µl	100µl	
1/5	50µl	50µl	50µl	

*Add 10% DMSO to each preparation just prior to cryopreservation

Monday afternoon - Palavas:

Concentration and motility studies of every sample of fresh sperm records

Tuesday - Palavas

Storage efficiency control
Motility check after 24 hours and recording of the different samples
CASA analysis with image J

Wednesday - Balaruc

Participation in the process of artificial fertilization of meager, including first sperm collection in addition to fresh sperm and egg collection and fertilization. Some eggs will be sampled for further control of fertilization in Palavas.
In addition, collection of sperm to complement previous dataset.

Thursday
Sperm analysis using CASA and “brain storm” meeting to discuss data meaning
Discussion about theory and applications to mullet

Friday
Conclusions

A glance at the results

The experiments were performed on 2 batches of 4 males coming from either a broodstock progressing towards the end of its reproductive season or at full spawning season. However, only some samples were analyzed to transfer the technical know-how and share the methodology of sperm analysis.

Preliminary results of sperm counting (from the 4 males of each group), by image analysis using imageJ software, revealed that at the end of reproduction season, sperm was 25% more concentrated (4.5 10^{10} vs 3 10^{10} spz/ml) and viscosity (subjective evaluation) was higher. A similar trend was already observed in seabass. Fig. 1 summarizes the analysis using the free software Image J.

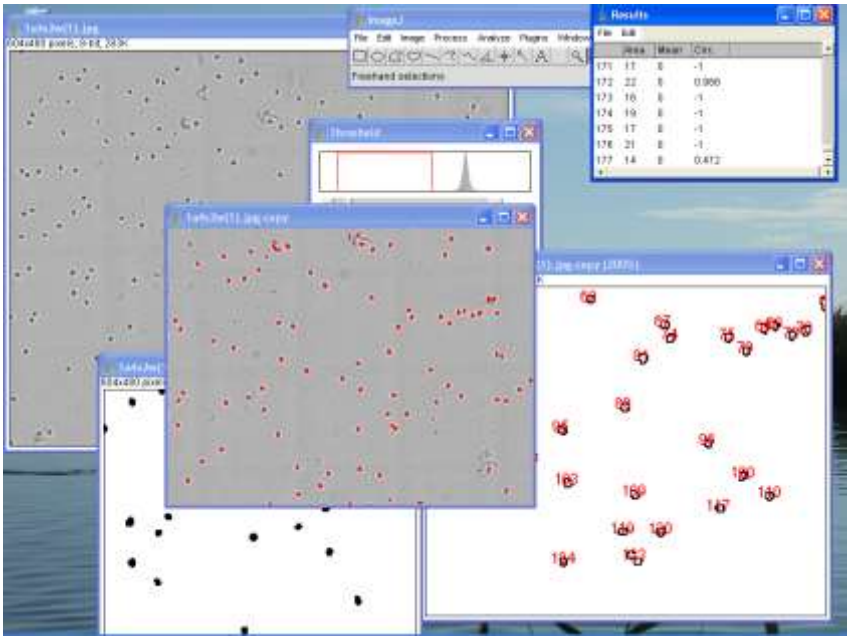

Figure1

For motility assessment, different trials drove us to use a concentration of 15mg (rather than 7.5mg) bovine serum albumin per ml of seawater to improve the capacity of sperm to swim in the special observation cell (Leja Cell, 10 µm depth). This improvement enabled us to record the mobility of sperm subjected to different conditions of storage.

After an introduction to CASA based on previous seabass recorded movies, we applied the protocols of sperm motility analysis on meager samples and adapted the settings for a sensitive analysis. Not all the sampled records could be analyzed during the stay in France, yet as an example we can illustrate the main trends of sperm ageing and sperm conservation in meager (Fig. 2).

At the end of spawning season, meager sperm may exhibit a lower percentage of motile cells as well as a lower duration of mobility; however we cannot exclude a problem related to sperm storage temperature.

At the conditions tested, meager fresh sperm is characterized by an initial motility (10 sec after activation) of around 65% of motile spermatozoa and an initial velocity (Average Path Velocity) of about 140µm/s. The velocity decreases regularly until all movement ceases in a time lap of 1 minute. Fig. 2 shows the CASA analysis of fresh-stored sperm of one male activated by seawater 3 hours after collection.

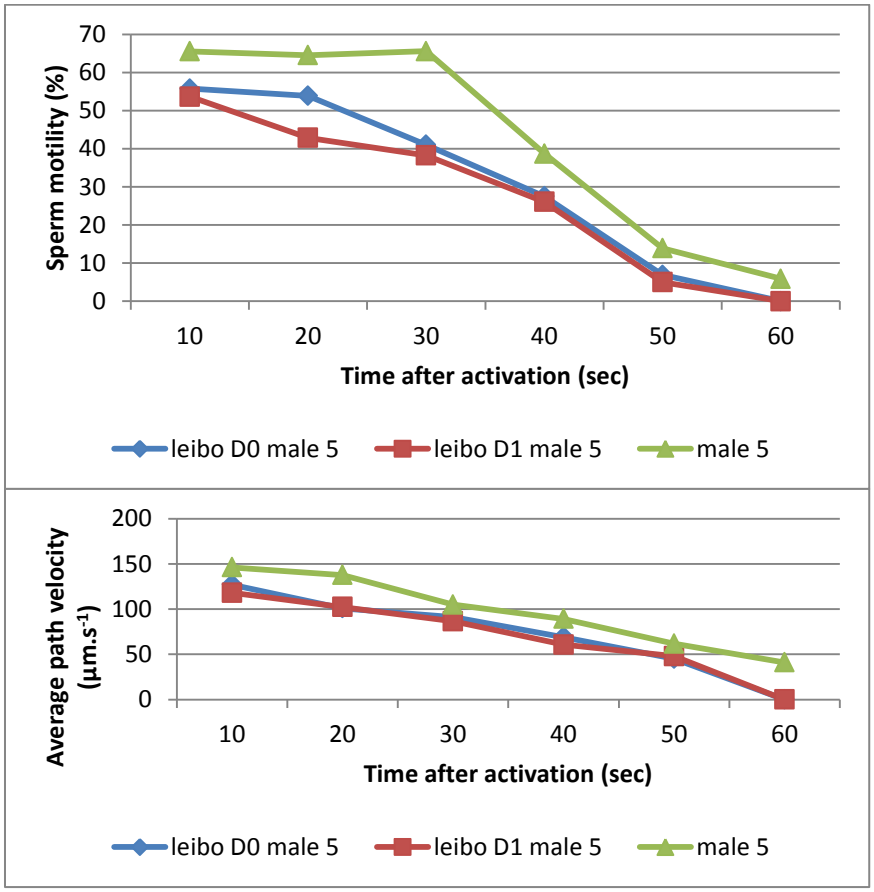

Figure 2

Sperm collected either during spawning season or at its end does not keep its ability to swim after 24 hrs of storage in raw state at 4°C. As expected the use of diluents can improve the survival of chilled sperm for at least 24hrs. In the current trials, a modified Leibovitz medium to which pyruvate (6mg/ml), glutamine (0.03mg/ml) and BSA 20 mg/ml were added, with 200mOsm and pH 8 allowed to store the sperm chilled for at least 24 hrs while still sustaining its motility. After 24h storage, attempts to sustain the survival of sperm using commercial products developed for salmonids and also used for seabream yielded lower performance of sperm motility. The names of the products Cryo and Store were modified since this work only provides preliminary results that need to be confirmed and strengthened. The differences in motility of sperm stored for 24 hrs in different media, 10 and 20 seconds after activation are shown in Figure 3.

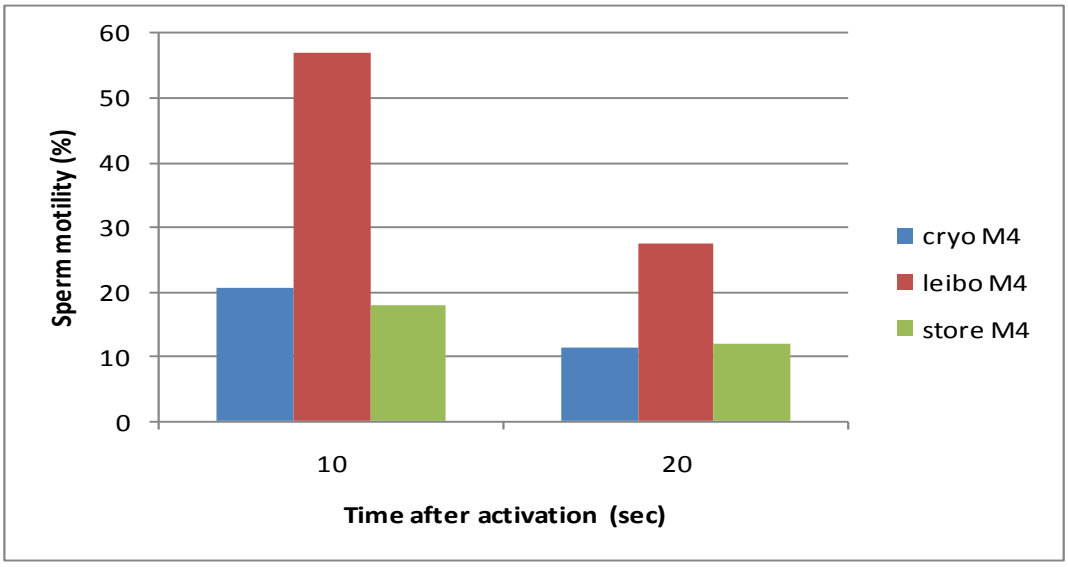

Figure 3

Fertilization ability

A protocol was designed to establish the fertilizing capacity of sperm subjected to Leibovitz and stored for 24h. We are unable to provide details of protocols and results since the work was conducted in a private company.

Six females were stimulated for ovulation and were stripped 36 hours later. Four females out of the 6 provided eggs that were dispatched independently into 7 beakers per female. Each beaker was inseminated by the addition of a known quantity of spermatozoa per egg according to a factorial design using four different; 24h chilled stored sperm and three fresh sperm. All sperm being diluted the same protocol in modified Leibovitz. (Fig 4)

As a result, the company reported that every cross provided progeny with variations linked to females. Sperm ability was not seen different according to their status (fresh/chilled stored)

Figure 4

Conclusion.

Due to the late date of the STSM the first choice of seabass as a model had to be abandoned and we had to turn to meager. The help of the private company “Les poissons du Soleil” was invaluable and we would like to express our sincere gratitude to its personnel who kindly welcomed us in their facilities and enabled us to develop the protocols.

The STSM was very fruitful since the collaboration allowed to exchange know-how and skills and share experience on sperm analysis and management between IOLR/NCM and Ifremer gamete quality researchers. Working paths for sperm count and sperm motility assessment were established with detailed explanations. These protocols will be used as a basis for experiments with mullet sperm.