**Deliverable Report**

| | | | |
|---|---|---|---|
| **Deliverable No:** | D2.6 | **Delivery Month:** | 36 |
| **Deliverable Title** | Description of sperm characteristics and cryopreservation protocol of meagre sperm | | |
| **WP No:** | 2 | **WP Lead beneficiary:** | P3. IRTA |
| **WP Title:** | Reproduction and Genetics – Meagre | | |
| **Task No:** | 2.3 | **Task Lead beneficiary:** | P14. IFREMER |
| **Task Title:** | Description of sperm characteristics and cryopreservation methods | | |
| **Other beneficiaries:** | P3. IRTA | | |
| **Status:** | Delivered | **Expected month:** | 36 |
| ……. | | | |

**Lead Scientist preparing the Deliverable:** Fauvel, C. (IFREMER),

**Other Scientists participating:** Duncan, N. (IRTA), Ramos, S. (IRTA), Gonzalez, W. (IRTA)

## 1. Objective:

The main goal of this deliverable is to provide 1) the motility features of a high quality meagre sperm based on the analyses of artificial fertilization results, 2) the characteristics of a cryopreservation media and improved fertilizing protocols (dilution rate, type of straw and cooling rate), and 3) the concentration of thawed sperm maximizing fertilization success.

## 2. Background:

The meagre *Argyrosomus regius* has been produced in aquaculture since 1997, but the species has remained a niche market product due to different causes of which:

- The small volume of meagre obtained by fisheries (6000t in 2015) and the seasonality of capture of wild fish did not allow a widespread and regular market, and, therefore, consumers did not develop a good knowledge of the product.
- The production of juveniles had been developed by only one European hatchery for more than 10 years since 1997 and the market for this production has increased slowly in relation to the lack of identified market.

However, the fish is a good candidate for further development of European aquaculture, since its reproduction in captivity is possible it grows fast, it presents high flesh quality and it may compete with market settled species such as European seabass *Dicentrarchus labrax* and gilthead seabream *Sparus aurata*, provided that some biological and socioeconomic bottlenecks are solved (Montfort, 2010). In terms of reproduction, the geographically limited origin of broodstocks and the difficulty to enrich the stocks by the introduction of wild fish presents a high risk of losing genetic variability and the possibility of the negative effects of inbreeding. Although the genetic variability of cultured meagre seems to be high (Haffray et al, 2012; Deliverable 2.1), there is a need to enrich the variability by the introduction of breeders from other genetic origins and the management of genetic variability through controlled multi-parental crosses on one hand and the start of genetic breeding programs that enhance the yield of this species in captivity on the

other hand. The development of reliable artificial fertilization will provide a powerful tool for facilitating genetic breeding programs. So far, induced spawning protocols have been developed in the last few years (Duncan et al., 2012; Mylonas et al., 2015) and paired-spawning methods have been established (***D2. Protocol for paired spontaneous tank spawning of meagre***). The next step is the development of reproducible protocols of artificial fertilization, which require a relevant assessment of quality and the adaptation of protocols for the management of gametes already existing for other species.

In captivity, fish reproduction can be impaired by uncontrolled external or internal factors modifying the neuro endocrine control of reproductive process at different levels. These endocrine disruptions have been successfully overcome by the application of homologous or heterologous hormones (Zohar and Mylonas, 2001, Mylonas et al., 2016a). In the case of spermiation, chronic stress from repeated sampling can induce decreased sperm availability and in extreme cases the complete lack of semen in species such as European seabass (Fauvel et al., 1999). In the case of meagre, a treatment by analogs of LHRH induced males to recover sperm production after a short time laps (Mylonas et al., 2016b), but it remains interesting to evaluate the quality of this newly produced sperm through the standardized protocols developed in the Diversify project in Deliverables D3.4 and D7.1.

The quality of sperm can be objectively described through different features accessible by field observations such as concentration, motility and fertility which were extensively described for different species in the literature and particularly in recent large reviews of applications to aquaculture (Cabrita et al. 2009, Bobe and Labbé, 2010, Fauvel et al., 1999, Fauvel et al. 2010). Fish spermatozoa are immobile in the genital tract and are activated to start swimming by the variation in osmotic pressure caused by contact with external medium at the moment of ejaculation (Cosson et al., 2008). If the dilution of sperm is adequate, this activation is global to activate all spermatozoa (Billard and Cosson, 1992) so that the maximal motility (% of motile spermatozoa) is usually observed in the first seconds. Due to a trade-off between weak respiration and the high energy cost of movement, the motility and the velocity of sperm rapidly decreases (Christen et al., 1987). The evaluation of concentration, initial motility, the velocity of sperm and the duration of progressive movement are interesting indexes of sperm quality, which must be confirmed by the fertility or ability of sperm to fertilize (Kime, 2001, Rurangwa et al., 2004)

The analysis of sperm just at collection reveals the intrinsic quality of gamete as a consequence of paternal physiological input (spermatogenesis, spermiogenesis and spermiation) and also of proper sperm physiology such as ageing process, which progressively affects sperm along the reproductive season (Dreanno et al., 1999). After collection the quality of sperm decreases in a species-specific way. For example, undiluted rainbow trout sperm presented high motility after 34 days (Stoss and Holt, 1983) while high variability was reported after only some hours in seabass (Fauvel et al., 2012). In artificial fertilization, the collection of male and female gametes may not be concomitant. In that case, according to species, sperm must be conditioned for chilled or cryogenic storage. It was interesting to test the capacity of meagre sperm to tolerate chilled conservation in adapted media and to establish the effects of cryopreservation using an optimized medium. Since a modified cell culture medium Leibovitz L15 efficiently protected sperm of seabass *Dicentrarchus labrax* (Fauvel et al., 2012), wreckfish *Polyprion americanus* (Deliverable D7.1) and greater amberjack *Seriola dumerili* (Deliverables D3.4), it was proposed to study its protective effect on meagre sperm.

## 3. Description:

In order to establish the most interesting features of meagre sperm quality and to test sperm storage media, preliminary studies were run in laboratory conditions in Ifremer facilities in 2014 using meagre sperm from the private company Les Poissons Du Soleil (LPDS, Balaruc, France), which is not a Diversify partner, but which actively and regularly collaborates with Ifremer (Partner 14). In a second step, the scientific team of IRTA went to Palavas where a short training of 2 days (from 14$^{th}$ to 16$^{th}$ of March 2016) was organized in order to practice on seabass sperm and prepare further meagre experiments on the IRTA site when reproductive season occurred (May-July). Finally, the results of these two preliminary steps were used to implement relevant experiments in IRTA facilities (with a participation on site of IFREMER (P14) team (6-

10th of June 2016) aiming at linking sperm quality parameters (concentration and motility) to its fertilization potential after setup and development of artificial fertilization protocols (see deliverable D2.7).

*Sperm collection and quality*

For sperm collection from males that were in full spermiation, the genital pore was carefully cleaned and dried and a gentle pressure was applied to the testes in order to obtain sperm. Urine was avoided to prevent sample contamination. Sperm was directly collected in 2-mL syringes immediately before the first stripping of females. Sperm samples were maintained on ice. Milt was diluted 1:4 (v:v) in Leibovitz cell culture medium modified; glutamine (0.3 mg/mL diluted Leibovitz), sodium pyruvate (6 mg/mL) and NaOH were added to the initially diluted medium of Leibovitz (350 mOSm and pH 7.3) to obtain a Leibovitz medium with pH 8 and 450 mOsm. In order to prevent sperm initial motility, the osmolarity was decreased to 250 mOSm by dilution in distilled water. Gentamycin sulphate (1 µL/mL) was added also to prevent any bacterial development and bovine serum albumin (BSA) (0.066 mL 30% BSA/mL dilution), to protect the plasma membrane and avoid sperm aggregation. For sperm sampling, positive displacement pipettes were used.

To assess sperm quality, aliquots of 10, 20 and 40 µL of diluted sperm (1:4), were mixed thoroughly with 1 mL of sea water with BSA (6.6 mL BSA for each 100 mL of sea water) in Eppendorf tubes for activation so that the observation dilution were 1:404, 1:204 and 1:104 depending on the density of initial dilution of sperm (1:4). A 1 µL sample of this dilution containing the activated sperm was immediately pipetted into ISAS (Integrated Sperm Analysis System) counting chamber. The progress or tracks of the activated sperm in the ISAS chamber were recorded as a digital video through a bright field equipped video microscope at 20x magnification (Leica DMLB Microscope and GigE Camera). The video recording was initiated at the moment the sperm was activated so as to provide a reliable reference start point and terminated when sperm ceased activity. The process of activation and filling the chamber allowed secured assessment of sperm activity from 15-25 s after activation. The videos were processed using Virtualdub free software, which transforms AVI movies into image sequences. Then the sequences were analysed with the Computer Assisted Sperm Analysis (CASA) plugin developed using open source software ImageJ (Wilson Leedy and Ingermann 2007). The parameters assessed using CASA were sperm motility (%), defined as the percentage of motile cells; average path velocity (VAP, µm/s), defined as the distance run by sperm head along its spatial average trajectory in a definite timelap; and duration of sperm motility (motility duration, min), defined as the period of time between activation and cessation of any cell displacement, sperm simply vibrating or turning on their axes were not considered. Motility parameters were evaluated each time sperm were used in fertilisation procedures to assess the changes in sperm quality and duplicate or triplicate analyses were made. The settings for the image analysis, which depend on the microscope and the movie quality, were as follows in IRTA facilities: brightness and contrast, -8 to 19/216 to 253; threshold, 0/231 to 254; minimum sperm size (pixels), 30; maximum sperm size (pixels), 200; then the CASA settings which depend both on camera characteristics and sperm features were the following: minimum track length (frames), 5; maximum sperm velocity between frames (pixels), 40; frame rate, 30; microns/1000 pixels, 301; and the rest of parameters set as default. Sperm concentration (number of spermatozoa/mL of milt) was also recorded for each male. In this case, previously diluted sperm (1:4) was diluted 1:125 in distilled water to obtain a 1:500 dilution from which 10 µL were pipetted into a THOMA cell counting chamber where it was allowed to sediment for 10 minutes, then observed under the microscope with 10x magnification. Quantification of sperm/mL milt was assessed using the particle analysis function of ImageJ adjusting image brightness to a range from 43 to 49, image contrast, 203 to 221, and image threshold from 225 to 238.

*Sperm storage*

For chilled storage, sperm was diluted immediately after collection as described above in modified Leibovitz medium and samples of 6 ml of diluted semen (1:4) were stored at 4°C for 6 to 24h in 15 ml test tubes (Falcon).

For cryoconservation, 10% dimethyl sulfoxide (DMSO) was added to diluted sperm (1:4 Leibovitz) and 200 µl aliquots were pipetted into 500µl straws (IMV, France) that were immediately placed on a Styrofoam floating rack that maintained the straws 6 cm above the liquid nitrogen (LN). The straws were kept for around 15 minutes above the LN before being plunged into LN. Finally, they were stored until use in large LN containers. The conditions of use of cryopreserved sperm were the following: straws were allowed to thaw for 15 seconds in 40°C water bath and immediately mixed with the ova. One straw of sperm (200 µl) was used for motility analysis after thawing following the same procedure used for the fertilization trials.

*Fertility assessment*

In order to assess the sperm ability to fertilize, sperm was deposited on batches of eggs at a ratio of 2 $10^5$ spz per egg and was activated following the procedure described in Deliverable 2.7. The fertilization rate was assessed under microscope after three hours of development when the embryos had reached or passed the four cell stage.

**Results**

*Quality assessment method setup Balaruc-Palavas, France (2014)*

The experiments were performed on 2 batches of 4 meagre males coming from either a broodstock progressing towards the end of its reproductive season or one at full spawning season but only some samples were analysed.

As preliminary results, sperm counting on the 4 males of each group, by image analysis using Image J software, found that at the end of the reproductive season, sperm was more concentrated by 25% (4.5 $10^{10}$ vs 3 $10^{10}$ spz/ml) while the viscosity (subjective evaluation) was higher than during the peak of the spawning period. The different steps of particle counting analysis using the free software Image J were the selection of particles, adjusting threshold levels and finally the individual identification of the particles (**Fig. 1**).

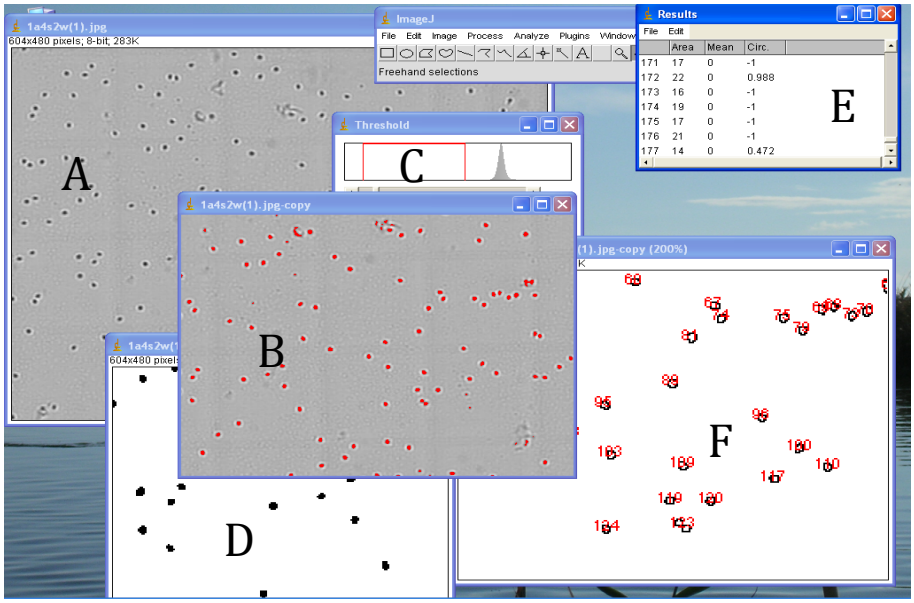



**Figure 1.** Summary of particle count with imageJ showing the original picture (A), the selection of particles (B) by thresholding on grey levels (C), the selected particles (D), The edition of results (E) and finally the outlines allowing the individual identification of the particles (F).

For motility assessment, different trials demonstrated that a concentration of 15mg $ml^{-1}$ Bovine serum albumin per ml of seawater was required to improve the capacity of sperm to swim in the observation cell (Leja Cell, 10 µm depth, IMV, France) compared to 7.5 mg $ml^{-1}$. In these optimized conditions, it was possible to record motility of sperm subjected to different situations and conditions of storage.

In these current conditions meagre fresh sperm was characterized by an initial motility (10 s after activation) of around 65% of motile spermatozoa and an initial velocity (Average Path Velocity) of about 140µm $s^{-1}$. The velocity decreases regularly until all movement ceases in a time lap of 1 minute (**Fig. 2**). These motility values being in the range of that of other marine fish sperm, the method was transferred to Partner 3 (IRTA, Spain) for field experiments.

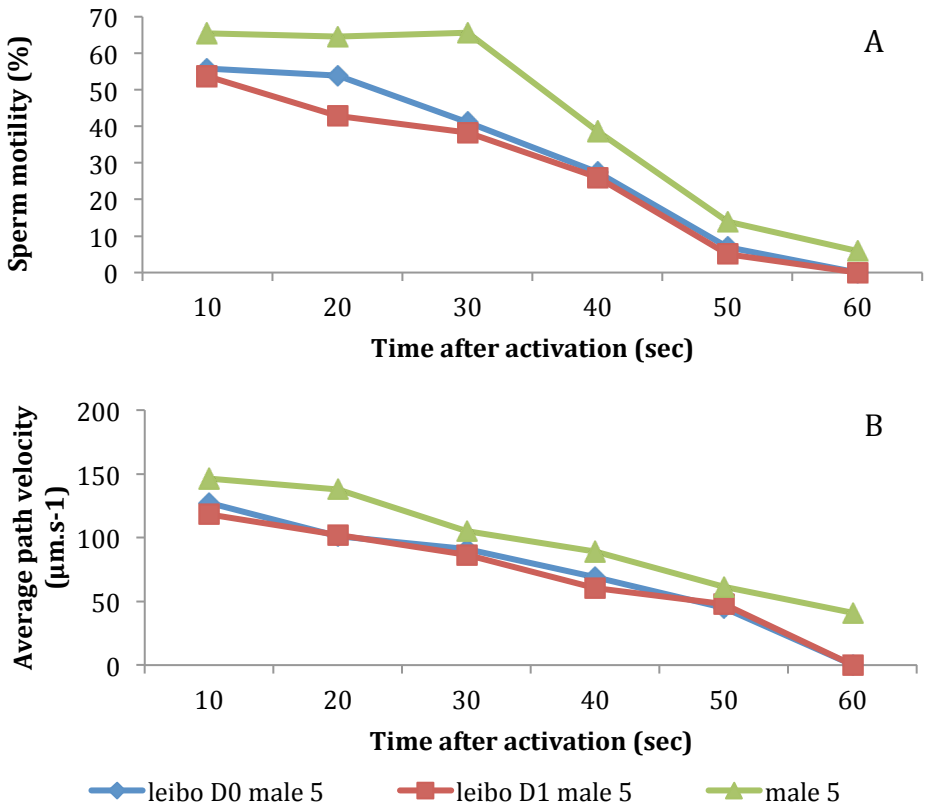



**Figure 2.** Mean sperm motility of a single male expressed as the percentage of motile spermatozoa at different times after activation (A) and mean average path velocities at the same intervals of time (B) showing the usual behavior of sperm i.e. high initial movement with high speed followed by a rapid decrease of both parameters driving to the cessation of any progressive movement by one minute.

*Maturity status of meagre males and sperm characterization in IRTA, San Carles de la Rapita, Spain (2016).*

Male genital state was regularly examined by the release of sperm upon gentle abdominal pressure and spermiation stage was determined on a scale from 0 to 3 (0 = not fluent, 1 = fluent but no sample can be obtained, 2 = fluent, 3 = very fluent). The available males used for sperm quality assessment and fertilization trials rapidly had low scores of spermiation at the beginning of season. In order to get sufficient volumes of sperm for both storage and artificial fertilization experiments, the males were fished 36 to 24h before sperm collection and a small sample was taken for initial quality assessment then they were immediately subjected

to a single intramuscular injection of 15 µL/kg sof GnRHa (des-Gly10, [D-Ala6]-gonadotropin releasing hormone, Sigma, Spain) in order to stimulate spermiation. The males were taken again and stripped 1-2 days later for efficient sperm collection.

The mean sperm density from the males used during the experimental period was $3.21 \cdot 10^{10} \pm 1.18$ spzoa/mL, the mean motility duration was 1.71± 0.29 min, the mean percentage of initial motility of spermatozoa was 48.17 ± 2.80 % and the mean initial VAP was 90.69 ± 5.76 µm/ s. Percentage of motility was maintained without variations until 55 s after activation and after which a quick decrease was observed with time until the movement ceased. In contrast, VAP values quickly decreased with time, so that initial values only remained without variation until 35 s after activation (**Fig. 3**). The shorter motility duration observed in in France during the setup of the protocol may be linked to the late collection in the season due to sperm ageing on one hand and to the delay/transportation between sperm collection and sperm analysis which took at least 3 hours.

The results demonstrated that some of the measured parameters changed due to the hormonal induction of males (duration of initial motility and VAP along time) and others did not (density, duration, initial motility and initial VAP). No significant differences were observed between sperm density of sperm obtained before GnRHa injection and after it and neither in duration, initial motility or in initial VAP (**Table 1**). However, higher values in initial motility were obtained after GnRHa injection. The percentage of motility was decreased gradually after activation, exhibiting ~50% of motility until 55 s after activation in samples obtained before GnRHa injection. However, in samples obtained after GnRHa injection, ~50% of motility was still exhibited after 75 s from activation. After 75 s, a drastic decrease in motility was observed. The VAP exhibited the same pattern; initial values lasted longer after GnRHa injection (45 s) than before (35 s) (**Fig. 3**).

The parameters (motility, VAP and duration of motility) of sperm did not significantly vary during a period of 7 hours of storage in Leibovitz culture medium in 4 of the 5 weeks tested (**Table 2**).

During the experimental period (May-June), no significant variation was observed between weeks in sperm concentration, but a weak increase was found around the sixth week and a decrease after it in sperm before GnRHa injection (**Fig. 4**). The number of males with a spermiation stage of 1 (fluent but no sample can be obtained) also increased in the latest weeks. Some differences were observed in the mean sperm initial motility (15 -25 s) of samples obtained both before and after hormonal treatment over the 9-week period. While before GnRHa treatment, initial motility decreased during the period, whilst initial motility was constant after GnRHa treatment. No significant differences were found within each week neither in VAP nor in sperm duration, with an exception that in week 8, VAP values significantly increased and duration decreased (**Table 4**).

**Table 1.** Sperm quality parameters of sperm samples obtained before and after GnRHa injection. Data from 5 males during the whole experimental period were used when data before and after were available. Values are expressed as mean ± standard deviation. Different letters mean significant differences between before and after hormonal treatment.

| Before/ after GnRHa injection | Sperm concentration (spermatozoa/ mL) | Sperm duration (min) | Initial motility (%) | Initial VAP (µm/s) |
|---|---|---|---|---|
| Before | $3.21 \cdot 10^{10} \pm 1.18^{a}$ | $1.71 \pm 0.29^{a}$ | $48.17 \pm 2.80^{a}$ | $90.69 \pm 5.76^{a}$ |
| After | $2.76 \cdot 10^{10} \pm 0.62^{a}$ | $1.57 \pm 0.50^{a}$ | $66.76 \pm 15.83^{a}$ | $98.07 \pm 11.68^{a}$ |

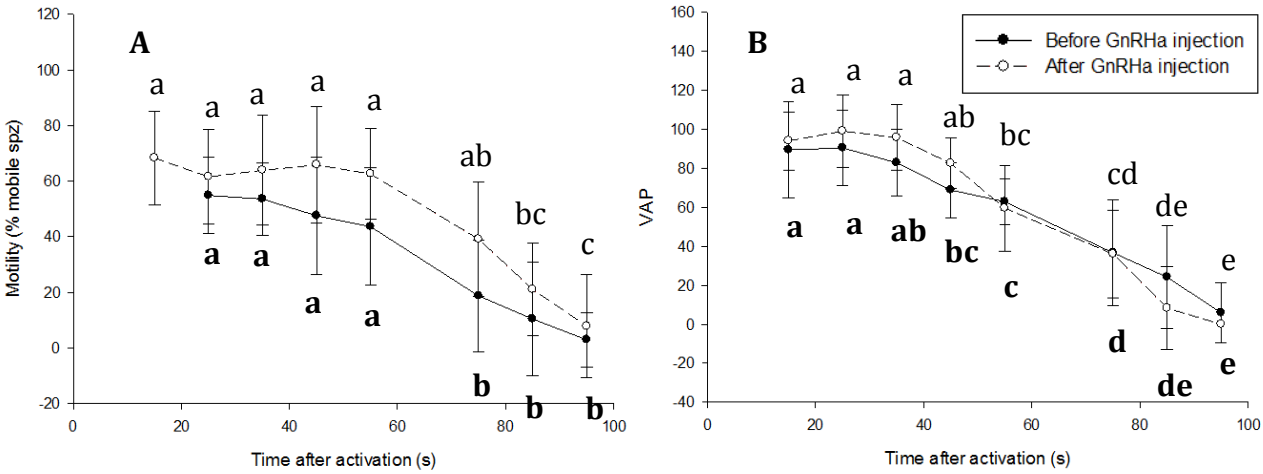



**Figure 3.** Effect of GnRHa treatment on (A) percentage of mobile spermatozoa (%) and (B) Average Path Velocity of spermatozoa (µm/ s) during the experimental period when data before and after treatment were available. Data are expressed as mean ± standard deviation. Different lowercase bold letters mean significant differences with time after activation before GnRHa injection. Different lowercase letters mean significant differences with time after activation after GnRHa injection. Data was from five different males with three repeated measures on different weeks for each male (n=15).

**Table 2.** Weekly sperm quality of sperm samples used to determine timing of stripping in the setup of artifical fertilization protocol. Data from 5 males were used and values are expressed as mean ± standard deviation. Different letters mean significant differences between different analysis times in the same week.

| Week | Analysis time (h) | Total duration(min) | Initial motility (%) | Initial VAP (µm/s) |
|---|---|---|---|---|
| 2 (11/May/16) | 10:30 | 1.51 ± 0.27[a] | 66.55 ± 4.38[a] | 116 ± 5.61[a] |
| | 13:30 | 1.40 ± 0.03[a] | 62.57 ± 10.46[a] | 100 ± 8.47[a] |
| | 17:30 | 1.39 ± 0.32[a] | 88.00 | 106.63 |
| 3 (19/May/16) | 10:30 | 1.49 ± 0.30[a] | 74.36 | 126 |
| | 13:30 | 1.73 ± 0.37[a] | 80.82 ± 1.88[a] | 94 ± 3.71[a] |
| | 17:30 | 1.64 ± 0.49[a] | 68.91 ± 10.29[a] | 94.49 ± 13.59[a] |
| 4 (25/May/16) | 10:30 | 1.81 ± 0.17[a] | 90.63 | 101.15 |
| | 13:30 | 1.55 ± 0.62[a] | 28.94 ± 6.32[a] | 86 ± 1.43[a] |
| | 17:30 | 1.34 ± 0.34[a] | 47.00 ± 23.53[a] | 92.75 ± 5.15[a] |
| 5 (08/June/16) | 10:30 | No data | | |
| | 13:30 | 1.84 ± 0.15[a] | 66.62 ± 3.95 | 79.08 ± 4.25 |
| | 17:30 | 1.25 ± 0.13[b] | 62.50 | 106.49 |
| 7 (15/June/16) | 10:30 | 1.70 ± 0.23 | 48.80 ± 8.08[a] | 84 ± 5.38[a] |
| | 13:30 | No data | | |
| | 17:30 | 0.48 | 6.31 ± 0.84[b] | 59.5 ± 4.94[b] |

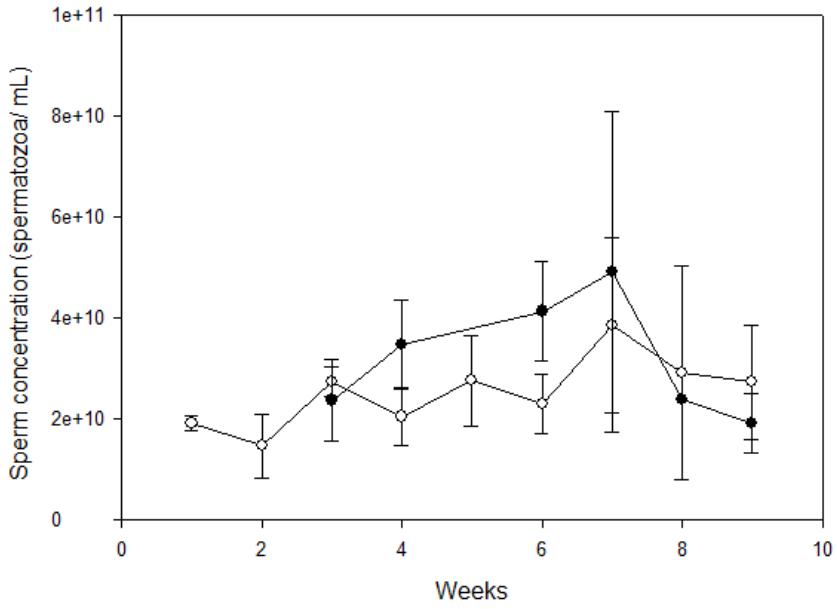



**Figure 4.** Variations of sperm concentration during the experimental weeks, week number corresponds to the dates expressed in **Table 2**.

**Table 3.** Weekly sperm quality parameters of sperm samples used in the experimental period. Data from 2-5 males, depending on week, were used and values are expressed as mean ± standard deviation. Different letters mean significant differences between weeks. N.D. = no data available.

| | After GnRHa injection | | | | | | Before GnRHa injection | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Week | VAP at different post-activation times (μm/s) | | Motility at different post-activation times (%) | | Duration (min) | concentration (10^10spz/ mL) | VAP at different post-activation times (μm/s) | | Motility at different post-activation times (%) | | Duration (min) | concentration (10^10spz/mL) |
| | 25 s | 15 s | 25 s | 15 s | | | 25 s | 15 s | 25 s | 15 s | | |
| 4-5May | N. D. | N. D. | N. D. | N. D. | N. D. | 1.92 ± 0.48$^{a}$ | N. D. | N. D. | N. D. | N. D. | N. D. | N. D. |
| 10 - 11 May | 109.96 ± 8.74$^{a}$ | 115.83 ± 5.61$^{a}$ | 70.72 ± 9.41$^{ab}$ | 66.54 ± 4.37$^{ab}$ | 1.51 ± 0.26$^{a}$ | 1.47 ± 0.62$^{a}$ | N. D. | N. D. | N. D. | N. D. | N. D. | N. D. |
| 18 - 19/May | 126.41 ± 1.36$^{a}$ | | 61.03 ± 4.72$^{ab}$ | 74.35 ± 0.00$^{ab}$ | 1.49 ± 0.30$^{a}$ | 2.75 ± 0.29$^{a}$ | 91.21 ± 6.03$^{a}$ | N. D. | 52.55 ± 3.12$^{a}$ | N. D. | 1.90 ± 0.41$^{a}$ | 2.37 ± 0.23$^{a}$ |
| 24 - 25 May | 104.47 ± 7.46$^{a}$ | 101.15 ± 0.0$^{a}$ | 74.77 ± 6.71$^{ab}$ | 90.62 ± 0.00$^{b}$ | 1.81 ± 0.17$^{a}$ | 2.05 ± 0.57$^{a}$ | 100.30 ± 6.04$^{a}$ | N. D. | 73.07 ± 5.43$^{b}$ | N. D. | 1.86 ± 0.49$^{a}$ | 3.48 ± 0.88$^{a}$ |
| 31 - 01 June | 81.52 ± 11.93$^{a}$ | 79.08 ± 4.25$^{a}$ | 65.60 ± 9.27$^{ab}$ | 66.61 ± 3.94$^{ab}$ | 1.84 ± 0.14$^{a}$ | 2.76 ± 0.89$^{a}$ | N. D. | N. D. | N. D. | N. D. | N. D. | N. D. |
| 07 - 08 June | 106.13 ± 15.13$^{a}$ | 109.37 ± 15.00$^{a}$ | 80.42 ± 40.43$^{b}$ | 80.50 ± 13.05$^{b}$ | 1.82 ± 0.29$^{a}$ | 2.3 ± 0.59$^{a}$ | 85.96 ± 12.52$^{a}$ | 81.29 ± 12.72$^{a}$ | 62.29 ± 7.09$^{a}$ | 60.89 ± 12.68$^{a}$ | 1.63 ± 0.6$^{a}$ | 4.14 ± 0.97$^{a}$ |
| 14 - 15June | 78.09 ± 7.41$^{a}$ | 83.65 ± 5.38$^{a}$ | 42.16 ± 21.95$^{a}$ | 48.80 ± 8.07$^{a}$ | 1.72 ± 0.24$^{a}$ | 3.85 ± 1.72$^{a}$ | 67.27 ± 9.21$^{a}$ | 72.28 ± 11.18$^{a}$ | 40.54 ± 1.84$^{c}$ | 42.65 ± 3.77$^{a}$ | 1.77 ± 0.32$^{a}$ | 4.92 ± 3.17$^{a}$ |
| 21 - 22/June | 108.93 ± 54.05$^{a}$ | 206.12 ± 7.77$^{b}$ | 40.53 ± 17.65$^{a}$ | 70.28 ± 7.30$^{ab}$ | 0.60 ± 0.09$^{a}$ | 2.92 ± 2$^{a}$ | N. D. | N. D. | N. D. | N. D. | 1.95 ± 0.20$^{a}$ | 2.40 |
| 28 - 29 June | 87.48 ± 3.55$^{a}$ | 108.13 ± 37.04$^{a}$ | 73.49 ± 5.55$^{ab}$ | 68.78 ± 5.50$^{ab}$ | 1.96 ± 0.63$^{a}$ | 2.73 ± 1.13$^{a}$ | 112.90 ± 20.27$^{a}$ | 116.04 ± 29.23$^{a}$ | 50.32 ± 2.30$^{d}$ | 43.89 ± 8.50$^{a}$ | 1.19 ± 0.40$^{a}$ | 1.91 ± 0.58$^{a}$ |

*Effect of storage and cryopreservation on sperm quality parameters*

The very simple system used to freeze sperm consisted in placing the straws at a distance 6 cm from the LN surface, which is a height where the vapors cool the sperm according to a relevant slope. The use of a 6cm high styrofoam device allowed obtaining an adapted cooling rate until -100°C after 10 min. After 10 min, the temperature was decreased down to -196°C by immersing the straws in LN (**Fig. 5**). The quality characteristics of sperm subjected to different conditions of storage are shown in **Table 4**. As regards analysis of sperm, there were no significant differences between the duration of motility nor initial VAP values from sperm subject to different kinds of storage, but initial motility of sperm stored in Leibovitz for 24 h was significantly the lowest.

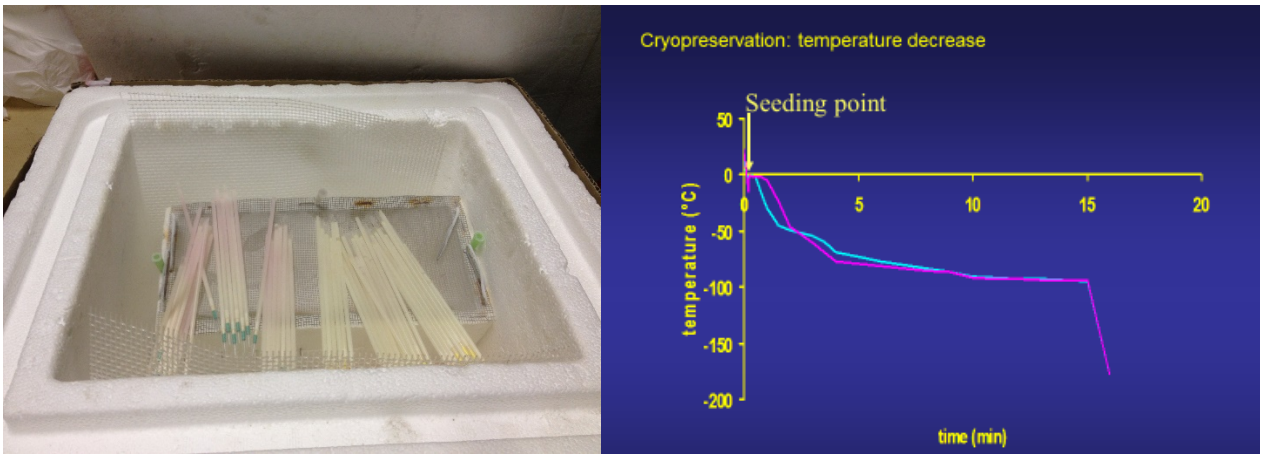



**Figure 5.** Cryopreservation device with straws on the floating device in liquid nitrogen vapors (left) and the temperature drop slopes measured by a thermocouple introduced in a filled straw i.e. in contact with sperm (right). An increase of temperature can be noticed at the time of crystallization of liquid phase, which is an exothermic reaction. This increase is called seeding point. The green curve corresponds to straw freezing and the purple one was obtained by placing 1.8 ml cryotube (Nalgene) at 2 cm over LN.

**Table 4** Weekly sperm quality of sperm samples used in experiment 3 (comparison of fertilisation by fresh, cryopreserved sperm and sperm stored in Leibovitz for 24h). Data from 5 males was used and values are expressed as mean ± SD. Different lowercase letters mean significant differences between each parameter from different storage types in the same week. Different capital letters mean significant differences between each parameter from fresh sperm used in fertilisation among three weeks (read in columns).

| Experiment | Sperm storage | Total duration (s) | Initial motility (%) | Initial VAP (µm/s) |
|---|---|---|---|---|
| 3a | Fresh for storage | 1.66 ± 0.59$^{a}$ | 55.16 ± 13.81$^{a}$ | 109.37 ± 15.00$^{a}$ |
| | Cryopreserved | 1.29 ± 0.32$^{a}$ | 50.24 ± 15.90$^{a}$ | 93.73 ± 15.59$^{ab}$ |
| | Chilled Stored for 24 h | 2.07 ± 0.51$^{a}$ | 8.91 ± 6.79$^{b}$ | 68.00 ± 3.73$^{b}$ |
| | Fresh for fertilisation | **1.82 ± 0.29$^{a\ A}$** | **80.51 ± 13.05$^{c\ B}$** | **81.29 ± 12.72$^{b\ A}$** |
| 3b | Fresh for storage | 1.77 ± 0.04$^{a}$ | 42.66 ± 3.78$^{a}$ | 72.28 ± 11.18$^{ab}$ |
| | Cryopreserved | 1.07 ± 0.31$^{b}$ | 24.16 ± 18.32$^{ab}$ | 50.26 ± 8.09$^{a}$ |
| | Chilled Stored for 24 h | 0.61 ± 0.04$^{c}$ | 2.60 ± 1.00$^{b}$ | 55.72 ± 5.1$^{a}$ |
| | Fresh for fertilisation | **1.34 ± 0.08$^{b\ B}$** | **48.80 ± 8.08$^{a\ A}$** | **83.65 ± 5.38$^{b\ A}$** |
| 3c | Fresh for fertilisation | **0.60 ± 0.09 C** | **70.29 ± 7.30$^{AB}$** | **260.15 ± 7.77 B** |

*Effect of storage and cryopreservation on sperm fertility*

Out of 4 experiments of artificial fertilization using both fresh and frozen samples of sperm, only one showed significant drop of fertility due to cryopreservation while in any case, chilled conservation did not allow to maintain high fertility until 24h. The results are not detailled in this deliverable since they are an important part of the development of artificial fertilization, which is the subject of deliverable D2.7.

**Discussion**

The values of meagre sperm concentration recorded in the present study are similar to others published for meagre (1.89 – 3.15 $\cdot 10^{10}$ spzoa/mL) (Mylonas et al., 2013) and to other marine fish such as the Atlantic croaker (2.34 – 3.64 $\cdot 10^{10}$ spzoa/mL) and the Atlantic halibut (1.19 – 3.72 $\cdot 10^{10}$ spzoa/mL) (Suquet et al., 1994). Similar values of sperm motility duration were also reported for meagre (0.78 – 1.27 min) (Mylonas et al., 2013) (0.56 – 1.33 min) (Schiavone et al., 2012). However, in other meagre studies, motility duration was longer (4.3 – 5.28 min) (Duncan et al., 2012) (6.59 – 7.18 min) (Fernández-Palacios et al., 2009). These differences could be due to the final dilution in which sperm is activated. In this study, for sperm assessment, a 2 step dilution was used first the sperm was pre-diluted at 1:4 in non-activating medium and a final ~1:100 dilution rate was used for activation and assessment. Schiavone et al. (2012) also utilized 1:1000 as a final dilution rate. Those pre-dilution steps probably offered better assessment of motility than in the other studies with lower dilutions, by preventing that undiluted sperm packs actuated as a store and released constantly newly activated cells (Fauvel et al.,1999). The percentage of initial motility was also similar to others reported for meagre (53 - 74%) (Schiavone et al., 2012) (44 - 80%) (Mylonas et al., 2013). Sperm did not show a higher slope of motility decay and the percentage of motile spermatozoa followed a gradual decline and exhibited ~50% of motility until 55 s after activation. On the other hand, the initial VAP measured for meagre spermatozoa in the present study was not the same as those measured in other studies of meagre sperm (17 – 24 µm/ s) (Schiavone et al., 2012). However, the present studies values were similar to other marine fish, such as the seabass (150 µm/ s) (Fauvel et al., 2012) and the European eel (118 µm/ s) (Pérez et al., 2016). These values are related to fast sperm (>100 µm/ s) (Gallego et al., 2013), but initial VAP had a quick decrease so that the initial VAP values remained only 35 s after activation. Sperm motility is an important aspect in fish breeding and has been directly related to fertilisation rates but spermatozoa velocities may also serve as a predictor of fertilisation ability. In fact, in some studies the highest coefficients of correlation were found for VAP (Gallego et al., 2013). Thus, if VAP is highly correlated with fertilising ability, the period of sperm fertility could be reduced to 35 s. The characteristics of the meagre sperm highlighted the need for an *in vitro* fertilisation protocol to mix and activate sperm at the moment of contact between eggs and sperm, as activated sperm will quickly lose motility and VAP resulting in low fertilisation.

This study showed that artificial induction of spermiation could affect the responsiveness of male fish and made a variation in quality parameters (Rurangwa et al., 2004) as observed in the duration of initial values of motility and VAP. Those values were longer after GnRHa injection than before injection, showing that hormonal treatment induced higher initial motilities and probably the fertilisation success. Therefore, the application of GnRHa was a reliable method that should be recommended to induce males to extend sperm motility and velocity and facilitate sperm collection, especially towards the end of the spawning season.

During the experimental period a gradual increase in sperm density from the beginning of the experimental weeks and a decline afterwards was observed, although no significant differences were found. However, the number of males from which no sample could be obtained, increased in the latest weeks. The spermiation period of meagre starts in March and finishes in July (Mylonas et al., 2013), so the total number of sperm more or less followed the expected trend of semen concentration. Thus, semen density was not reduced excessively and, if desired, males could be stripped weekly provided a regular hormonal support was applied.

The last factor that could facilitate the broodstock management for the creation of gene banks in breeding programs is the ability to conserve sperm in large volumes, without losing quality and fertility. The fertilisation ability of fish sperm can be maintained for different intervals of time after collection if the

conditions of conservation are adequate. For long-term preservation, cryopreservation is the best solution. Cryopreservation causes cell dehydration and concentrates the cytosol to reduce ice crystallisation during the contact with liquid nitrogen. The most cell damage occurs during pre-freezing and post-thawing and could be due to a sum of factors, such a decrease in ATP production, mitochondrial membrane depolarisation, osmotic stress and intracellular ice formation. So, cryoprotectants are needed to protect the sperm cell from cold and heat shock, but the same cryoprotectant can be lethal to the unfrozen cell. For short term use or before cryopreservation, the sperm can be stored for various freezing durations with the utilisation of an extender. An extender is a medium similar to the seminal fluid composition used to dilute sperm preventing any motility. Thus, the appropriate choice of the extender, the cryoprotectant for cryopreservation as well as the cooling and thawing conditions, are some of the most important aspects to establish storage protocols (Cabrita et al., 2005; Muchlisin, 2005; Fauvel et al., 2012; Gallego, 2012). Up to now, there are no reports on fertility of stored meagre sperm both cryopreserved and diluted or about a development of these protocols. The suitability of extenders and cryoprotectants differs from one fish to another (Gallego, 2012). In the present work, we have used the protocol proposed by Fauvel et al. (2012): the use of an adapted medium of Leibovitz as an extender in a dilution 1:4 (sperm: extender) and 10% DMSO as cryoprotectant.

In the present study, sperm motility parameters of interest to characterize the fertilizing ability such as initial motility, velocity and duration of progressive swimming of fresh and cryopreserved spermatozoa were not significantly different whereas there was a significant decay of initial motility in chilled stored sperm.

As a conclusion, the standardized analysis of sperm quality using the opensource ImageJ CASA provided a complete dataset for meagre sperm characterisation that was used to estimate quality during the reproductive season and to implement protocols for sperm storage either chilled or cryopreserved. It reveals that it was possible to collect good quality sperm during a 2 month period and to store it for artificial fertilization. The modified Leibovitz medium is well adapted to meagre sperm conservation. However, it must be noticed that the use of this medium for chilled sperm storage must be refined in order to avoid uncontrolled result discrepancies. Finally, the current characteristics of sperm are in accordance with the fertilization success obtained from the different experiments developed in the DIVERSIFY deliverable 2.7 (setup of meagre artificial fertilization).

**Acknowledgments**

Some of the text used in this report has been modified from the MSc Thesis of Sandra Ramos Judez, which was completed as a research project within the framework of Diversify and to fulfil the MSc of the Autonomous University of Barcelona.

**Deviations:** There were no deviations from the DOW

**References**

Billard R. and Cosson M.P. 1992. Some Problems Related to the Assessment of Sperm Motility in Freshwater Fish. J. EXP. ZOOL. 261, 122-131.

Bobe J., Labbé C.2010.Egg and sperm quality in fish. Gen Comp Endocrinol. 165(3), 535-48.

Cabrita, E., Robles, V., Cuñado, S., Wallace, J. C., Sarasquete, C., Herráez, M. P. 2005. Evaluation of gilthead sea bream, *Sparus aurata*, sperm quality after cryopreservation in 5 ml macrotubes. Cryobiology, 50, 273-284.

Cabrita E., Robles V., Herraez P. 2009. Sperm quality assessment. Methods in Reproductive Aquaculture, Marine and freshwater species pp 93-147. Cabrita,E, Robles,V. and Herraez P. eds, CRC Press Taylor and Francis Group, Boca Raton, London New York .

Christen R., Gatti J.L., Billard R. 1987.Trout sperm motility: The transient movement of trout sperm is related to changes in the concentration of ATP following the activation of the flagellar movement. Eur J Biochem. 166(3), 667-671.

Cosson J. 2008. The motility apparatus of fish spermatozoa. Fish spermatology. Alpha Science International Ltd Oxford (Alavi S.M.H., Cosson J.,Coward K. and Rafiee G. eds) pp 281-316.

Dreanno C., Suquet M., Fauvel C., Le Coz J.R., Dorange G., Quéméner L. & Billard R. 1999. Effect of aging process on the quality of seabass (*Dicentrarchus labrax*) semen. Journal of Applied Ichthyology 15, 176-180.

Duncan N., Estévez A., Porta J., Carazo I., Norambuena F., Aquilera C., Gairin I., Bucci F., Valles R., Mylonas C. C. 2012. Reproductive development, GnRHa-induced spawning and egg quality of wild meagre (*Argyrosomus regius*) acclimatised to captivity. Fish Physiol Biochem, 38, 1273–1286.

Fauvel C., Savoye O., Dreanno C., Cosson J.and Suquet M. 1999. Characteristics of sperm of captive seabass in relation to its fertilization potential. J Fish Biol. 54, 356-369.

Fauvel, C., Suquet, M., Cosson, J. 2010. Evaluation of fish sperm quality. J. Appl. Ichthyol., 26 (5), 636-643.

Fauvel C., Boryshpolets S., Cosson J., Wilson Leedy J. G., Labbé C., Haffray P., Suquet M. 2012. Improvement of chilled seabass sperm conservation using a cell culture medium. Journal of applied Ichthyology, 28 (6), 961-966.

Fernández-Palacios H., Schuchardt D., Roo J., Hernández C.M., Duncan N. 2009. Eficacia de la inducción hormonal con distintas dosis de GnRHa en corvina (*Argyrosomus regius*). En: D. Beaz, M. Villaroel y S. Cardenas (Eds): Madrid, España. Actas del XII Congreso Nacional de Acuicultura, 556-557.

Fernández-Palacios H., Schuchartd D., Roo J., Izquierdo M., Hernandez-Cruz C., Duncan N. 2014. Dose-dependent effect of a single GnRHa injection on the spawning of meagre (*Argyrosomus regius*) broodstock reared in captivity. Spanish Journal of Agricultural Research, 12 (4), 1038-1048.

Gallego V., Pérez L., Asturiano J.F., Yoshiba M. 2013. Relationship between spermatozoa motility parameters, sperm/egg ratio, and fertilization and hatching rates in pufferfish (*Takifugu niphobles*). Aquaculture, 416-417, 238-243.

Haffray P., Malha R., Ould Taleb Sidi M., Prista N., Hassan M., Castelnaud G., Karahan-Nomm B., Gamsiz K, Sadek S., Bruant J.S., Balma P., Bonhomme F. 2012. Very high genetic fragmentation in a large marine fish, the meagre *Argyrosomus regius* (Sciaenidae, Perciformes): impact of reproductive migration, oceanographic barriers and ecological factors. Aquatic Living Resources 25 (2), 173-183.

Kime D. E., Van Look K. J., McAllister B. G., Huyskens G., Rurangwa E., Ollevier F. 2001. Computer assisted sperm analysis (CASA) as a tool for monitoring sperm quality in fish. Comp. Biochem.physiol. 130, 425-433.

Lahnsteiner F, Berger B, Weismann T., Patzner R.A. Determination of semen quality of the rainbow trout,Oncorhynchus mykiss, by sperm motility, seminal plasma parameters, and spermatozoal metabolism. Aquaculture, 163 (1-2), 163–181.

Monfort M.C., 2010. Present market situation and prospects of meagre (Argyrosomus regius), as an emerging species in Mediterranean aquaculture, Studies and Reviews, General Fisheries Commission for the Mediterranean No. 89, FAO, Roma, pp. 28.

Muchlisin Z. A. 2005. Review: Current status of extenders and cryoprotectants on fish spermatozoa cryopreservation. Biodiversitas, 6, 12-15.

Mylonas C. C., Duncan N. J., Asturiano J. F. 2016a. Hormonal manipulations for the enhancement of sperm production in cultured fish and evaluation of sperm quality. Aquaculture (in press).

Mylonas C. C. Salone S., Biglino,T., de Mello P. H., Fakriadis I., Sigelaki I., Duncan N. J. 2016b. Enhancement of oogenesis/spermatogenesis in meagre *Argyrosomus regius* using a combination of temperature control and GnRHa treatments. Aquaculture, 464, 323-330.

Mylonas, C.C., Fatira, E., Karkut, P., Sigelaki, I., Papadaki, M., Duncan, N., 2015. Reproduction of hatchery-produced meagre *Argyrosomus regius* in captivity III. Comparison between GnRHa implants and injections on spawning kinetics and egg/larval performance parameters. Aquaculture 448, 44-53.

Mylonas C. C., Mitrizakis N., Papadaki M., Sigelaki I. 2013. Reproduction of hatchery-produced meagre *Argyrosomus regius* in captivity I. Description of the annual reproductive cycle. Aquaculture, 414–415, 309–317.

Pérez L., Vílchez M.C., Gallego V., Morini M., Peñaranda D. S., Asturiano J. F. 2016. Role of calcium on the initiation of sperm motility in the European eel. Comparative biochemistry and Physiology, Part A, 191:98-106.

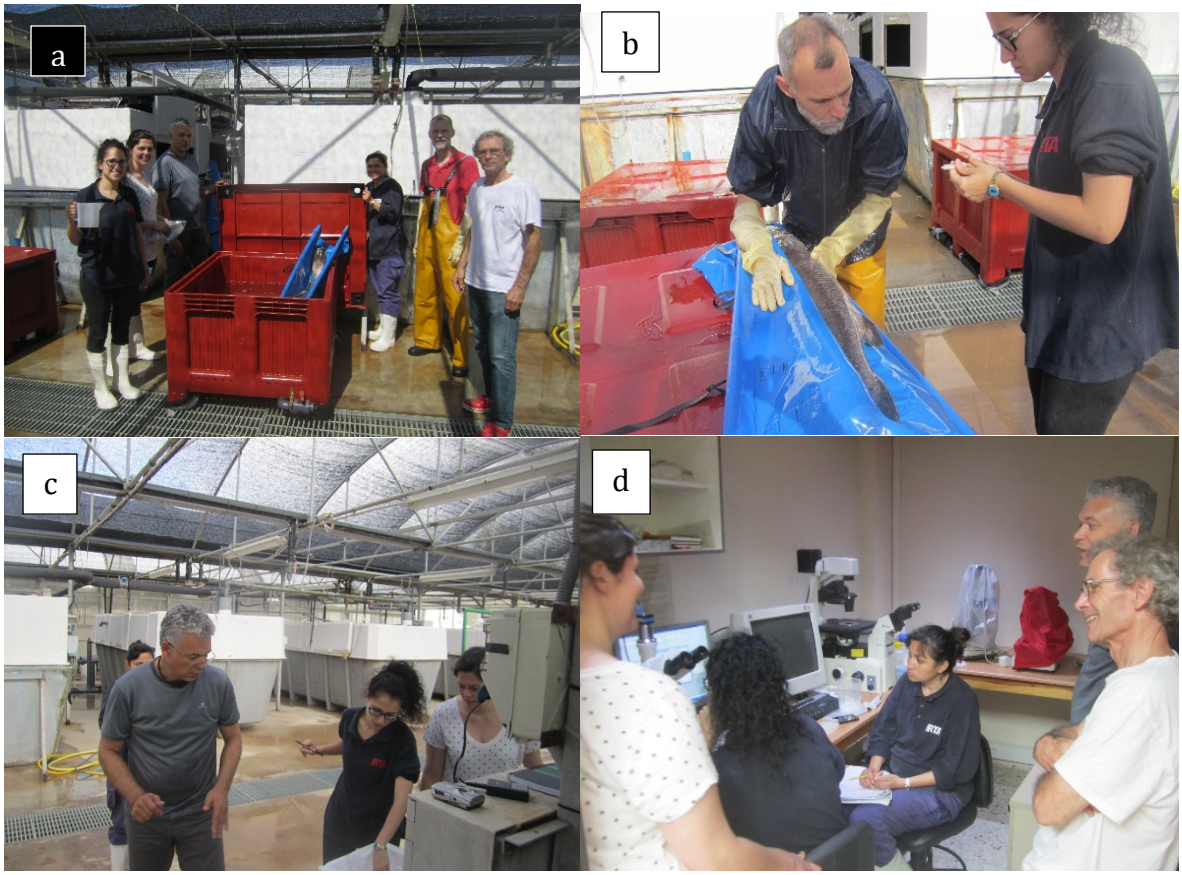
Rurangwa E., Kime D. E., Ollevier F., Nash J. P. 2004. The measurement of sperm motility and factors affecting sperm quality in cultured fish. Aquaculture, 234, 1-28.

Schiavone R., Zilli L., Storelli C., Vilella S. 2012.Changes in hormonal profile, gonads and sperm quality of Argyrosomus regius (Pisces, Scianidae) during the first sexual differentiation and maturation. Theriogenology, 77 (5), 888-898.

Stoss J and Holtz W.1983. Successful storage of chilled rainbow trout (*Salmo gairdnerii*) spermatozoa for up to 34 days. Aquaculture 31, 269.

Suquet M., Billard R., Cosson J., Dorange G., Chauvaud L., Mugnier C., Fauvel C. 1994. Sperm features in turbot (*Scophthalmus maximus*): a comparison with other freshwater and marine fish species. Aquat. Living Resour., 7, 283 – 294.

Wilson-Leedy J.G.and Ingermann R.L. 2007. Development of a novel CASA system based on open source software for characterization of zebrafish sperm motility parameters. Theriogenology. 67 (3), 661-72.




Sequence of sperm analysis experiments in IRTA Spain: Fish anesthesia before stripping (a), Sperm collection (b), sample management (c), sperm analysis (d)

Co-funded by the Seventh Framework Programme of the European Union