

Gamete quality and management for in vitro fertilisation in meagre (*Argyrosomus regius*)

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Abstract :

The aquaculture of meagre (*Argyrosomus regius*) requires methods for the control of reproduction that enable the production of families from specific individuals for selective breeding programs. We experimentally determined the parameters required for an in vitro fertilisation protocol. A total of 14 females and 5 males (mean \pm S.D. weights of 20.45 ± 6.22 and 15.94 ± 2.75 kg, respectively) were used. Selected females had vitellogenic oocytes $>550 \mu\text{m}$ in diameter and males had fluid sperm upon application of abdominal pressure. Both sexes were treated with an injection of $15 \mu\text{g kg}^{-1}$ of gonadotropin-releasing hormone agonist (GnRHa) to induce oocyte maturation/ovulation and enhance sperm production. To determine the timing of ovulation and window of high egg viability, females were stripped serially every 2.5 h beginning 35 h after GnRHa treatment. Sperm was obtained 24 h after GnRHa treatment and was diluted 1/4 in modified Leibovitz for storage at 4°C until use. Sperm quality parameters such as percentage initial spermatozoa motility, duration of motility, velocity and density were determined using computer assisted sperm analysis (CASA). In vitro inseminations were made in duplicate or triplicate batches of eggs from each spawn by mixing 0.5–1 mL of eggs, 20–40 μL diluted sperm (pooled from two males) and 100 mL of seawater. Fertilisation success was examined at spermatozoa (spz): egg ratios between ~ 2000 and $400,000$ spz egg $^{-1}$. The optimal time for stripping ovulated females was ≤ 3 h after ovulation, which was the window of optimal egg viability. Ovulation under the conditions of this study was close to 38 h after GnRHa treatment, with a range from 35 to 41 h. Beginning from 3 h after ovulation, egg viability declined probably due to overripening. Sperm diluted in Leibovitz maintained motility and velocity for as long as 7 h after collection. Spermatozoa motility (%) and average path velocity (VAP, $\mu\text{m/s}$) of sperm samples obtained from males before GnRHa injection declined rapidly after activation compared to the samples obtained 24 h post-injection, with significant decreases respectively after 75 and 45 s. A minimum ratio of 150,000 spermatozoa egg $^{-1}$ was necessary to ensure high fertilisation success. The acquired knowledge of the present study will aid the aquaculture industry and future research on selective breeding programs for meagre.

Highlights

► An *in vitro* fertilisation protocol has been developed for meagre (*Argyrosomus regius*). ► Ovulation took place close to 38 h and in the range of 35–41 h after GnRHa induction at 18 °C. ► Good quality eggs were collected within a three-hour window from ovulation. ► The spermatozoa: egg ratio for high fertilisation success was 150,000 spermatozoa egg⁻¹.

Keywords : *Argyrosomus regius*, meagre, reproduction, artificial fertilisation, gamete management, GnRHa

1. Introduction

The meagre (*Argyrosomus regius*) is a sciaenid fish found in the Mediterranean and Black Sea, and along the eastern coast of the Atlantic Ocean. This species is a suitable candidate for the diversification of aquaculture in the Mediterranean, due to a number of attractive market and biological attributes. Market attributes include a large size, good processing yield, low fat content, excellent taste and firm texture (Monfort, 2010). The biological characteristics include fast growth of $\sim 1 \text{ kg year}^{-1}$ (Duncan *et al.*, 2013a), low feed conversion ratio of 0.9-1.2, relatively easy larval rearing (Vallés and Estévez, 2013) and an effective spawning protocol based on multiple injections of a synthetic agonist of gonadotropin-releasing hormone (GnRH α) (Duncan *et al.*, 2012; 2013a; Mylonas *et al.*, 2013a; 2015; 2016; Fernández-Palacios *et al.*, 2014).

These positive attributes for aquaculture have resulted in Mediterranean production rising to 7488 tons in 2016, representing a 25% increase from 2015 (APROMAR 2017). To increase the production further, enhance growth rates and reduce costs, while avoiding inbreeding problems, the industry needs to implement selective breeding programs to select heritable traits that improve production and product quality for the market (Duncan *et al.*, 2013b). The implementation of breeding programs and the reproductive control necessary was identified as a bottleneck for meagre aquaculture (Estévez *et al.*, 2015). Although meagre broodstocks had adequate genetic variation, it was apparent that broodstocks across the Mediterranean region had been taken from few individuals from just three wild populations (Estévez *et al.*, 2015). Therefore, the industry requires methodologies that can be used within the framework of a breeding program to (a) produce families from selected breeders with desired traits and (b) maintain the genetic variability through generations of selection to avoid inbreeding.

There are different ways to obtain families from selected breeders, such as tank spawning, paired spawning and *in vitro* fertilisation. Tank spawning is the simplest method, but there is little control over the crosses and families produced, which are often unbalanced in relation to the entire broodstock. For example, in gilthead seabream (*Sparus aurata*) a few breeders dominated the spawning and the families produced, whilst some breeders did not participate in any families (Brown *et al.*, 2005; Chavanne *et al.*, 2012). Paired spawning in separate tanks was successfully carried out with meagre to produce progeny of the same families every week (Mylonas *et al.*, 2016). However, when combined with a cross mating design with male rotation to obtain a higher number of different families every week, a change in spawning kinetics appeared to limit the contribution of females that often lost maturity and spawning potential after being crossed sequentially with three males, thus producing only three half-sib families (Duncan *et al.*, 2018). Therefore, an alternative method is necessary to overcome the drawbacks of breeding in pairs or communal tank spawning. Artificial (*i.e. in vitro*) fertilisation of eggs is the optimal method employed to obtain planned crosses that enable the production of a larger number of families at the same time. In addition, no reproductive mate preference has to be considered and no separate facilities are required.

Protocols of *in vitro* fertilisation have been established for several teleost species that do not spawn spontaneously in culture (Colombo *et al.*, 1995; Rasines *et al.*, 2012; Sanches *et al.*, 2011a), and have formed the bases of reproductive control for many genetic breeding programs (Mañanós *et al.*, 2008; Duncan *et al.*, 2013b). To develop a protocol for successful *in vitro* fertilisation it is necessary to control reproduction and manage the gametes to obtain high quality ova and sperm at the same time for fertilisation. To obtain high quality ova, the time of ovulation and window of optimal ova quality after

ovulation must be established. Hormone inductions are often used to ensure that ovulation time can be synchronized and predicted accurately among females (Mañanós *et al.*, 2008; Rasines *et al.*, 2012; Duncan *et al.*, 2013b). During ovulation, mature oocytes are released as ova that are maintained in the ovarian cavity until abdominal pressure is used to manually strip the ova from the fish or spontaneous spawning is stimulated by social and environmental factors. Delayed stripping can result in obtaining ova that have overripened, which results in low fertility, high occurrence of embryo malformation (Mañanós *et al.*, 2008) and high mortality rates of embryos and larvae (Flett *et al.*, 1996). Sperm production and storage must be managed to ensure the availability of high quality sperm when ova are available (Mylonas *et al.*, 2017). The sperm should be obtained before the ova and stored to ensure successful fertilisation during *in vitro* fertilisation (Rurangwa *et al.*, 2004; Mylonas *et al.*, 2017). Hormone inductions are often used to ensure adequate sperm production, quantity and quality (Mylonas *et al.*, 2017). Lastly, and especially when large quantities of ova are being produced, it is necessary to know the optimal ratio of spermatozoa to egg to estimate how much sperm is required to ensure high rates of fertilisation (Fauvel *et al.*, 1999).

To facilitate the implementation of selective breeding programs in meagre using *in vitro* fertilisation methods, this study determined (a) the time of ovulation and appropriate time window for stripping after hormone treatment to obtain high quality eggs, (b) a suitable short term sperm storage method, (c) the optimum spermatozoa: egg ratio to obtain a high fertilisation and (d) various quantitative spermatozoa parameters that will be useful for sperm quality assessment. This knowledge will aid the aquaculture industry and future research on selective breeding for meagre.

2. Materials and methods

2.1. Broodstock management

The broodstock consisted of wild specimens caught off the south coast of Portugal and cultured specimens reared in the Canary Islands and IRTA Sant Carles de la Ràpita. In the years 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 $18.6 \pm 0.5^{\circ}\text{C}$. The broodstock was fed four days a week on a commercial broodstock diet (MAR VITALIS REPRO, Skretting, Spain) and one day a week with frozen sardines and squid.

During the first week of July 2015 and from the first week of May to the last week of June 2016, the maturity status of randomly selected males and females was examined on a weekly basis. On each date, ovarian and sperm samples were obtained after anaesthetizing the fish with 70.6 mg L^{-1} MS-222 (Tricaine methane-sulfonate). Ovarian biopsies were obtained by cannulation according to the protocol of Duncan *et al.* (2012). Briefly, a plastic catheter (2 mm x 470 mm) was inserted approximately 10-15 cm into the gonopore and a slight suction was applied. Fresh ovarian samples were examined initially at 5x magnification in clearing solution (6 mL absolute ethanol, 3 mL formalin, 2 mL glycerol) and the diameter of the 10 largest (most advanced) vitellogenic oocytes was recorded. Females were considered eligible for spawning induction if they contained oocytes in full vitellogenesis with a diameter $>550 \mu\text{m}$ (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 $>550 \mu\text{m}$ were selected and placed in 16-m³ tanks and were induced with an intramuscular injection of $15 \mu\text{g kg}^{-1}$ of the GnRHa des-Gly¹⁰, [D-Ala⁶]-gonadotropin releasing hormone (Sigma, Spain), as proposed by Fernández-Palacios *et al.* (2014). 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 = only a few drops of sperm released and no sample can be obtained, 2 = fluent, 3 = very fluent with minimal abdominal pressure). Males were also subject to stimulation by GnRH α intramuscular injection ($15 \mu\text{g kg}^{-1}$) to ensure sperm availability, which was particularly necessary towards the end of the trial when quantities of sperm produced decreased. Females were injected between 8 and 10 p.m. and males between 9 and 10 a.m. on the day after the females were injected. Females were held in constant darkness after GnRH α was applied and while checking for ovulation, and males were placed together in a separate tank. Constant darkness was used, to reduce possible effects of the photoperiod control on ovulation time, as meagre ovulate and spawn during the night in the wild (Gil, 2013) or when induced to spawn in captivity (personal observation). A total of 14 females and 5 males were used in the experiments, having mean (\pm S.D.) weights of 20.45 ± 6.22 and 15.94 ± 2.75 kg, respectively. Both females and males were used multiple times in the different years or weeks of the experiment. In a past study, Mylonas *et al.* (2016) induced meagre females to spawn each week for up to 17 consecutive weeks. Therefore, in the present study some females were used more than once, these females were checked each time and eligible females selected as described above.

The broodstock was handled (routine management and experimentation) in agreement with European regulations on animal welfare (Federation of Laboratory Animal Science Associations, FELASA, <http://www.felasa.eu/>).

2.2. Sperm collection and quality

Before sperm collection, the genital pore was cleaned and dried, and gentle abdominal pressure was applied in order to obtain sperm, avoiding any urine contamination. Sperm was collected directly in one or 2-mL syringes immediately before the first checking for ovulation of females was made. Sperm samples were maintained 3 cm above ice. Milt was diluted 1/4 in modified Leibovitz L-15 cell culture medium as described by Fauvel *et al.* (2012) for European seabass (*Dicentrarchus labrax*). Briefly, Leibovitz L-15 was supplemented with 300 μg glutamine mL^{-1} , 6 mg sodium pyruvate mL^{-1} and 1 M NaOH 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 spermatozoa activation in the medium, the osmolarity was decreased to 250 mOSm by dilution in distilled water (Wayman *et al.*, 1998). Gentamycin sulphate (1 mg mL^{-1}) was also added to prevent any bacterial development and bovine serum albumin (BSA, 20 mg mL^{-1}), to protect the plasma membrane and avoid sperm aggregation. For sperm sampling, positive displacement pipettes were used. Diluted sperm samples were stored 3 cm above ice or at 4°C until required for *in vitro* fertilisation.

Analyses of spermatozoa motility were conducted using ImageJ (Image processing and Analysis in Java, <https://imagej.nih.gov/ij/>) with the CASA plugin described by Wilson-Leedy and Ingermann (2007). Aliquots of 10, 20 or 40 μL of Leibovitz-diluted sperm (1/4), were mixed thoroughly with 1 mL of seawater with BSA (1/16) in 1.5 mL Eppendorf tubes for activation, so that the observation dilution were 1/404, 1/204 and 1/104 depending on the density of the initial sperm sample (1/4). A 1 μL sample of this dilution containing the activated spermatozoa was pipetted directly into an ISAS counting chamber (Integrated Sperm Analysis System, Spain). The tracks of the activated spermatozoa in the ISAS chamber were recorded as a digital video through a bright field equipped video microscope at 20x magnification (Leica DMLB Microscope

and DMK 22BUC03 Camera with 744 x 480 “0.4 MP” resolution at 60 FPS, The Imaging Source Europe GmbH, Bremen, Germany). The video recording was initiated at the moment the spermatozoa was activated to provide a reliable reference start point, and was ended when spermatozoa ceased activity. The process of activation and filling the chamber allowed secured assessment of spermatozoa activity in <15 s after activation. The videos were processed using VIRTUALDUB 1.9.11 (virtualdub.org) free software which transforms *.avi movies into image sequences (*.jpg). The image sequences were analysed with the CASA plugin. The sperm parameters assessed were spermatozoa motility (%), defined as the percentage of motile cells; average path velocity (VAP, $\mu\text{m/s}$), defined as the distance moved by the spermatozoa head along its spatial average trajectory in a definite time lap (Gallego *et al.*, 2013); and duration of spermatozoa motility (motility duration, min), defined as the time between activation and end of all spermatozoa movement. Motility parameters were evaluated (in triplicate) each time sperm was used in fertilisation procedures to assess the changes in sperm quality. The settings for the image analysis, which depend on the microscope and the movie quality, were as follows: brightness and contrast, -8 to 19 and 216 to 253, respectively; threshold, 0/231 to 254; minimum spermatozoa size (pixels), 30; maximum spermatozoa size (pixels), 200; then the CASA settings that depend both on camera characteristics and spermatozoa features were the following: minimum track length (frames), 5; maximum spermatozoa velocity between frames (pixels), 40; frame rate, 30; microns/1000 pixels, 301; and the rest of parameters set as default.

Sperm concentration (spermatozoa mL^{-1}) was also recorded for each sperm sample used. 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 settle for 10 min, and was then observed under the

microscope at 10x magnification. Quantification of spermatozoa density of the collected sperm was assessed using the particle analysis function of ImageJ, adjusting image brightness to a range from 43 to 49, image contrast from 203 to 221, and image threshold from 225 to 238. These results were used to calculate the concentration of sperm used to fertilise ova and in particular in the spermatozoa : egg ratio experiments.

2.3. Timing of ovulation and ova viability

To determine the time of ovulation after the administration of $15 \mu\text{g kg}^{-1}$ of GnRHa (Fernández-Palacios *et al.*, 2014), females were first examined for ovulation 35 h after the GnRHa injection and every 2.5 h thereafter using abdominal pressure. The initial evaluation time was decided in accordance with previous observations suggesting that meagre ovulated 36 - 37 h after GnRHa treatment at 19.2°C (Fatira, 2013) and that tank spawning by meagre was at 36 - 39 h after GnRHa application (personal observations). Ovulation was confirmed when ova were stripped from the abdominal cavity. The time ovulated ova 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 ova were collected into a dry bowl, avoiding contamination with faeces or urine. The ova were inseminated with a pool of sperm from two males. The spermatozoa concentration of each sperm sample was measured. Duplicates of 1 mL of eggs (~1600 eggs) from each spawn were inseminated using $40 \mu\text{L}$ of mixed diluted sperm (approximately $230,000 \text{ spermatozoa egg}^{-1}$) followed by the addition of 100 mL of seawater for spermatozoa activation. After 3 min, all batches of inseminated eggs were placed into separate small incubators (1.5 L capacity) with recirculating water at a temperature range of $17.8 - 18.4^{\circ}\text{C}$ with the exception of one week when the water was at 20.7°C (this temperature change did not appear to affect the egg quality). Thirty hours

after fertilisation, 400 eggs from each incubator were examined under a binocular microscope to determine fertilisation success (percentage of eggs with developing embryos). To assess the time stripped ova could be preserved prior to fertilisation without compromising fertilisation success, duplicate batches of ova stored at $\sim 20^{\circ}\text{C}$ (temperature of the room where the bowls were left) were inseminated every 0.5 h after stripping and fertilisation success was determined as described above.

2.4. Minimum and optimal spermatozoa : egg ratio

Once the optimal timing of stripping to obtain the highest quality eggs was established, insemination was carried out at different spermatozoa : egg ratios in order to establish the minimum number of spermatozoa required to obtain maximal fertilisation. Freshly collected sperm from individual fish was diluted 1/4 in modified Leibovitz culture medium with BSA (as described above). Aliquots of 0.5 mL ova (approximately 800 eggs) stripped from a single female were placed in beakers together with sperm in different insemination doses (each dose in triplicate) and 100 mL of seawater for activation and fertilisation. The experiment was repeated with ova from two females and sperm from two males, resulting in four different male-female combinations of each spermatozoa : egg ratio. Three to 5 min after fertilisation an additional 200 mL of seawater was added to each beaker and the eggs were allowed to develop (at $\sim 20^{\circ}\text{C}$). After 2 h, fertilisation success (percentage of eggs in the 4-8 cell stage) of 100 randomly chosen eggs from each batch was assessed under a binocular microscope. Sperm quality and concentration was evaluated (as described above) and the amount of motile spermatozoa per egg was estimated according to this evaluation.

2.5. Statistical analysis

Data normality and homogeneity of variance were analysed using the Shapiro-Wilk and Levene tests, respectively. Analyses of spermatozoa density, motility and VAP of sperm collected before and after the GnRHa treatment, and in sperm samples stored during the inseminating protocols were made through one-way repeated measures ANOVA using SigmaPlot version 12.0 (Systat Software Inc., Richmond, CA, USA). Regression and correlation analysis were made using SigmaPlot. Differences in mean oocyte diameter were examined using a t-student or the equivalent Mann-Whitney test when data was not normally distributed. Fertilisation success in relation to different spermatozoa: egg ratio and variation of sperm quality parameters in relation to time after activation were examined using one-way ANOVA, followed by the post hoc Tukey's HSD test (for data with equal variances) or Games-Howell's test (for data that did not meet the homogeneity of variances assumption). The distribution of spermiation stage before and after GnRHa application was compared with the Chi-squared test against expected values. The expected values were that GnRHa had no effect (null hypothesis) and that the spermiation stage was the same before and after GnRHa application. Analyses were performed using SPSS software version 20.0 (Armonk, NY: IBM Corp). A level of $P < 0.05$ was considered to be significant and the results are expressed as mean \pm standard deviation of the mean.

3. Results

3. 1. Sperm characterization, storage and quality

During the experimental period (May-June), no significant variation was observed in sperm parameters and generally, spermiation stage was classified as fluent (stage 2) and

very fluent (stage 3). However, at the end of the spawning season (the 21st of June), three of the four males examined had only a few drops of sperm and no sample could be obtained (spermiation stage 1) whilst just one male was fluent (stage 2). At the examination 24 hours after the application of GnRH α , the spermiation stage of these four males was very fluent (stage 3). Over the entire spawning period, 18 applications of GnRH α were made to the males and after 24 h more fluent males were observed. The distribution after GnRH α application of 17 males at stage 3 and one at stage 2 was significantly ($P < 0.0001$) different from before GnRH α application with a distribution of three males at stage 1; nine at stage 2 and six at stage 3.

There were no significant differences in spermatozoa density, motility percentage, motility duration or VAP between sperm samples obtained before and after GnRH α treatment (Table 1). However, there were differences in the rate of decline in motility and VAP after sperm activation between sperm samples obtained before and after GnRH α application (Fig. 1). The decline was described by two different cubic polynomial functions. The decline in motility of samples collected before GnRH α injection was described by the equation: $y = 0.0003x^3 - 0.0517x^2 + 2.362x + 23.7591$ ($R^2 = 0.9933$). The equation indicated that for samples obtained before GnRH α injection, the percentage of spermatozoa motility decreased quickly after activation, exhibiting 50% of motility 43 s after activation and a significant decrease from initial values after 55 seconds. On the contrary, in samples obtained after the GnRH α injection the decrease in motility was more gradual and 50% of motility was still exhibited 62 s after activation, as described by the following equation: $y = -0.0001x^3 + 0.0131x^2 - 0.6617x + 74.0331$ ($R^2 = 0.9683$). After 75 s, a significant decrease in motility was observed compared to initial values. The VAP exhibited the same pattern; initial values

of VAP were maintained without significant differences for a longer period in sperm obtained after GnRHa injection (45 s) compared to sperm obtained before (35 s).

Storage of sperm up to 7 h in modified Leibovitz did not affect sperm quality characteristics. Duration of motility, percentage of motility and VAP of fresh diluted sperm immediately after collection (1.67 ± 0.23 min, 63.49 ± 16.68 % and 101.39 ± 18.51 $\mu\text{m/s}$, respectively) did not vary significantly either after 3 h (1.62 ± 0.33 min, 59.73 ± 20.92 % and 93.03 ± 13.82 $\mu\text{m/s}$) or 7 h (1.19 ± 0.43 min, 49.36 ± 31.18 % and 88.32 ± 19.56 $\mu\text{m/s}$) of storage at 4°C.

3.2. Timing of ovulation and ova viability

A total of 23 GnRHa applications were made to eligible females to induce ovulation. The success to induce ovulation was 57% (13 ovulations from 23 inductions) with two ovulations in 2015 and 11 ovulations in 2016. In 2016, ovulation was detected at 35 - 39 h after injection depending on the female. A total of three females had ovulated at 35 - 36 h and a further eight females had ovulated at 38 - 39 h. The two inductions in 2015 had later ovulation times from 39 - 41 h. The mean diameter of the most advanced vitellogenic oocytes in females that ovulated in 2016 (670 ± 39 μm) was significantly greater ($P = 0.015$) than in females that ovulated in 2015 (590 ± 2 μm). There was no correlation between oocyte diameter and ovulation (latency) time. The eggs from the 13 successful ovulations were stripped in a time series every 2.5 h to evaluate changes in fertilisation success after ovulation. Three types of regressions, sigmoid, Gaussian peak and lineal described the subsequent changes in fertilisation rates over time for each ovulation (Fig. 2). There was a high variability in the fertilisation success of ova obtained from 35 to 36 h between females (33 ± 43.5 %) while from 38 to 39 h ($51 \pm$

28.0 %) this variability was reduced. The fertilisation success during the period 35 - 36 h that represented the first examination for ovulation was clustered in two groups (bimodal) with poor eggs (<20% fertilisation) and good eggs (>60% fertilisation). The good eggs were obtained from females that had fully ovulated and ova was easily obtained applying little abdominal pressure. The fertilisation rates of the subsequent stripping of ova from these females were described by Sigmoidal functions that represented initial high fertilisation rates that were maintained for at least 2.5 h with a subsequent linear decrease in fertilisation rates. The two inductions in 2015 were also described by the Sigmoidal function. The poor eggs from the 35 - 36 h appeared to be related to incomplete ovulation as all five ova batches were of small volumes as few ova were free in the abdominal captivity. In all cases, in the subsequent examination for ovulation (38 to 39 h), ova were more fluid and fertilisation rates increased before a drastic decrease appeared after 40 h. The fertilisation success of the subsequent examinations after the initial poor ova were described by either a Gaussian peaked curve or a linear regression. The Gaussian peak, described a progression from low to high fertilisation, followed by a decrease in fertilisation success. The linear regressions described a linear increase in fertilisation, which was similar to the increase in fertilisation rates observed in the Gaussian curves. All females induced in 2016 exhibited a decrease in fertilisation after 40 h. Although there were differences in the initial time of ovulation both in 2016 and especially between 2016 and 2015, the relationship between fertilisation and time was represented by a Sigmoidal regression ($P = <0.0001$ and $R^2 = 0.9175$) (Fig. 3). The maximum period of egg viability after ovulation was 3 h when the first revision coincided with ovulation. Therefore, the window to obtain viable eggs extends from ovulation to 3 h after ovulation. After this period, fertilisation decreased drastically.

Ova that were stored *in vitro* at 20°C maintained viability for up to 2 h. There was an initial small decline in fertilisation during the first 30-60 min, fertilisation stabilised at approximately 80% after 2 h, after which there was a rapid decline (Fig. 4).

3.3. Optimal spermatozoa : egg ratio

Spermatozoa motility for males was 70.71 ± 5.37 % and was used to adjust the dosage of spermatozoa per egg. There was no significant difference in the fertilisation success between the two males used for each female ($21 \pm 11.2\%$ and $25 \pm 14.3\%$). However, there were significant differences in fertilisation success between different females. The data for the two males used to fertilise eggs from each female was, therefore, combined to obtain two logarithmic regressions with a $R^2 = 0.964$ and $R^2 = 0.937$, respectively. A progressive rise in the fertilisation rates with the increase in spermatozoa : egg ratio with a final stabilisation was observed in both cases. Both females exhibited an increase in fertilisation from a ratio of approximately 2,000 motile spermatozoa : egg until 70,000 motile spermatozoa : egg. After 70,000 spermatozoa : egg the regression equations levelled off and further increases in fertilisation rates were low. This indicated that a spermatozoa : egg ratio in excess of 70,000 gave optimal fertilisation. However, comparing means indicated a significant difference for one female from 70,000 to 180,000 spermatozoa : egg ratio indicating that a spermatozoa : egg ratio in excess of 150,000 may be the most desirable ratio to ensure optimal fertilisation (Fig. 5).

4. Discussion

The present study has established a protocol for the *in vitro* fertilisation of meagre with a description of the management of female and male gametes. The window of

availability of optimal ova quality extended from when ovulation was complete until 3 h after ovulation. At 18°C with the described stock, maturity stage, handling and holding conditions, ovulation occurred within 35 to 41 h after GnRH α injection at a dose of 15 $\mu\text{g kg}^{-1}$, and at 38 - 39 h the majority of fish had completed ovulation and exhibited a peak in ova quality. Sperm could be stripped prior to the expected timing of ovulation of females, diluted in an adapted medium of Leibovitz (1/4) and stored for up to 7 h without affecting spermatozoa motility or velocity. A spermatozoa : egg ratio of 70,000 to 150,000 gave the highest fertilisation success with good quality sperm (>60% spermatozoa motility) after the administration of GnRH α , which was shown to enhance spermiation (spermiation stage) and extend the period of time that spermatozoa maintained high levels of motility and velocity before a significant decrease.

Meagre is an anadromous species that migrates to estuaries to spawn (Haffray *et al.*, 2012). Females have a group-synchronous ovarian development that means that two or more groups of oocytes are present in the ovary during the spawning period (Duncan *et al.*, 2012; Duncan *et al.*, 2018) to prepare for different spawning events during the spawning season. This configuration is typical of iteroparous species that spawn two or more times with a separation of a few days or weeks between each spawn. This spawning strategy in the meagre was manipulated with repeated injections of GnRH α to induce weekly spontaneous tank spawning of captive stocks (Mylonas *et al.*, 2016; Duncan *et al.*, 2018). The present study aimed to take this a step further to strip the ovulated ova for *in vitro* fertilisation procedures.

Determining the time of ovulation and the period of optimum egg ripeness during which eggs have maximum fertilisation success is important when *in vitro* fertilisation is necessary or required for breeding programs (Samarin *et al.*, 2015). Ovulated ova age over time: exhibiting ripening with improving viability, ripeness with optimal viability

and then overripening when the ova lose viability (Bromage, 1995; Mañanós *et al.*, 2008; Samarin *et al.*, 2015). Overripening has been described as morphological, physiological, biochemical, histological, cellular and molecular changes in the egg (see review Samarin *et al.*, 2015) and the associated decrease in fertilisation success has been attributed to hypoxia, changes in membrane permeability or a decrease in egg ATP stores. The period of egg ripeness and maximum fertilisation success varies amongst species and with temperature (Bromage, 1995; Samarin *et al.*, 2015). For example, it was reported to be 3 - 6 h for Senegalese sole (*Solea senegalensis*) at 16 °C (Rasines *et al.*, 2012), 5 - 15 days over a temperature range of 10 - 17°C for rainbow trout (*Oncorhynchus mykiss*) (Samarin *et al.*, 2008), but only 30 min in white bass (*Morone chrysops*) at 22°C (Mylonas *et al.*, 1996). In the present study with meagre, there appeared to be a sequence, starting with egg ripening followed by a window of optimal ripeness and finally overripening when eggs lost viability, similar to that described for rainbow trout (Bromage, 1995). The ripening period appeared to extend from the initiation of ovulation until ovulation was complete as some females that had incomplete ovulation in the first revision (35 - 36 h) when a low quantity of ova with low fertilisation was obtained, had completed ovulation by the time of the subsequent revision (38 - 39 h). In this revision, greater quantities of ova with higher fertilisation were obtained to indicate that the ova had ripened. Once ovulation was complete, a window of optimal ripeness with high fertilisation rates was maintained for at least 2.5 h as shown in females with high fertilisation in both the initial and the second examination. A sigmoidal regression ($R^2 = 0.9175$) based on all these females (Fig. 3) described a 3 h period of high fertilisation rates before a reduction in fertilisation rates was observed. The rapid decline in fertilisation rates after the window of egg ripeness

was indicative of the overripening process that has been described in many species (Bromage, 1995; Samarin *et al.*, 2015).

This short 3 h period of ripeness in meagre, highlights the importance of identifying the exact timing of ovulation, in order to time stripping to optimize the acquisition of good quality ova. Identification of the correct time for stripping also leads to a reduction in multiple handling of fish to detect ovulation, which can have severe effects in the egg quality (Zohar and Mylonas, 2001). The timing of ovulation in other finfish species has been shown to be affected by different causes, such as the hormone dose (Rasines *et al.*, 2012), the time of day of the hormone administration (Shiraishi *et al.*, 2008), the rearing temperature (Samarin *et al.*, 2008) and the stage of ovarian development at the time of treatment (Duncan *et al.*, 2003). In the present study, under the described holding conditions, ovulation occurred within 35 to 41 h after GnRH α injection. Ovulation was detected at different times (a) in females stripped in different years and (b) in females stripped in the same year. Perhaps the most notable observation was, in 2015, ovulation was detected 39 to 41 h post-injection, while in 2016 ovulation occurred within 35 to 39 h after GnRH α injection. The study was performed in both years with the same GnRH α dose, administered at the same time of the day and the same rearing temperature. Therefore, the different stage of gonadal development of females between the two years may be one reason for the different timing of ovulation observed. Females in 2016 that had a higher mean diameter of the largest vitellogenic oocytes ($670 \pm 39 \mu\text{m}$) prior to GnRH α injection ovulated earlier than females that contained oocytes close to $550 \mu\text{m}$ (2015). The observation that larger oocytes matured and were ovulated earlier agrees with the positive correlation between oocyte diameter and ovulation time observed in the bullseye puffer (*Sphoeroides annulatus*) (Duncan *et al.*, 2003). However, there was no correlation between oocyte diameter and time to ovulation in the present study,

which may be because a narrow range of oocyte diameter existed among the different eligible females. In 2016, no differences in initial gonadal stage were registered, as females were selected with similar oocyte diameter, and oocyte size does not appear to explain the differences in ovulation responses that were observed. At 35 h post-injection, in some females, the first ova that were obtained by hand-stripping had high fertilisation success which suggests that ovulation was complete before the females were examined. In other females, at this same time, ovulation was incomplete as the number of ova collected was low and those ova from partially ovulated females had low fertilisation. However, at the subsequent examination for ovulation (38 - 39 h post-injection) the highest fertilisation was obtained in almost all females. The females with good egg quality at 35 h maintained high fertilisation and those that gave at first low quantity and quality ova completed ovulation to give ova in greater quantities with higher fertilisation rates. The combination of a 3 h window for the highest ova quality and the poor quality of ova stripped in the early stages of ovulation (at 35 h) that subsequently improved, indicated that females that ovulated close to 35 - 36 h were perhaps best left and stripped at 38 - 39 h. Therefore, altogether the present study has identified that most fish had ovulated and had highest egg quality at 38 - 39 h after GnRH α treatment. However, it is clear that many factors influence the timing of ovulation and future studies with different stocks, husbandry conditions, stages of maturity, etc. must expect deviations from the stripping time in the present study, which can be used as a reference point.

Variations in egg quality between females is another factor that can influence fertilisation rates (Springate *et al.*, 1984). Fertilisation depends both on ova and sperm quality. Ova quality refers to the eggs developmental competence that means the ability of the ova to be fertilised and to develop into normal embryo. Sperm quality refers to

the ability of spermatozoa to fertilise an egg and allow the development of a normal embryo (Migaud *et al.*, 2013). To avoid the effect of sperm quality on fertilisations, sperm from two different males was mixed to fertilise all batches of eggs from different retention times and females. In addition, sperm characteristics were evaluated to ensure the same fertilising ability. Thus, the differences observed may be due to the different quality of ova that has been observed in fish from the same stock (Springate *et al.*, 1984).

Once the gametes have been obtained from the fish for *in vitro* fertilisation, the length of time spermatozoa and oocytes survive is an important logistic factor to consider (Billard, 1988). The viability of ova *in vitro* depends on temperature and has been reported to be species-specific. For instance, in curimata (*Prochilodus marggravii*), fertilisation success of eggs stored at 18°C was drastically reduced when compared with storage at 26°C. The eggs stored at 26°C in curimata lost viability almost completely 2 h after stripping (Rizzo *et al.*, 2003) while in kutum (*Rutilus frisii kutum*) at the same temperature viability was lost after 4 h (Samarin *et al.*, 2015). According to the results obtained in this study, eggs from meagre have a relatively short period of viability after stripping, during which successful fertilisation can occur while eggs are maintained at hatchery temperature. Therefore, we suggest that meagre eggs should be fertilised within the first 50 min post-stripping to achieve high fertilisation success, as a decrease in fertilisation rates were detected with time with a rapid decrease in viability occurring from 2.5 to 4 h post-stripping.

Gamete survival with respect to sperm quality must also be controlled for a proper *in vitro* fertilisation. The experiment conducted in this study showed that when sperm was stored in modified Leibovitz culture medium, the spermatozoa of meagre retained the same initial motility percentage, duration of motility and velocity (which relates to

fertilizing capacity) within at least 7 h at 4°C. A recent study demonstrated the possibility to store meagre sperm at a 1/4 dilution in 0.9% NaCl at 4°C for up to 10 days (Santos *et al.*, 2018) and would improve artificial fertilisation techniques. The values of meagre sperm concentration ($3.21 \pm 1.18 \cdot 10^{10}$ spz mL⁻¹), spermatozoa motility duration (1.71 ± 0.29 min) and initial motility (58 ± 12 %) recorded in the present study were similar to other recent values published for meagre (Mylonas *et al.*, 2013a; Schiavone *et al.*, 2012; Santos *et al.*, 2018). Initial velocities recorded here (90.69 ± 5.76 µm/s) were lower, but in the range to those obtained by Santos *et al.* (2018) (140.90 ± 7.75 µm/s) and were a characteristic of fast sperm (Gallego *et al.*, 2013). In the present study, the decay in initial motility and velocity values with time after activation was also examined as fertilising ability could be limited to the first seconds after activation. The percentage of motility gradually declined and became significantly lower than initial values after 55 s and exhibited $\geq 50\%$ motility until 43 s after activation. The initial velocity values for meagre declined after 35 s post-activation. Spermatozoa 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 spermatozoa velocity (Gallego *et al.*, 2013). Thus, if spermatozoa velocity is highly correlated with fertilising ability, the period of sperm fertility could be reduced to 35 s in meagre. However, as seen in the present study, GnRHa administration to males maintained initial values of motility percentage (with $\geq 50\%$ until 62 s) and velocity for a longer period, 75 and 45 seconds after activation, respectively, thus potentially increasing the period of fertilising ability. Therefore, male therapy with GnRHa is recommended because (a) the period in which sperm of good motility and velocity was enhanced or lengthened and (b) spermiation stage -and thus sperm production or availability for

stripping- was enhanced, especially towards the end of the season when spermiation stage declined naturally. Mylonas *et al.* (2016) also reported a decline in meagre sperm availability at the end of the spawning season.

Assuming both high quality male and female gametes have been obtained and managed correctly, in order to ensure the success of *in vitro* fertilisation protocols, an important aspect is to determine the appropriate spermatozoa : egg ratio for maximum fertilisation success. Usually, an excess of sperm is used in insemination procedures (Gallego *et al.*, 2013), but an appropriate combination of the numbers of spermatozoa per oocyte should improve the fertilisation outcome and significantly reduce sperm waste. The spermatozoa : egg ratio is different between species. For example, the optimal spermatozoa : egg ratio can range from 66,000 in European seabass (*Dicentrarchus labrax*) (Fauvel *et al.*, 1999) to 1,000,000 in northern pike (*Esox Lucius*) (Zhang *et al.*, 2011). The present study, showed that maximum fertilisation in meagre occurred within a wide range of spermatozoa : egg ratios. Different characteristics, such as spermatozoa swimming distance (Sanches *et al.*, 2016), micropyle closing time (Suquet *et al.*, 1995), properties in eggs that may attract spermatozoa, as well as egg size are determinants in the spermatozoa : egg ratios among fishes (Bombardelli *et al.*, 2013). Meagre eggs are small, 0.90 mm in diameter (Cárdenas, 2010), compared to the 2.32 mm of northern pike (Murry *et al.*, 2008) so a lower spermatozoa : egg ratio should be needed in the case of meagre since at a fixed spermatozoa density per egg the probability of a spermatozoa reaching the micropyle should be higher. However, both meagre and European seabass have similar egg size (Froese and Pauly, 2015), so the other factors mentioned above may have relevance. Therefore, in the case of meagre, with initial velocities values no more than 35 s after activation, if velocity is highly correlated with fertilising ability, the period of sperm fertility could be short and a high amount of

spermatozoa per egg should be needed. In the present study, both males had good sperm quality (> 60% spermatozoa motility) and the same regression was obtained in the fertilisation rate obtained for each female. This suggests that the wide range found in optimal spermatozoa to egg ratio was due to a difference in the quality of the female gametes, which has been previously observed to be crucial in the fertilisation success achieved in the timing of an ovulation experiment. Bombardelli *et al.* (2013) observed in cascudo-preto (*Rhinelepis aspera*) that more sperm was needed for inseminating batches of ova of lower viability to reach the maximum fertilisation success. However, in the present study, the opposite was found and ova with higher quality required more sperm than ova of poorer quality. This observation would suggest that sperm were not attracted to poor ova and that poor quality batches that have less viable ova need less sperm to achieve maximum rates of fertilisation. Although, in the present study there were only two batches of ova (with duplicate males), this appears to be true as maximum fertilisation of the eggs was approximately double (35% compared to 70% fertilisation) indicating that twice as many ova were available to be fertilised in the good quality batch of ova. Therefore, spermatozoa requirements were double (70,000 compared to 180,000) for the good quality batch of ova, as double the number of ova required fertilisation. These arguments would indicate that 150,000 spermatozoa were required per egg of good quality. It appears that the sperm requirement of eggs depends on the quality of ova. Therefore, both sperm quality and ova quality should be taken into account to determine spermatozoa egg ratio making it possible to obtain high fertilisation rates. This also highlights that the use of high-quality gametes, both from males and females, is an essential factor to reach proper fertilisation both for aquaculture and scientific purposes. However, to have high quality viable gametes at the moment of fertilisation to obtain suitable fertilisation rates in a protocol for *in vitro*

fertilisation requires attention to many factors including those researched in the present study, reproductive traits, individual variability, timing of taking the gametes from the fish, protocols for ova and sperm storage before fertilisation and characteristics of ova and sperm quality.

5. Conclusion

The present study has determined the parameters required for a protocol for *in vitro* fertilisation for meagre. The window of optimal ova quality extended from when ovulation was complete until 3 h after ovulation. Under the described experimental conditions, the optimal time for stripping females during this 3 h window was 38 - 39 h after a single GnRHa injection, which represents a reference time for future studies. Once stripped, meagre ova should be fertilised within the next 50 min. Sperm diluted in an adapted medium of Leibovitz (1/4) can be stored for up to 7 h without affecting spermatozoa motility percentage or velocity. Although sperm quality overall was similar before and after GnRHa treatment, the use of GnRHa is recommended in order to increase sperm availability and increase the period of high spermatozoa motility and velocity after activation. A minimum of 150,000 spermatozoa per egg was optimal to ensure high fertilisation success. This knowledge will aid the aquaculture industry and future research in using *in vitro* fertilisation for meagre selective breeding programs.

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Figure legends

Table 1. Sperm quality parameters of sperm samples obtained immediately before and 24 hours after a GnRH α injection. Mean (\pm standard deviation) values of sperm from 5 males that were used to fertilise eggs during the weekly fertilisation trials. There were no significant differences in any of the parameters before and after hormonal treatment.

Figure 1. Mean (\pm standard deviation, n = 2-5) of meagre (*Argyrosomus regius*) sperm quality parameters. (A) Percentage of motile spermatozoa (%) and (B) Average Path Velocity (VAP) of spermatozoa ($\mu\text{m/s}$) over time before (back circles) and after (open white circles) GnRH α injection during the experimental period. Different capital and small letters indicate significant differences ($P < 0.05$) over time after spermatozoa activation of samples collected before and after GnRH α injection, respectively. The decline in sperm quality parameters is represented by cubic regressions with the following equations $y = 0.0003x^3 - 0.0517x^2 + 2.362x + 23.7591$ ($R^2 = 0.9933$) and $y = -0.0001x^3 + 0.0131x^2 - 0.6617x + 74.0331$ ($R^2 = 0.9683$) for motility decay before and after GnRH α injection, respectively. The horizontal line indicates the 50% of motility. Regression coefficients for VAP are $R^2 = 0.9951$ and 0.9895 .

Figure 2. Fertilisation success of eggs stripped at different times after GnRH α injection for individual meagre (*Argyrosomus regius*) ovulations. Different symbols represent different ovulations and the change in fertilisation success over time after induction for each ovulation is represented by either a sigmoid, Gaussian peak or lineal regression. Thick lines represent ovulations from 2015. All regressions were significant ($P < 0.05$) and regression coefficients ranged from $R^2 = 0.773$ to 0.990 .

Figure 3. Evolution of egg fertilisation success of meagre (*Argyrosomus regius*) eggs obtained by stripping after GnRH α treatments, over time after the occurrence of optimal fertilisation success (time 0). In females stripped close to ovulation (0 time point) eggs retained high fertilisation (viability) for 3 hours. Fertilisation decreased drastically to 0% between 3 and 6 hours after ovulation, which is indicative of the overripening period. The black lines represent the Sigmoidal regression ($P < 0.0001$ and $R^2 = 0.9175$) that described the relationship between fertilisation and time, the blue lines represent 95% confidence intervals and the red lines represent 95% prediction of values.

Figure 4. The fertilisation success over time of maintaining meagre (*Argyrosomus regius*) ova in the laboratory at 20°C before fertilisation. The relationship was described by a quadratic regression ($P < 0.05$, $R^2 = 0.927$).

Figure 5. Mean fertilisation of meagre (*Argyrosomus regius*) eggs fertilised *in vitro* at different spermatozoa : egg ratios. The error bars indicate the standard deviation of mean. Significant differences are indicated by different letters (capital letters for fertilisation of eggs obtained by the combination of female 1 with males 1♂ and 2♂, and lowercase letters for the combination of female 2 and males 1♂ and 2♂) ($P < 0.05$).

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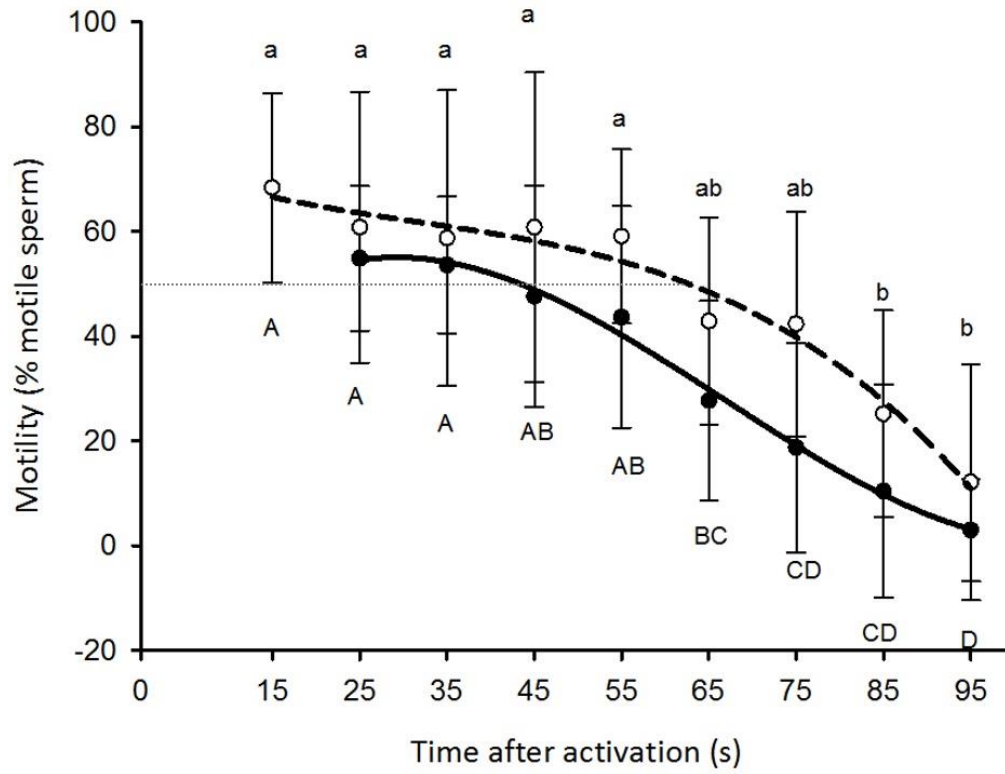
Table 1.

Sampling time in relation to GnRHα injection	Spermatozoa concentration (10^{10} spz ml$^{-1}$)	Spermatozoa duration of motility (min)	Initial motility (%)	Initial velocity (VAP, μm/s)
Before	3.21 ± 1.18	1.71 ± 0.29	58.17 ± 12.80	90.69 ± 5.76
After	2.76 ± 0.62	1.57 ± 0.50	66.76 ± 15.83	98.07 ± 11.68

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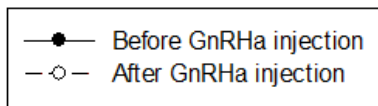
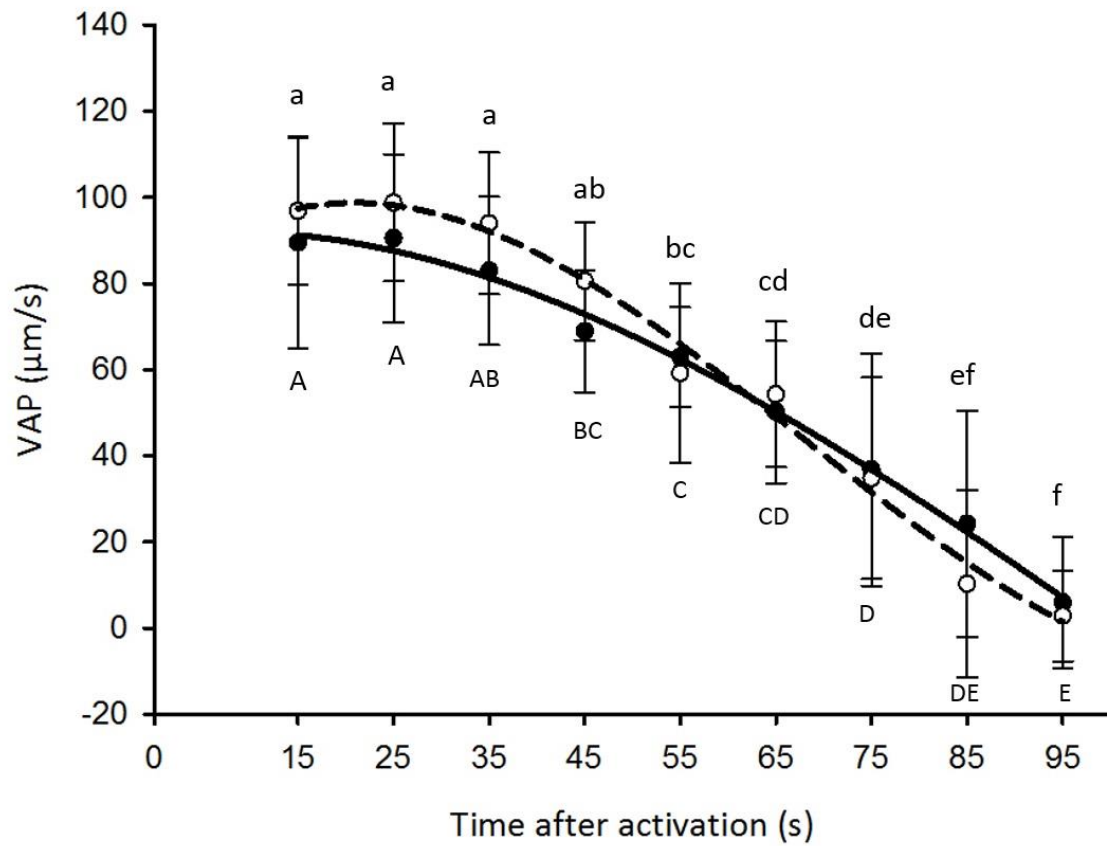
Figure 1

A



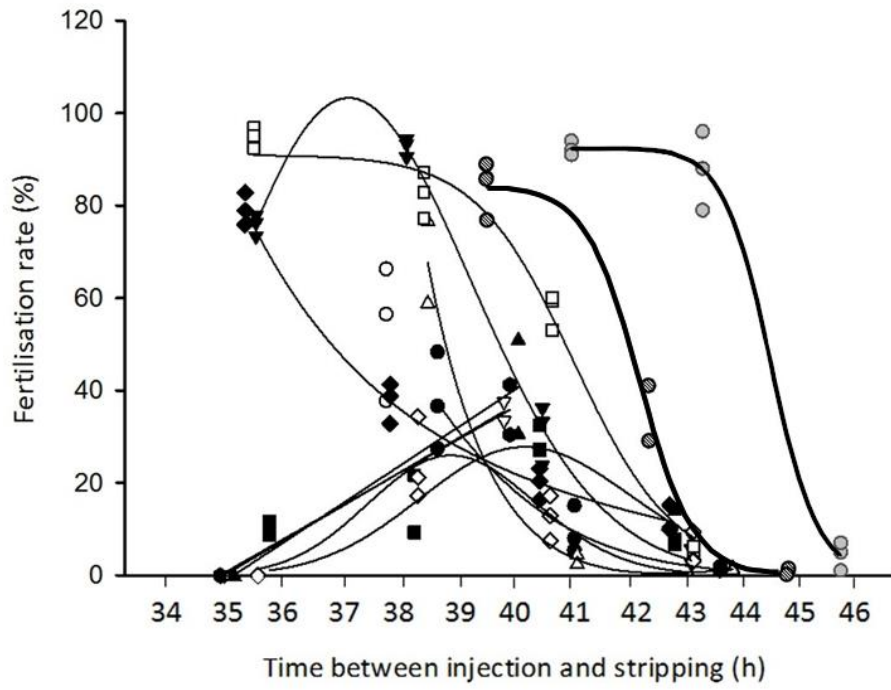
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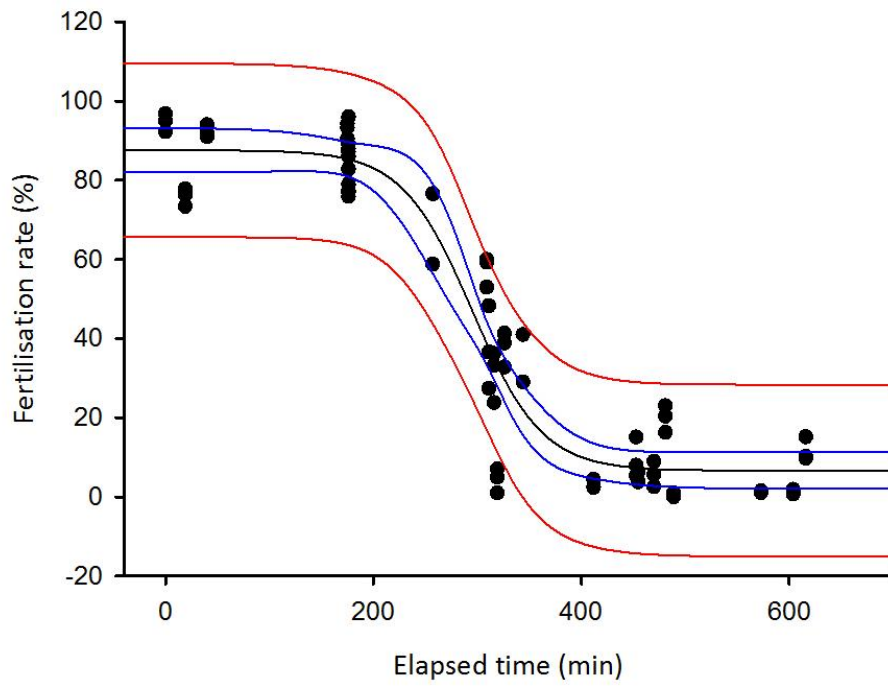
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Figure 2.



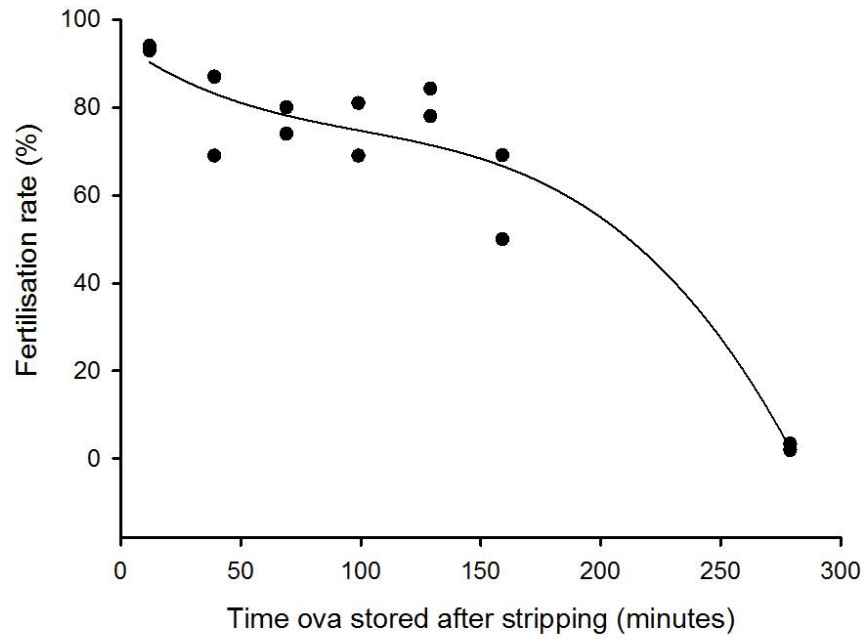
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Figure 3.



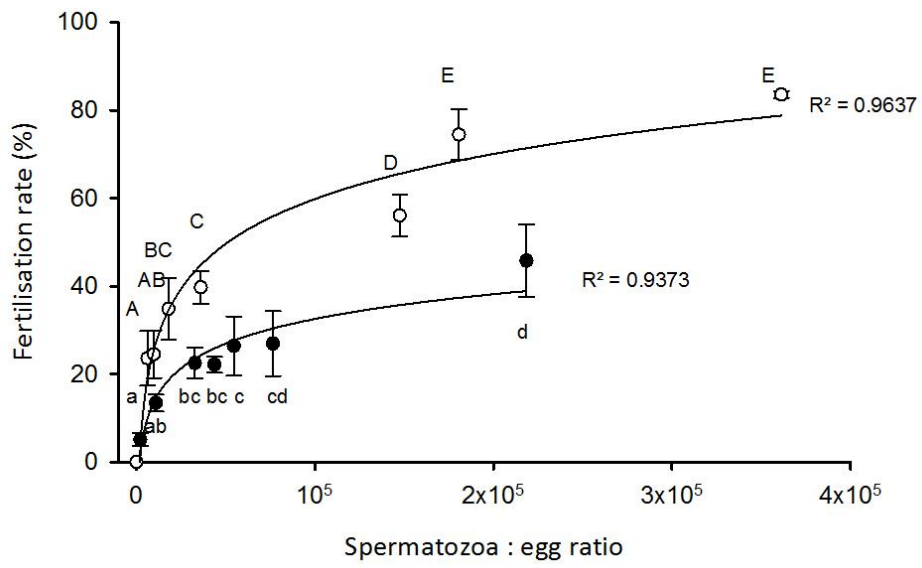
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Figure 4.



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Figure 5.



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Highlights of the manuscript

- An *in vitro* fertilisation protocol has been developed for meagre (*Argyrosomus regius*).
- Ovulation took place close to 38 h and in the range of 35 - 41 h after GnRH α induction at 18°C.
- Good quality eggs were collected within a three-hour window from ovulation.
- The spermatozoa : egg ratio for high fertilisation success was 150,000 spermatozoa egg⁻¹.

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