Deliverable Report

Deliverable No:	D2.7	**Delivery Month:**	36
Deliverable Title	Protocol for the strip spawning of meagre females and *in vitro* fertilisation		
WP No:	2	**WP Lead beneficiary:**	P3. IRTA
WP Title:	Reproduction and Genetic-meagre		
Task No:	2.4	**Task Lead beneficiary:**	P3. IRTA
Task Title:	Development of in vitro fertilisation methods for planned crosses		
Other beneficiaries:	P14. IFREMER		
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Objective:
A protocol was developed for the strip spawning of meagre females and *in vitro* fertilisation. The deliverable presents the procedures for the extensive handling and manipulation of large meagre breeders, stage of maturity required of breeders, dosage of hormone, timing for hormone application, timing of ovulation, timing of stripping eggs and sperm and methods for *in vitro* fertilisation. In addition, the deliverable includes the results from repeated trails that determine and validate the procedures and include the following data: relative fecundity (number of total eggs obtained per kilo of female), latency period (timing of application of hormones in relation to egg collection), and egg quality parameters.

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Summary

The development of strip spawning with *in vitro* fertilisation methods is necessary for the meagre (*Argyrosomus regius*) aquaculture industry, in order to facilitate planned crosses between selected breeders to aid the implementation of genetic breeding programs. This task used the sperm management protocols developed in ***Deliverable D2.6 Description of sperm characteristics and cryopreservation protocol of meagre sperm*** to obtain high quality sperm, which was stored for a short time (1-7 hours) in modified Leibovitz medium until eggs were obtained from the female meagre. Females with advanced stages of maturity were induced to ovulate with a single 15 µg/kg GnRHa injection. The injections were applied at 20:00-22:00 hours and the females held separate from males in darkness until being checked for ovulation. Checks for ovulation were made every 2.5 hours from 35 to 45 hours post GnRHa injection. When ovulated eggs were obtained, *in vitro* fertilisation was made and egg quality assessed by determining the percentage of developing eggs. An injection of GnRHa was also applied to males, and sperm requirements and quality were assessed. Ratios of sperm to eggs were tested from approximately 3,000 to 500,000 sperm per eggs. Ovulated eggs were observed from 35 hours onwards. Optimal eggs quality was observed at 38-39 hours after the GnRHa injection. From 35 hours to 38-39 hours there was a slight increase in eggs quality and the ease with which eggs could be stripped indicating that from 35-38 hours there was a possibility that eggs were not fully ovulated. After 38-39 hours, there was a decline in eggs quality to 43 – 44 hours. Sperm quality was maintained without decline for up to 7 hours in Leibovitz medium and sperm quality did not appear to affect fertilisation success. The *in vitro* fertilisation was made by rapidly mixing eggs, sperm and sea water at the same time to ensure sperm were activated and in contact with eggs during the first 30 seconds after activation, which was identified as the optimal period for fertilisation in ***Deliverable D2.6***. The optimal ratio of sperm to eggs to obtain high percentage of fertilisation was above 200,000 sperm per egg. The protocol was successfully used in a large factorial cross of 120 *in vitro* fertilisations using either fresh or cryopreserved sperm.

Different phases of artificial fertilisation experiments in P3. IRTA, San Carles de la Rapita (Spain): Top left) sperm collection; Top right) egg collection; Bottom left) gamete mixing and activation, Bottom middle) view of 134 individual fertilised batches of eggs resulting from a factorial cross with 3 females, 4 males and 3 different types of sperm storage (fresh, chilled stored and frozen) in triplicates; Bottom right) devices for the assessment of embryo development after artificial fertilisation.

Introduction

The meagre is a sciaenid fish found in the Mediterranean and Black Sea, and along the eastern Atlantic coast (Haffray et al., 2012). This fish has attractive attributes for the market that include large size, good processing yield, low fat content, excellent taste and firm texture (Monfort, 2010). The species also has the biological characteristics required for commercial aquaculture using well-established gilthead sea bream (*Sparus aurata*) and European sea bass (*Dicentrarchus labrax*) culture technologies (Duncan et al., 2013a). These characteristics include a fast growth of ~1 kg per year (Duncan et al., 2013a), a low feed conversion ratio of 0.9-1.2 (Monfort, 2010; Duncan et al., 2013a), relatively easy larval rearing (Roo et al., 2010; Vallés and Estévez, 2013) and established induced spawning protocols for the production of viable eggs (Duncan et al., 2012, 2013a; Mylonas et al., 2013a; 2013b; 2015; Fernandez-Palacios et al., 2014). Meagre was first produced in 1997 in a commercial hatchery in France and since then it has exhibited annual production increases as high as 7 fold (FAO). In 2010, European meagre aquaculture production was 2,387 t, produced

mainly in Spain, with smaller quantities produced in France, Portugal, Italy, Greece, Cyprus and Croatia (FAO).

A survey of meagre producers that was conducted in preparation of **DIVERSIFY,** identified that one of the major bottlenecks to the expansion of the industry was that the distribution of this fish only in specific areas in the Mediterranean region has resulted in the acquisition of fish for developing broodstocks from a limited number of sources (mainly a hatchery in France), resulting perhaps in a limited genetic variation of the available broodstocks. This was recently demonstrated by **DIVERSIFY** (see ***Deliverable D2.2 Genetic characterization of different meagre captive broodstocks and evaluation of available variability***), which confirmed that the breeders being used in aquaculture have originated from two different wild populations. Although adequate genetic variation exists in the broodstocks being held by aquaculture companies and research centres, the few populations providing breeders (two) and in some cases few families from these populations clearly highlight the needs to establish genetic breeding programs that will avoid the problems associated with inbreeding. The control of reproduction is an essential part to a genetic breeding program (Duncan et al., 2013b).

Breeding in pairs is one way to create families in breeding selection programs. This approach was examined in ***Deliverable D2.3 Protocol for paired spontaneous tank spawning of meagre***. Four paired spawning experiments were completed to determine the potential of paired spawning inductions with male rotation to perform a dialled cross mating design as the basis of a breeding program. In the experiments, three 4 x 4 and one 6 x 6 dialled crosses of pairs with weekly male rotation and induced spawning were completed. Fecundity and percentage fertilisation were determined when eggs were collected and percentage hatching and 5-d (day) larval survival were determined by incubating eggs from each spawn in replicated 96-well microtiter plates. The efficacy of spawning pairs with male rotation was high (76%) and across the four experiments a total of 61 families out of 84 (full and half-sib) were produced that had >200,000 eggs of >80% fertilisation success. However, not all paired crosses with male rotation were successful and a number of females after consecutive successful spawning inductions either failed to spawn or did not present vitellogenic oocytes and could not be induced as planned in the dialled cross design. This failure to spawn or maintain maturity status after successive successful spawning inductions appeared to represent a change in spawning kinetics from the prolonged (up to 17 weeks) induced spawning period observed in experiments with the same-pair (Mylonas et al., 2016).

This change in kinetics may be attributed to the stress of male rotation and consideration should be made that as the number of rotations increases, spawning pairs may fail or induced spawning may not be possible. Together these experiments were a successful "proof of concept" that has shown that paired spawning of meagre is possible for the production of known families from parents with known phenotypes as is necessary for the implementation of a breeding program. However, the study also highlighted possible drawbacks that a number of females can only be induced a limited number of times to produce a limited number of families. In addition the families are produced over a longer period of time and differences in egg quality with time and conditions for larval and juvenile culture may affect future assessment of family performance. Therefore, the present **Deliverable D2.7** examines an alternative method, the use of strip spawning with *in vitro* fertilisation with the aim to develop protocols that can be used to make planned crosses to produce at a time desired families for a genetic improvement program.

Methods

BROODSTOCK MANAGEMENT

The broodstock of meagre consisted of wild specimens caught off the south of Portugal and cultured specimens reared in the Canary Islands and in IRTA Sant Carles de la Ràpita. In the months prior to the experiment, fish were held in two thermally isolated 70 m³ circular tanks in a recirculating system with a natural photoperiod and a controlled natural temperature cycle. The mean temperature during the experiment was 18.6ºC, although after sampling the temperature in the main holding tank (not tanks containing fish selected for trials) rose briefly (to a maximum of 20.5ºC) due the temperature of replacement

water. The broodstock was fed four days a week on a commercial broodstock diet and one day a week with frozen sardines (Family Clupeidae) and squid.

From the first week of May to the last week of June, the maturity status of randomly selected males and females was examined on weekly bases. On each date, ovarian and sperm samples were obtained after anaesthetizing the fish with 70.6 mg/l MS-222 (Tricaine methane-sulfonate). Ovarian biopsies were obtained by cannulation according to the protocol of Duncan et al. (2012). A plastic catheter (2mm x 470 mm) was inserted approximately 10-15 cm into the gonopore and a slight suction was applied. Fresh ovarian samples were examined at 5x magnification in clearing solution (6 ml absolute ethanol, 3 ml formalin, 2 ml glycerol) and the diameter of the 10 largest and most advanced vitellogenic oocytes was recorded. Females were considered eligible for spawning induction if they contained oocytes in full vitellogenesis with a diameter greater than 550 µm (Duncan et al., 2012). From the chosen females, the diameter was recorded of 100 randomly chosen oocytes and 35 of the largest oocytes. In this way, every week females with oocytes with a diameter >550 µm were selected and placed in 16 m³ tanks and were stimulated with an intramuscular injection of 15 µg/kg of gonadotropin releasing hormone analogue (GnRHa) des-Gly10, [D-Ala6]-gonadotropin releasing hormone, (Sigma, Spain). Maturation of the males was examined by the release of sperm upon application of 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 chosen males were placed together in a separate tank from the females and injected with a dose of 15 µg/kg of GnRHa. Injections to females were made between 8 and 10 p.m. and to males, between 9 and 10 a.m. on the day after the females were injected. Females were held in constant darkness until ovulation was observed. A total of 14 females and 5 males were used in the experiments. The eligible females and males in the whole experiment had mean weights of 20.45 ± 6.22 and 15.94 ± 2.75 kg, respectively. As a precaution, after hormonal treatment, external egg collectors connected to the tanks of the induced females were examined on a daily basis. Spawned eggs were retrieved during the period from 50 h after hormone treatment and the volume and number of eggs were recorded. To determine the number of eggs/ml, triplicates of 0.1 ml were taken and eggs were counted under a binocular microscope.

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 1-ml or 2-ml syringes immediately before the first stripping of females. Sperm samples were maintained above 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 also added to prevent any bacterial development and bovine serum albumin (BSA) (0.066 ml BSA/ml dilution), to protect the plasma membrane and avoid sperm aggregation. For sperm sampling, positive displacement pipettes were used. Sperm collected before the first revision was made of ovulated females was diluted (as above) and stored above ice or at 4ºC until required for *in vitro* fertilisation. Sperm quality was assessed with Computer Assisted Sperm Analysis (CASA) using open source software ImageJ as described in **Deliverable D2.6**. The quality of sperm did not deteriorate during the maximum 7 hours of storage required to complete the *in vitro* fertilisation experiments (**Deliverable D2.6)** and sperm quality was not a factor that affected egg quality parameters. Cryopreservation techniques described in **Deliverable D2.6** were also used in the *in vitro* fertilisation experiments.

EXPERIMENT 1: Timing of ovulation and viability of eggs

Injected females, received abdominal massages every two and a half hours initiating 35 hours after the GnRHa injection. Ovulation was detected when eggs were easily stripped from the abdominal cavity. The time ovulated eggs were first detected from a female was taken as the time of ovulation and used to calculate the latency time, the time elapsed between hormonal treatment and ovulation. The stripped eggs were collected into a dry bowl, avoiding contamination by faeces and urine. The eggs were fertilised with mixed

milt obtained from two males. Duplicates of 1 ml of eggs from each spawn were separately fertilised using 40 µl of mixed diluted milt (approximately 200,000 sperm per egg) followed by the addition of 100 ml of seawater for sperm activation. After 3 or 4 minutes, all batches of fertilised eggs were placed into separate small incubators (1.5 l capacity) with recirculating water at a range from 17.8ºC to 18.4 ºC with the exception of one week that water was at 20.7ºC. A number of 400 eggs from each incubator were examined under a binocular microscope approximately 30 hours after fertilisation to determine survival rate (percentage of number of eggs with embryos). To determine the viability of eggs stored at room temperature, duplicates of batches of eggs were fertilised every half hour after being removed from the ovarian cavity and survival rates were determined.

EXPERIMENT 2: Optimal sperm:egg ratio

Once the optimal timing of stripping to obtain the highest quality eggs was established, fertilisation was carried out at different sperm concentrations in order to establish the minimum number of sperm to obtain maximal success in fertilisation. Freshly collected sperm from individual fish was diluted 1:4 in a diluted medium of Leibovitz with BSA. The number of sperm used to fertilise egg batches ranged from 2,675,000 to 407,500,000. Aliquots of 0.5 ml eggs (approximately 850 eggs) stripped from a single female were placed in beakers and fertilised with each sperm concentration and 100 ml of seawater was added for activation and fertilisation. Eggs were used from two females, and two males were used to fertilise the eggs from each female to give four different male-female combinations of each sperm to egg ratio. Each combination of fertilisation was made in duplicate or triplicate. Finally, an additional amount of 200 ml of seawater were added for the early embryonic development stages. After 2 hours, the content was poured onto a 200 µm sieve and both floating and sinking eggs were placed into a petri dish. Fertilisation rate was assessed under a binocular on 100 randomly chosen eggs of each batch.

EXPERIMENT 3: Comparison of sperm storage (fresh, Leibovitz and cryopreserved)

Sperm samples were obtained from GnRHa induced males. Meagre sperm was cryopreserved using the extender and cryoprotectant proposed by Fauvel et al. (2012) for European seabass (diluted sperm in cell culture medium Leibovitz plus 10% dimethyl sulfoxide (DMSO)). However, in this case, one sperm dilution 1:4 (sperm: extender) was used. Straws were filled with the dilution and placed 5-6 cm above the liquid nitrogen surface for 15 min before being introduced into the liquid nitrogen (**Deliverable 2.6**). Sperm was stored 1:4 in Leibovitz for 24 hours before fertilisation and fresh samples were diluted 1:4 and kept above ice (**Deliverable 2.6**).

To assess fertilisation ability, eggs were stripped from 4 GnRHa induced females and 5 ml of eggs were placed in each beaker. Fertilisation was performed by two ways: (1) by the protocol applied by Fauvel et al. (2012); in triplicates using 200 µl of each male sperm both fresh and cryopreserved and adding 2.5 ml of seawater filling it up to 100 ml, or (2) in duplicates using 100 ml of seawater to allow fertilisation instead of 2.5 ml and filling it up to 300 mL. Fertilisation rate was assessed under a binocular microscope on 100 randomly chosen eggs. The experiment was repeated twice, experiments 3a and 3b. Experiments 3a tested fresh, in Leibovitz for 24 h and cryopreserved sperm, while experiment 3b only tested fresh and cryopreserved.

EXPERIMENT 4: Large scale production trial

In order to test the production of large number of different families for a genetic breeding program, a large scale factorial cross using *in vitro* fertilisation was attempted in a private farm. The sperm of 33 males from 2 different broodstocks were individually collected, immediately diluted to 1/3 (v/v) in modified Leibovitz, and distributed in 500 µl straws. The straws were cooled and frozen according to the protocol described in **Deliverable D 2.6**. Seven more males were sampled the day that the females were stripped. Eight females at the right genital state were stimulated by a 20 µg/kg GnRHa and maintained at 18°C for 36h-43h before being examined for ovulation when available eggs were stripped. After each successful stripping of each female, the eggs were dispatched into 40 aliquots of 10 ml of eggs (around 15000 eggs) and fertilised individually with selected sperm from individual males. In this way *in vitro* fertilisation was completed with 33 cryopreserved and 7 fresh batches of sperm from 40 different males. Straws were first thawed in warm

(approximately 40ºC). Each fertilisation was made with 500 µl (straw or fresh) of sperm (around 300,000 spz/egg), which was rapidly activated by the addition of 5 ml seawater. Each fertilisation trial took around 2 min including egg dispatching, straw thawing, deposition of sperm and activation so that for one female the process lasted 80 minutes. The sperm of the different males were deposited in the same order onto the samples of the different females. Samples of 500 µl of each cross of the 3 first females of the 5 successfully spawning were taken apart for fertilisation rate assessment while the rest were mixed and placed in 5 large incubators (1 per female). The fertilisation rates were assessed of 100 eggs, 1-2 hours after the end of the fertilisation of the three first spawns, when eggs presented varied development stages from 16-32 cells to morula.

STATISTICAL ANALYSIS

The results are expressed as mean ± standard deviation of the mean. Data normality and homogeneity of variance were analysed using the Kolmogorov-Smirnov and Levene tests, respectively. Differences were examined using ANOVA or the equivalent nonparametric Kruskal-Wallis. Significant differences between means were compared using Tukey HSD test when equality of variances was assumed and Games-Howell test when not. Analyses were performed using SPSS software version 20.0 (Armonk, NY: IBM Corp). Analyses of density, motility and VAP along the experimental period were made though one way ANOVA repeated measures using SigmaPlot version 12.0 (Systat Software Inc., Richmond, CA, USA). Significant differences between means were compared using Holm-Sidak test. A level of $P < 0.05$ was considered to be significant. Linear regressions of sperm to egg ratio were made with Excel (Microsoft, USA) and regressions between fertilisation rate and the time of ovulation were made with SigmaPlot version 12.0.

Results

EXPERIMENT 1: Timing of ovulation and viability of eggs

The oocytes diameter from induced females was significantly different between some females. However, no significant differences were observed in the stage of maturity among females that were observed to ovulate and females that did not ovulate, either from 100 random oocytes or 35 of the largest post-vitellogenic oocytes. Vitellogenic oocyte diameter ranged from 617.14 ± 23.33 µm to 747.11 ± 56.12 µm in females that ovulate and from 606.85 ± 49.21 µm to 715.50 ± 61.61 µm in females that did not ovulate (**Table 1**).

A total of 14 females were induced to ovulate with some females being induced on more than one occasion to give a total of 24 different applications of GnRHa to induce ovulation. A total of 9 fish given a GnRHa injection did not ovulate within the period of 35-45 hours. These included 4 females that never responded to the GnRHa injection and 5 females that did not respond on the second application of GnRHa. A total of 15 GnRHa injections induced a complete or partial ovulation. Eleven ovulations from six different females were stripped in a time series every 2.5 hours to evaluate changes in egg quality after ovulation (over-ripening effect). Ovulation was detected at 35-39 h (2,100 – 2,340 min) after injection depending on the female. However, there was a high variability in the fertilisation rate of eggs obtained from 35 to 36 hours between females (32.5 ± 43.50 %) while from 38 to 39 hours (51.11 ± 28.04 %) this variability was reduced (**Fig. 1**). This variability was still lower in the fertilisation rate of eggs obtained from 40 to 41 hours (27.49 ± 16.39 %) and from 43 to 44 hours (5.72 ± 3.90 %) after GnRHa injection. The variability during the period 35-36 hours that represented the first revision for ovulation was clustered in two groups (bimodal) with very poor eggs (<20% fertilisation) and good eggs (>60% fertilisation). The poor eggs (<20% fertilisation) appeared to be related to incomplete ovulation as in 5 of the 8 batches with low fertilisation during the period 35-36 hours only small volumes of eggs could be obtained. In all cases in subsequent revisions for ovulation, egg quality improved, indicating a period when egg quality improved to a maximum. If this initial variability is removed to consider only eggs that had completed ovulation to give an initial maximum value, the distribution of the averages of the fertilisation rate of each group of eggs compared to the time between injection and stripping can be described by a Hill regression with $R^2 = 0.8167$ (**Fig. 2**). The Hill regression identified a linear decrease after 40 hours between injection and stripping in the fertilisation rates of eggs from all the females.

Table 1. Oocytes diameter from induced meagre females (*Argyrosomus regius*) (from 100 random oocytes and 35 of the largest oocytes and the percentage of oocytes larger than 550 µm). Shaded cells show females that did not ovulate. The data in columns with different letter superscripts were significantly different ($P < 0.05$). Females with numbers in brackets indicate the 2nd, 3rd or 4th induction for the particular female, all females without a bracketed number were the 1st induction.

Female	Oocyte diameter (µm) from 100 random oocytes	% oocyte > 550 µm	Oocyte diameter (µm) from the 35 biggest ones
8♀	129.40 ± 121.78^{a}	3	606.85 ± 49.21^{a}
16(2)♀	134.94 ± 133.56^{ab}	4	723.42 ± 65.34 ^{hi}
11(2)♀	162.60 ± 160.67^{abc}	10	617.14 ± 23.33^{ab}
12♀	198.80 ± 200.78^{abcd}	15	633.71 ± 64.12^{abc}
10♀	211.81 ± 139.74^{abcd}	7	645.73 ± 43.56^{abcd}
4♀	212.33 ± 185.78^{abcd}	13	674.87 ± 66.50^{cdefg}
2(2)♀	220.00 ± 223.80^{abcde}	22	621.71 ± 19.62^{ab}
5(2)♀	226.56 ± 158.33^{abcdef}	8	618.91 ± 50.84^{ab}
2♀	233.40 ± 206.24^{bcdef}	14	694.85 ± 74.41^{efgh}
9♀	233.60 ± 175.36^{bcdef}	7	614.98 ± 37.12^{ab}
14(2)♀	240.82 ± 171.51^{cdef}	9	655.20 ± 30.75^{bcdef}
6(2)♀	242.10 ± 183.52^{cdef}	14	676.10 ± 70.37^{cdefg}
13(4)♀	246.49 ± 181.35^{cdef}	17	648.80 ± 34.50^{abcde}
3(2)♀	258.43 ± 182.34^{cdef}	14	658.09 ± 71.08^{bcdef}
13♀	271.80 ± 246.85^{def}	21	710.85 ± 56.63^{ghi}
5♀	281.95 ± 183.18^{def}	14	659.50 ± 58.09^{bcdef}
11♀	286.00 ± 243.16^{def}	24	660.57 ± 48.86^{bcdef}
13(3)♀	290.81 ± 195.21^{defg}	15	658.80 ± 74.54^{bcdef}
13(2)♀	294.07 ± 194.08^{defg}	15	747.11 ± 56.12^{i}
28♀	295.39 ± 194.30^{defg}	16	699.65 ± 45.49^{fgh}
14♀	314.86 ± 215.41^{fg}	24	646.11 ± 59.38^{abcd}
3♀	318.37 ± 211.91^{fg}	22	715.50 ± 61.61^{ghi}
16♀	325.80 ± 246.38^{fg}	31	681.14 ± 40.27^{efgh}
6♀	386.58 ± 214.11^{g}	33	697.31 ± 28.67^{fgh}

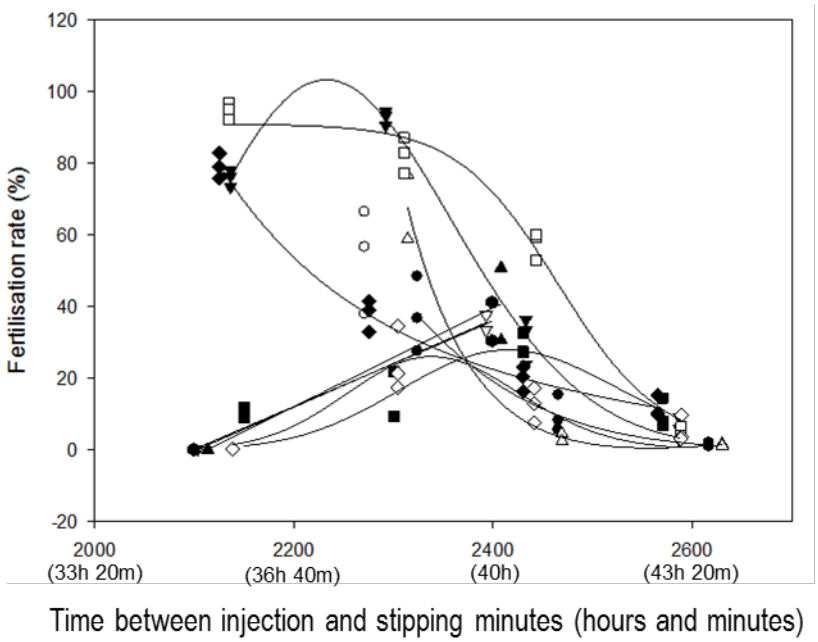

Figure 1. The fertilisation rate (%) of each batch of eggs stripped from each female meagre (*Argyrosomus regius*) at different times after the GnRHa (15 µg/kg) injection. n = 6 females and 11 ovulations.

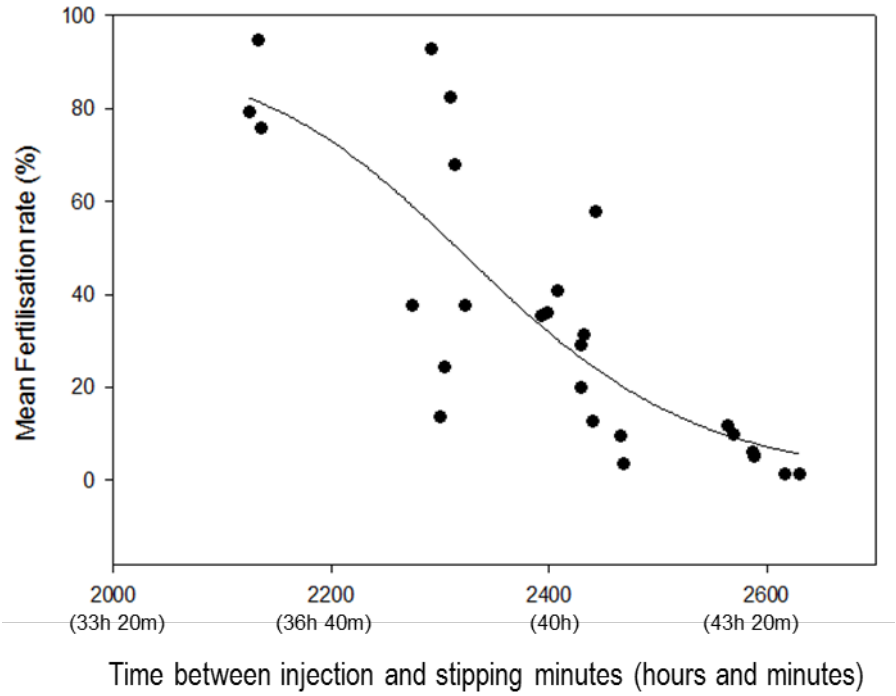

Figure 2. The fertilisation rate (%) of each batch of eggs stripped from each female meagre (*Argyrosomus regius*) at different times after the GnRHa (15 µg/kg) injection. The line represents a Hill regression (R^2 = 0.8167). Data represents the decline from maximum fertilisation rates of 10 ovulations from 5 different females i.e. the overripening period. Initial low fertilisation levels that appeared to represent a maturing period of improving egg quality that are displayed in **Fig. 1** were removed from the data set for **Fig. 2**.

Eggs maintained at room temperature (approximately 20ºC) maintained viability for up to 2 hours (**Fig. 3**). There was an initial small decline in fertilisation rate during the first 30-60 minutes, fertilisation stabilised at approximately 80% until after 120 minutes when a rapid decline in the fertilisation of the eggs was observed. Asynchronous divisions in many of the fertilised eggs were observed at 4 h.

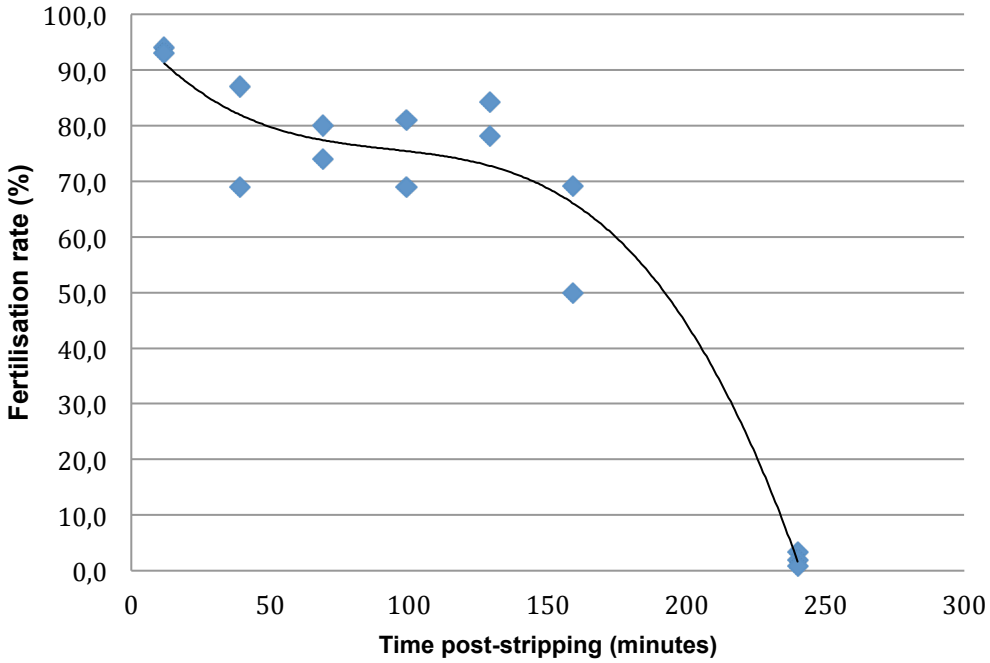

Figure 3. The fertilisation rate (%) of stripped meagre (*Argyrosomus regius*) eggs held at room temperature for different times after stripping. The line represents a polynomial regression with an equation expressed: $y = -2E\text{-}05x^3 + 0{,}0058x^2 - 0{,}5891x + 97{,}447$ ($R^2 = 0.9523$).

EXPERIMENT 2: Optimal sperm:egg ratio

There was no significant difference in the fertilisation rates between the two males used for each female (21.10 ± 11.2 % and 25.26 ± 14.26%). However, there were significant differences in fertilisation rates between each female (indicating differences in egg quality between females). The data for the two males used to fertilise eggs from each female was therefore combined. Both pairs of males were combined to obtain a logarithmic regression with a $R^2 = 0.8437$ and $R^2 = 0.958$, respectively. A progressive rise in the fertilisation rates with the increase in the sperm to egg ratio with a final stabilisation was observed in both cases (**Fig. 4**). Both pairs of males exhibited an increase in fertilisation from a ratio of approximately 3,000 sperm:egg until 100,000 sperm:egg. After 100,000 sperm:egg the regression equations levelled off and further increases in fertilisation rates were low. This indicated that a sperm to egg ratio in excess of 100,000 gave optimal fertilisation. However, comparing means indicated a significant difference for males 17(2)♂ and 23♂ was obtained from 100,000 to 240,000 sperm:egg ratio indicating that a sperm to egg ration in excess of 200,000 may be the most desirable ratio to ensure optimal fertilisation (**Fig. 4**).

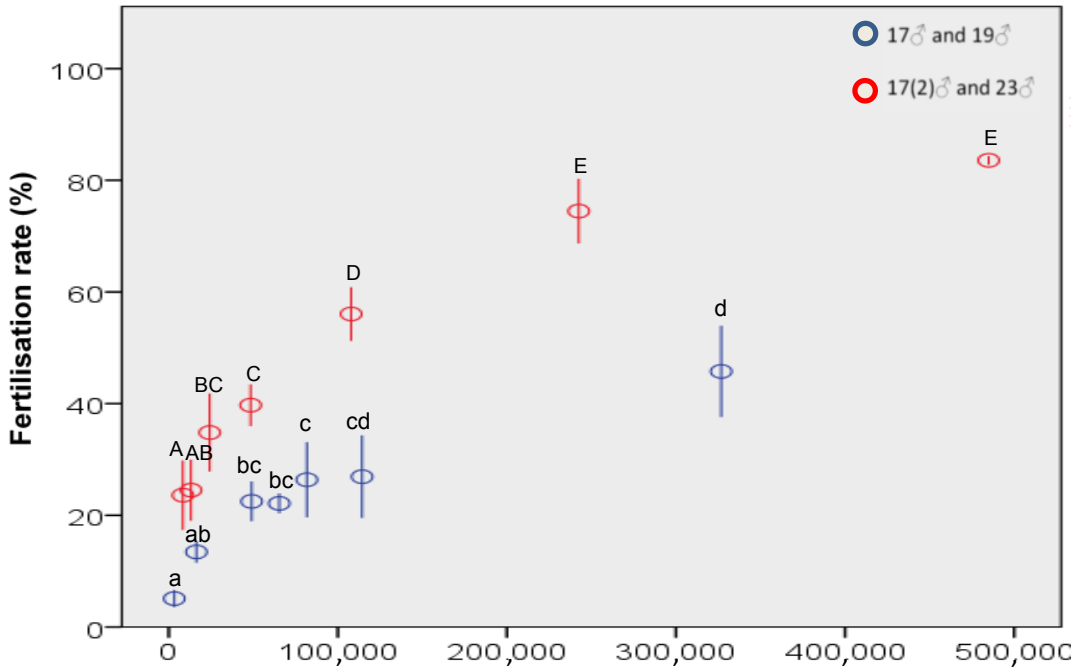

Figure 4. Mean fertilisation rates of meagre (*Argyrosomus regius*) eggs fertilised *in vitro* at different sperm: egg ratios. The horizontal error bars indicate the standard deviation of mean. Significant differences are indicated by different letters (capital letters for males 17♂ and 19♂, and lowercase letters for males 17(2) ♂ and 23♂) ($P < 0.05$).

EXPERIMENT 3: Comparison of sperm storage (fresh, Leibovitz and cryopreserved)

Experiment 3a

The effect on fertilisation rate was examined in relation to three factors; types of conservation (fresh, stored in Leibovitz for 24h or cryopreserved) in different females that provided eggs and different males that provided sperm. There were no significant differences due to the conservation method or individual male sperm used to fertilise each batch of eggs. However, significant differences were found between different females ($P = 0.000$), which was due to the variability in the quality of the eggs provided by each females and there was a significant interaction between female and the conservation medium ($P = 0.008$). The eggs from females 13♀ and 28♀ had better quality as shown by the higher fertilisation rates for each kind of stored sperm than the eggs from female 6♀ (**Fig. 5**). The highest fertilisation rates obtained were only 41%.

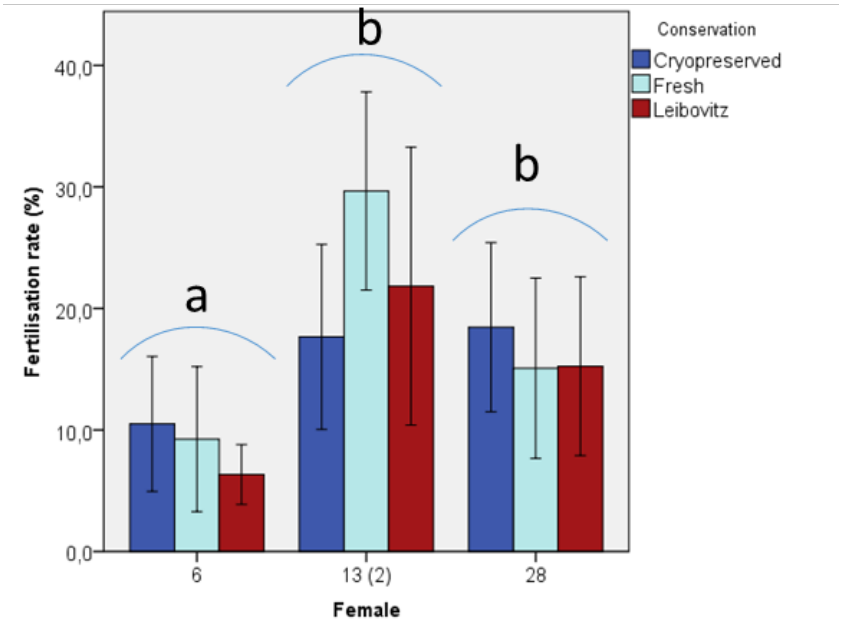

Figure 5. Fertilisation rate of different batches of meagre (*Argyrosomus regius*) eggs from different females (6, 13(2) and 28) fertilised with cryopreserved sperm (dark blue), fresh sperm (light blue), and sperm stored for 24 h in Leibovitz (red). Fertilisation rates from female 6 were significantly lower than from females 13(2) and 28 ($P < 0.05$) as indicated by different letters.

Experiment 3b

There was no significantly difference in fertilisation rate between sperm subject to two different kinds of storage, fresh and cryopreserved ($P = 0.715$) (**Fig. 6**) or sperm from different males ($P = 0.968$). Higher fertilisation rates were found in this experiment (86%) compared to fertilisations in experiment 3a and this was attributed to the quality of the eggs.

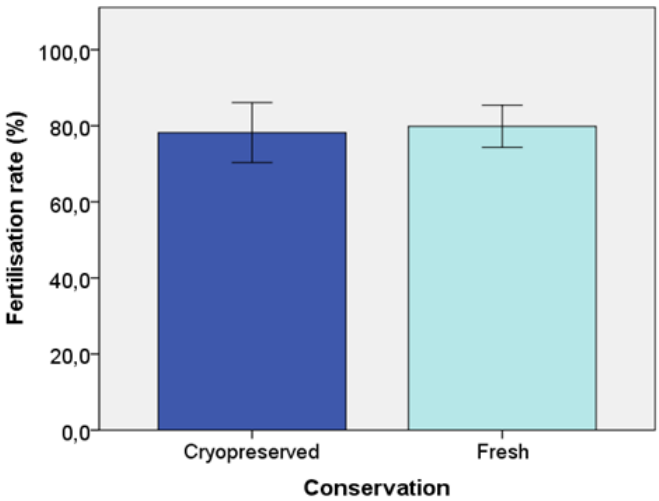

Figure 6. Fertilisation rate of different batches of meagre (*Argyrosomus regius*) eggs fertilised with cryopreserved sperm (dark blue) and fresh sperm (light blue).

EXPERIMENT 4: Large scale production trial

A total of 5 females ovulated from the 8 females that were injected and the eggs were stripped for in vitro fertilisation. The large factorial cross of these 5 females with 40 male sperms (of which 33 were cryopreserved and 7 fresh) was performed within 7h. Embryos and larvae were only obtained from the eggs from the first 3 females that were stripped. The delay to fertilise the eggs from the first 3 females probably resulted in the eggs from the 2 last females being overripe. A total of 800,000 larvae were obtained from the 3 females. Female 3 had poorer egg quality than females 1 and 2 (**Table 2, Fig. 7**) that were stripped first. Fertilisation rates for females 1 and 2 fertilised with cryopreserved and fresh sperm ranged from 49.4 to 79.3 % and fertilisation rates for female 3 ranged from 26.9 to 37.2%.

Table 2. Mean fertilisation rates for meagre (*Argyrosomus regius*) eggs from 3 females fertilised with cryopreserved sperm (n=33 males) and fresh sperm (n=7 males).

		Female 1	Female 2	Female3
Global	**mean**	**62,9**	**72,1**	**35,4**
	St deviation	10,9	13,3	18,0
	coef-variation	17,3%	18,4%	50,7%
	minimum	30	33	9
	maximum	78	96	65
Detailed				
Cryo-	**mean**	**65,3**	**70,6**	**37,2**
preserved	St deviation	9,0	13,3	18,6
	coef-variation	13,8%	18,9%	50,1%
	minimum	38	33	9
	maximum	78	89	65
Fresh	**mean**	**49,4**	**79,3**	**26,9**
	St deviation	11,1	11,0	11,9
	coef-variation	22,6%	13,9%	44,2%
	minimum	30	65	15
	maximum	61	96	43

The ANOVA using the general linear model with females as the replicates for male effect and males as the replicates for female effect of the fertilisation rates for individual crosses revealed that both male and female had a significant effect on fertilisation rate (respectively $F(2,77) = 85.2$, $p = 0.00000$, and $F(39,77) = 1.61$ $p=0.037$) (**Fig. 7**). However, there was no significant ($P=0.087$) effect of storage method of sperm, cryopreserved or fresh on fertilisation rate after allowing for the female effect. The analysis of covariance of the variation of fertilisation rate with time (with 2 minutes for each individual fertilisation revealed that the variation of fertilisation with time was significant ($p<0.01$) and the slope of this variation was significantly different between females ($p< 0.001$). In detail females 1 and 2 exhibited a limited decay in fertilisation with time and had with similar slopes ($p=0.68$) while a significantly negative slope was observed for female 3 (**Fig. 7**).

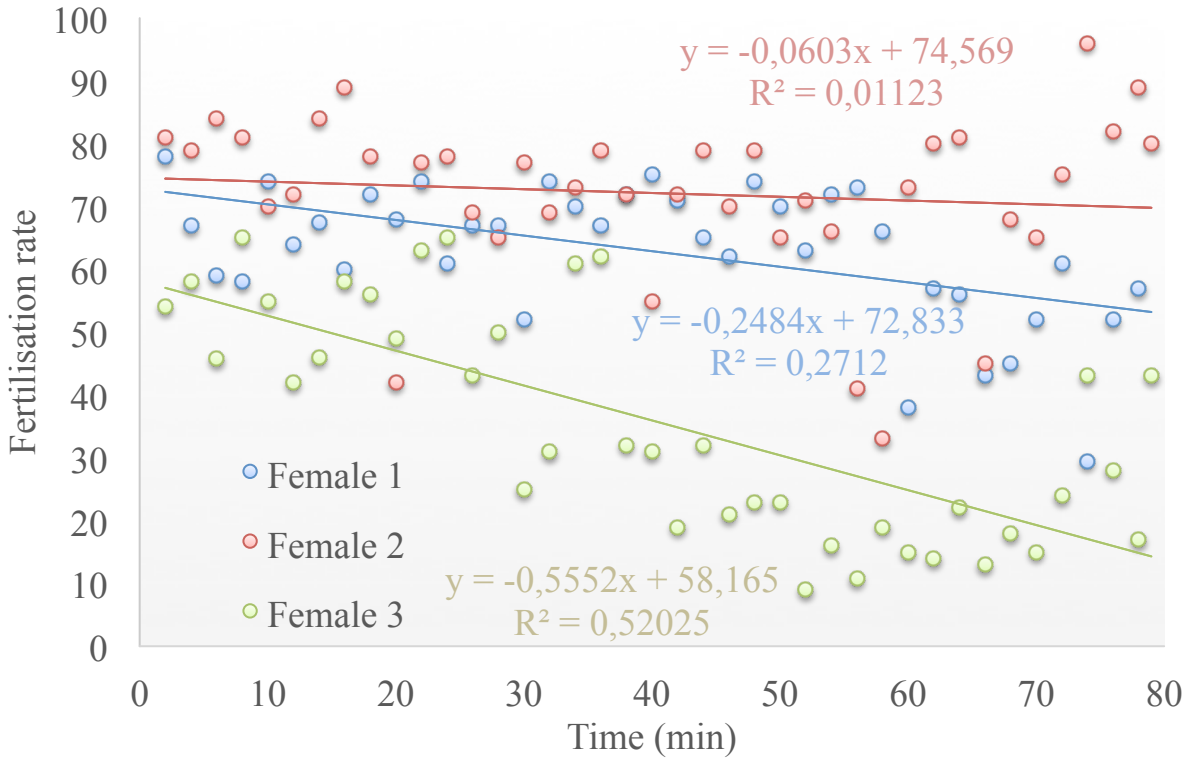

Figure 7. Variations of individual fertilisation rates of meagre (*Argyrosomus regius*) eggs with time in a factorial cross of 3 females X 40 males. The artificial fertilisation of the egg batches from each of the three females (1, 2 and 3) were made at the same time and in the same order and each artificial fertilisation was completed in 2 minutes. Therefore, each time point represents a sperm sample from a different male.

Discussion

These experiments have identified the information necessary for a protocol for the strip spawning of meagre and *in vitro* fertilisation. The protocol was used to successfully fertilise large numbers of eggs with sperm from many different males to produce a large number of families. A protocol for the strip spawning and *in vitro* fertilisation needs to define the following steps (a) handling and anaesthesia of breeders, (b) timing of stripping to obtain gametes of good quality (c) timing (storage), mixing and proportion of gametes during *in vitro* fertilisation to obtain high fertilisation rates. These practices are more common in the mass production of freshwater species, but recently are also being applied to marine species important in aquaculture. For example European seabass have offered challenges to develop strip spawning (Fauvel et al., 1999; 2012) and were reported as difficult to strip spawn (Duncan et al., 2013b).

Meagre breeders are large (in the present study females = 20.45 ± 6.22 kg and males = 15.94 ± 2.75 kg) and excessive handling will have detrimental affects on ovulation and gamete quality. Handling the females every 2.5 hours to examine changes in egg quality will have affected egg quality and ovulation (experiment 1). Therefore, breeders should if possible only be handled twice, once to make a hormone induction (if necessary) and a second time to strip gametes at the appropriate time. Generally sperm can be obtained and stored, whilst eggs have a short period of viability after stripping, which should be defined in a protocol. This highlights the importance of defining the most reliable and optimal time for stripping eggs from females.

In the present study, sperm was easily obtained throughout the spawning season and stored with few problems or loss in quality, and further details on handling sperm for *in vitro* fertilisation are available in **Deliverable D2.6**. Briefly, males were injected with 15 µg/kg GnRHa 24 hours before females were

expected to ovulate. The sperm was stripped immediately before the first females were checked for ovulation (optimal time would be 35 hours after females were injected). The sperm was mixed at a ratio of 1:4 in Leibovitz and stored above ice or at 4 ºC until eggs were available to make *in vitro* fertilisation. Sperm was also successfully stored short-term for 24h in Leibovitz medium and cryopreserved with no loss of fertilisation ability compared to fresh sperm (Experiments 3 and 4). Across all experiments sperm quality was shown not be a factor that affected the fertilisation rates.

In the present study, females that were treated with a GnRHa injection of 15 µg/kg at 18°C were induced to ovulate from 35-39 h after the GnRHa injection. The variability in the ovulation time and in fertilisation rates obtained could be due to slight differences in the stage of gonadal maturation among females at the time of the GnRHa injection or just a difference in the viability of eggs from fish of the same stock (Springate et al., 1984). There was a high variability in the fertilisation rate of eggs obtained from 35 to 36 hours and during this time period two groups (bimodal) of egg quality were observed, a group with poor eggs (<20% fertilisation) and a group with good eggs (>60% fertilisation). The poor eggs (<20% fertilisation) appeared to be related in incomplete ovulation as in 5 of the 8 batches with low fertilisation only small volumes of eggs could be obtained. The batches with good eggs all exhibited flowing eggs that was easily stripped. An increase in viability was observed with time (from 35-36 to 38-39 hours) in the group of eggs with low egg quality and it appeared that there was a period of improving egg quality as ovulation progressed, which has been referred to as ripening (Duncan et al 2013b). Eggs from 38 to 39 hours exhibited a more uniform egg quality with the highest mean rates of fertilisation (51.11 ± 28.04 %). The fertilisation rates then deteriorated rapidly with time to 40-41 hours (27.49 ± 16.39 %) and then 43-44 hours (5.72 ± 3.90 %) after GnRHa injection. This was clearly the overripening period that has been identified in a number of different species (Springate et al., 1984; Legendre and Otémé, 1995; Samarin et al., 2008; Rasines et al., 2012; Samarin et al., 2015). Viability of meagre eggs after ovulation was short. Eggs were observed to maintain viability over a 2.5 hour period between two revisions, however, in some females viability was only observed at one time point and was lost during the following 2.5 hours. No eggs were observed to maintain viability over three revisions or 5 hours. These observations indicate that eggs can retain viability for 2.5 hours, but not longer. Together, this study clearly indicated that under the conditions reported (18ºC) eggs have optimal viability and should be stripped during a short window close to 38-39 hours after females were injected with GnRHa. This window of opportunity and deterioration due to overrippening was also demonstrated in Experiment 4 where egg quality and fertilisation rates deteriorated as females were stripped in sequence. In the current trial, a trend towards degradation occurred in eggs from female 1 compared to that of female 2 that was collected one and a half hour later, while female 3 exhibited a lower initial fertilisation rate and a more acute (significant) loss of egg fertility. In addition, fertilisation was not possible with the eggs obtained from the last two females. These later observations suggest that ovulation occurs within a time interval linked to individual female reaction to heterologous stimulation, while stripping is applied at fixed times leading to different egg overripening states when collected. In the future, attempts to better control ovulation (more acute choice of female state at stimulation, adjustment of external factors after stimulation, better detection of individual ovulation, etc.) would allow minimizing egg quality variation at collection.

Interestingly the period of eggs viability was similar when eggs were left in the abdomen and when eggs were stored at room temperature outside of the abdomen. Eggs stored at room temperature exhibited an initial small decline in fertilisation rate during the first 30-60 minutes, fertilisation stabilised at approximately 80% until 120 minutes, when a rapid decline in the fertilisation of the eggs was observed.

Once eggs have been obtained and sperm stored in an appropriate manner, it is important that *in vitro* fertilisation is executed quickly by mixing eggs and sperm in the correct ratio with sea water. The sperm exhibited a rapid decline in motility and velocity (see **Deliverable D2.6**), which indicated that the optimal period for fertilisation by sperm was the first 30 seconds after the sperm was activated. It is also important that the number of sperm per eggs is above a minimum ratio at which the rate of fertilisation begins to decline. For instance, the optimal sperm to egg ratio range was 66,000 spzoa/egg in European seabass (Fauvel et al., 1999), 1,000 and 100,000 in pufferfish (*Takifugu niphobles*) (Gallego et al., 2013) and 1,000,000 in northern pike (*Esox lucius*) (Zhang et al., 2011). In meagre, this study shows that the maximum

fertilisation rates occur within a wide insemination dose range from 100,000 to 240,000 spzoa/egg. It is known that other characteristics, such as spermatozoa swimming distance, micropyle closing time and oocyte size, are determinants in the insemination doses. Meagre eggs are small (0,90 mm in diameter, Cárdenas, 2010), so at a fixed sperm density per egg the probability of a sperm reaching the micropyle would appear to be higher than in other fish species with bigger eggs. Therefore, that is why less sperm/egg ratio was expected, but if VAP is highly correlated with fertilising ability, the period of sperm fertility could be short and a high amount of sperm per egg should be needed. Similar results were obtained in both weeks that the experiment was done. However, it appears to be that higher sperm/egg ratios were needed in one of the two experimental weeks when higher VAP values of sperm were obtained. It could be explained as the sperm requirement of eggs also depends on the quality of eggs. As Bombardelli et al. (2013) observed in cascudo-preto (*Rhinelepis aspera*), more sperm should be needed inseminating batches of eggs of lower viability to reach the maximum fertilisation success. Therefore, it seems to be critical to maintain identical fertilisation conditions to estimate sperm:egg ratio in order to avoid hiding effects through the experimental variables (Gallego et al., 2013).

Conclusion

The results obtained in the present study have established a protocol for the optimum artificial fertilisation of meagre in the current state of knowledge. The broodstock should be examined at 38 h post-injection at 18ºC to obtain optimum egg quality. For conventional production, a minimum of 200,000 spermatozoa per egg is recommended to ensure high fertilisation rates. Furthermore, it is recommended that eggs, sperm and water are mixed to coordinate activation with the sperm coming into contact with the eggs, and that meagre eggs should be fertilised within the first 50 min post-stripping at least to maintain a high capacity to be fertilised until more research is done to identify which delay in activation can be assumed not to affect fertilisation success or until extenders that mimic the ovarian fluid are developed. The up scaling to a large factorial cross of 120 artificial fertilisation using either fresh or cryopreserved sperm confirmed the feasibility of the protocol.

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Deviations: The were no deviation from the DOW

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