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

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16 **Abstract**

17 The aquaculture of meagre (*Argyrosomus regius*) requires methods for the control of
18 reproduction that enable the production of families from specific individuals for selective
19 breeding programs. We experimentally determined the parameters required for an *in vitro*
20 fertilisation protocol. A total of 14 females and 5 males (mean ± S.D. weights of 20.45 ±
21 6.22 and 15.94 ± 2.75 kg, respectively) were used. Selected females had vitellogenic
22 oocytes >550 µm in diameter and males had fluid sperm upon application of abdominal
23 pressure. Both sexes were treated with an injection of 15 µg kg^{-1} of gonadotropin-
24 releasing hormone agonist (GnRHa) to induce oocyte maturation/ovulation and enhance
25 sperm production. To determine the timing of ovulation and window of high egg viability,
26 females were stripped serially every 2.5 h beginning 35 h after GnRHa treatment. Sperm
27 was obtained 24 h after GnRHa treatment and was diluted 1/4 in modified Leibovitz for
28 storage at 4ºC until use. Sperm quality parameters such as percentage initial spermatozoa
29 motility, duration of motility, velocity and density were determined using computer
30 assisted sperm analysis (CASA). *In vitro* inseminations were made in duplicate or
31 triplicate batches of eggs from each spawn by mixing 0.5 - 1 mL of eggs, 20 - 40 µL
32 diluted sperm (pooled from two males) and 100 mL of seawater. Fertilisation success was
33 examined at spermatozoa (spz): egg ratios between ~ 2,000 to 400,000 spz egg^{-1}. The
34 optimal time for stripping ovulated females was ≤3 h after ovulation, which was the
35 window of optimal egg viability. Ovulation under the conditions of this study was close
36 to 38 h after GnRHa treatment, with a range from 35 - 41 h. Beginning from 3 h after
37 ovulation, egg viability declined probably due to overripening. Sperm diluted in Leibovitz
38 maintained motility and velocity for as long as 7 h after collection. Spermatozoa motility
39 (%) and average path velocity (VAP, µm/s) of sperm samples obtained from males before
40 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.

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

48 **Highlights of the manuscript**

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

55

56 **1. Introduction**

57 The meagre (*Argyrosomus regius*) is a sciaenid fish found in the Mediterranean and Black
58 Sea, and along the eastern coast of the Atlantic Ocean. This species is a suitable candidate
59 for the diversification of aquaculture in the Mediterranean, due to a number of attractive
60 market and biological attributes. Market attributes include a large size, good processing
61 yield, low fat content, excellent taste and firm texture (Monfort, 2010). The biological
62 characteristics include fast growth of ~ 1 kg year $^{-1}$ (Duncan *et al.*, 2013a), low feed
63 conversion ratio of 0.9-1.2, relatively easy larval rearing (Vallés and Estévez, 2013) and
64 an effective spawning protocol based on multiple injections of a synthetic agonist of
65 gonadotropin-releasing hormone (GnRHa) (Duncan *et al.*, 2012; 2013a; Mylonas *et al.*,
66 2013a; 2015; 2016; Fernández-Palacios *et al.*, 2014).

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

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

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

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

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

127

128 2. Materials and methods

129 **2.1. Broodstock management**

130 The broodstock consisted of wild specimens caught off the south coast of Portugal and
131 cultured specimens reared in the Canary Islands and IRTA Sant Carles de la Ràpita. In
132 the years prior to the experiment, fish were held in two thermally isolated 70-m³ circular
133 tanks in a recirculating system with a natural photoperiod and a controlled natural
134 temperature cycle. The mean temperature during the experiment was 18.6 ± 0.5ºC. The
135 broodstock was fed four days a week on a commercial broodstock diet (MAR VITALIS
136 REPRO, Skretting, Spain) and one day a week with frozen sardines and squid.

137 During the first week of July 2015 and from the first week of May to the last week of
138 June 2016, the maturity status of randomly selected males and females was examined on
139 a weekly basis. On each date, ovarian and sperm samples were obtained after
140 anaesthetizing the fish with 70.6 mg L^{-1} MS-222 (Tricaine methane-sulfonate). Ovarian
141 biopsies were obtained by cannulation according to the protocol of Duncan *et al*. (2012).
142 Briefly, a plastic catheter (2 mm x 470 mm) was inserted approximately 10-15 cm into
143 the gonopore and a slight suction was applied. Fresh ovarian samples were examined
144 initially at 5x magnification in clearing solution (6 mL absolute ethanol, 3 mL formalin,
145 2 mL glycerol) and the diameter of the 10 largest (most advanced) vitellogenic oocytes
146 was recorded. Females were considered eligible for spawning induction if they contained
147 oocytes in full vitellogenesis with a diameter >550 µm (Duncan *et al*., 2012). From the
148 chosen females, the diameter was recorded of 100 randomly chosen oocytes and 35 of the
149 largest oocytes. In this way, every week females with oocytes with a diameter >550 µm
150 were selected and placed in 16-m³ tanks and were induced with an intramuscular injection
151 of 15 µg kg^{-1} of the GnRHa des-Gly^{10}, [D-Ala^{6}]-gonadotropin releasing hormone (Sigma,
152 Spain), as proposed by Fernández-Palacios *et al*. (2014). Maturation of the males was
153 examined by the release of sperm upon application of gentle abdominal pressure and

154 spermiation stage was determined on a scale from 0 to 3 (0 = not fluent, 1 = only a few
155 drops of sperm released and no sample can be obtained, 2 = fluent, 3 = very fluent with
156 minimal abdominal pressure). Males were also subject to stimulation by GnRHa
157 intramuscular injection (15 µg kg^{-1}) to ensure sperm availability, which was particularly
158 necessary towards the end of the trial when quantities of sperm produced decreased.
159 Females were injected between 8 and 10 p.m. and males between 9 and 10 a.m. on the
160 day after the females were injected. Females were held in constant darkness after GnRHa
161 was applied and while checking for ovulation, and males were placed together in a
162 separate tank. Constant darkness was used, to reduce possible effects of the photoperiod
163 control on ovulation time, as meagre ovulate and spawn during the night in the wild (Gil,
164 2013) or when induced to spawn in captivity (personal observation). A total of 14 females
165 and 5 males were used in the experiments, having mean (± S.D.) weights of 20.45 ± 6.22
166 and 15.94 ± 2.75 kg, respectively. Both females and males were used multiple times in
167 the different years or weeks of the experiment. In a past study, Mylonas *et al*. (2016)
168 induced meagre females to spawn each week for up to 17 consecutive weeks. Therefore,
169 in the present study some females were used more than once, these females were checked
170 each time and eligible females selected as described above.

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

174

175 **2.2. Sperm collection and quality**

176 Before sperm collection, the genital pore was cleaned and dried, and gentle abdominal
177 pressure was applied in order to obtain sperm, avoiding any urine contamination. Sperm

178 was collected directly in one or 2-mL syringes immediately before the first checking for
179 ovulation of females was made. Sperm samples were maintained 3 cm above ice. Milt
180 was diluted 1/4 in modified Leibovitz L-15 cell culture medium as described by Fauvel
181 *et al.* (2012) for European seabass (*Dicentrarchus labrax*). Briefly, Leibovitz L-15 was
182 supplemented with 300 µg glutamine mL^{-1}, 6 mg sodium pyruvate mL^{-1} and 1 M NaOH
183 added to the initially diluted medium of Leibovitz (350 mOSm and pH 7.3) to obtain a
184 Leibovitz medium with pH 8 and 450 mOsm. In order to prevent spermatozoa activation
185 in the medium, the osmolarity was decreased to 250 mOSm by dilution in distilled water
186 (Wayman *et al.*, 1998). Gentamycin sulphate (1 mg mL^{-1}) was also added to prevent any
187 bacterial development and bovine serum albumin (BSA, 20 mg mL^{-1}), to protect the
188 plasma membrane and avoid sperm aggregation. For sperm sampling, positive
189 displacement pipettes were used. Diluted sperm samples were stored 3 cm above ice or
190 at 4ºC until required for *in vitro* fertilisation.

191 Analyses of spermatozoa motility were conducted using ImageJ (Image processing and
192 Analysis in Java, https://imagej.nih.gov/ij/) with the CASA plugin described by Wilson-
193 Leedy and Ingermann (2007). Aliquots of 10, 20 or 40 µL of Leibovitz-diluted sperm
194 (1/4), were mixed thoroughly with 1 mL of seawater with BSA (1/16) in 1.5 mL
195 Eppendorf tubes for activation, so that the observation dilution were 1/404, 1/204 and
196 1/104 depending on the density of the initial sperm sample (1/4). A 1 µL sample of this
197 dilution containing the activated spermatozoa was pipetted directly into an ISAS counting
198 chamber (Integrated Sperm Analysis System, Spain). The tracks of the activated
199 spermatozoa in the ISAS chamber were recorded as a digital video through a bright field
200 equipped video microscope at 20x magnification (Leica DMLB Microscope and DMK
201 22BUC03 Camera with 744 x 480 "0.4 MP" resolution at 60 FPS, The Imaging Source
202 Europe GmbH, Bremen, Germany). The video recording was initiated at the moment the

203 spermatozoa was activated to provide a reliable reference start point, and was ended when
204 spermatozoa ceased activity. The process of activation and filling the chamber allowed
205 secured assessment of spermatozoa activity in <15 s after activation. The videos were
206 processed using VIRTUALDUB 1.9.11 (*virtualdub.org*) free software which transforms
207 *.avi movies into image sequences (*.jpg). The image sequences were analysed with the
208 CASA plugin. The sperm parameters assessed were spermatozoa motility (%), defined as
209 the percentage of motile cells; average path velocity (VAP, µm/s), defined as the distance
210 moved by the spermatozoa head along its spatial average trajectory in a definite time lap
211 (Gallego *et al.*, 2013); and duration of spermatozoa motility (motility duration, min),
212 defined as the time between activation and end of all spermatozoa movement. Motility
213 parameters were evaluated (in triplicate) each time sperm was used in fertilisation
214 procedures to assess the changes in sperm quality. The settings for the image analysis,
215 which depend on the microscope and the movie quality, were as follows: brightness and
216 contrast, -8 to 19 and 216 to 253, respectively; threshold, 0/231 to 254; minimum
217 spermatozoa size (pixels), 30; maximum spermatozoa size (pixels), 200; then the CASA
218 settings that depend both on camera characteristics and spermatozoa features were the
219 following: minimum track length (frames), 5; maximum spermatozoa velocity between
220 frames (pixels), 40; frame rate, 30; microns/1000 pixels, 301; and the rest of parameters
221 set as default.

222 Sperm concentration (spermatozoa mL^{-1}) was also recorded for each sperm sample used.
223 In this case, previously diluted sperm (1/4) was diluted 1/125 in distilled water to obtain
224 a 1/500 dilution from which 10 µL were pipetted into a THOMA cell counting chamber
225 where it was allowed to settle for 10 min, and was then observed under the microscope at
226 10x magnification. Quantification of spermatozoa density of the collected sperm was
227 assessed using the particle analysis function of ImageJ, adjusting image brightness to a

228 range from 43 to 49, image contrast from 203 to 221, and image threshold from 225 to
229 238. These results were used to calculate the concentration of sperm used to fertilise ova
230 and in particular in the spermatozoa : egg ratio experiments.

231

232 **2.3. Timing of ovulation and ova viability**

233 To determine the time of ovulation after the administration of 15 µg kg^{-1} of GnRHa
234 (Fernández-Palacios *et al.*, 2014), females were first examined for ovulation 35 h after
235 the GnRHa injection and every 2.5 h thereafter using abdominal pressure. The initial
236 evaluation time was decided in accordance with previous observations suggesting that
237 meagre ovulated 36 - 37 h after GnRHa treatment at 19.2ºC (Fatira, 2013) and that tank
238 spawning by meagre was at 36 - 39 h after GnRHa application (personal observations).
239 Ovulation was confirmed when ova were stripped from the abdominal cavity. The time
240 ovulated ova were first detected from a female was taken as the time of ovulation and
241 used to calculate the latency time: the time elapsed between hormonal treatment and
242 ovulation. The stripped ova were collected into a dry bowl, avoiding contamination with
243 faeces or urine. The ova were inseminated with a pool of sperm from two males. The
244 spermatozoa concentration of each sperm sample was measured. Duplicates of 1 mL of
245 eggs (~1600 eggs) from each spawn were inseminated using 40 µL of mixed diluted
246 sperm (approximately 230,000 spermatozoa egg^{-1}) followed by the addition of 100 mL of
247 seawater for spermatozoa activation. After 3 min, all batches of inseminated eggs were
248 placed into separate small incubators (1.5 L capacity) with recirculating water at a
249 temperature range of 17.8 - 18.4ºC with the exception of one week when the water was
250 at 20.7ºC (this temperature change did not appear to affect the egg quality). Thirty hours
251 after fertilisation, 400 eggs from each incubator were examined under a binocular
252 microscope to determine fertilisation success (percentage of eggs with developing

253 embryos). To assess the time stripped ova could be preserved prior to fertilisation without
254 compromising fertilisation success, duplicate batches of ova stored at ~20ºC (temperature
255 of the room where the bowls were left) were inseminated every 0.5 h after stripping and
256 fertilisation success was determined as described above.

257

258 **2.4. Minimum and optimal spermatozoa : egg ratio**

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

274

275 **2.5. Statistical analysis**

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

294

295 **3. Results**

296 **3. 1. Sperm characterization, storage and quality**

297 During the experimental period (May-June), no significant variation was observed in
298 sperm parameters and generally, spermiation stage was classified as fluent (stage 2) and
299 very fluent (stage 3). However, at the end of the spawning season (the 21st of June), three

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

308 There were no significant differences in spermatozoa density, motility percentage,
309 motility duration or VAP between sperm samples obtained before and after GnRHa
310 treatment (Table 1). However, there were differences in the rate of decline in motility and
311 VAP after sperm activation between sperm samples obtained before and after GnRHa
312 application (Fig. 1). The decline was described by two different cubic polynomial
313 functions. The decline in motility of samples collected before GnRHa injection was
314 described by the equation: $y = 0.0003x^3 - 0.0517x^2 + 2.362x + 23.7591$ ($R^2 = 0.9933$).
315 The equation indicated that for samples obtained before GnRHa injection, the percentage
316 of spermatozoa motility decreased quickly after activation, exhibiting 50% of motility 43
317 s after activation and a significant decrease from initial values after 55 seconds. On the
318 contrary, in samples obtained after the GnRHa injection the decrease in motility was more
319 gradual and 50% of motility was still exhibited 62 s after activation, as described by the
320 following equation: $y = - 0.0001x^3 + 0.0131x^2 - 0.6617x + 74.0331$ ($R^2 = 0.9683$). After
321 75 s, a significant decrease in motility was observed compared to initial values. The VAP
322 exhibited the same pattern; initial values of VAP were maintained without significant
323 differences for a longer period in sperm obtained after GnRHa injection (45 s) compared
324 to sperm obtained before (35 s).

325 Storage of sperm up to 7 h in modified Leibovitz did not affect sperm quality
326 characteristics. Duration of motility, percentage of motility and VAP of fresh diluted
327 sperm immediately after collection (1.67 ± 0.23 min, 63.49 ± 16.68 % and 101.39 ± 18.51
328 µm/s, respectively) did not vary significantly either after 3 h (1.62 ± 0.33 min, 59.73 ±
329 20.92 % and 93.03 ± 13.82 µm/s) or 7 h (1.19 ± 0.43 min, 49.36 ± 31.18 % and 88.32 ±
330 19.56 µm/s) of storage at 4°C.

331

332 **3.2. Timing of ovulation and ova viability**

333 A total of 23 GnRHa applications were made to eligible females to induce ovulation. The
334 success to induce ovulation was 57% (13 ovulations from 23 inductions) with two
335 ovulations in 2015 and 11 ovulations in 2016. In 2016, ovulation was detected at 35 - 39
336 h after injection depending on the female. A total of three females had ovulated at 35 - 36
337 h and a further eight females had ovulated at 38 - 39 h. The two inductions in 2015 had
338 later ovulation times from 39 - 41 h. The mean diameter of the most advanced vitellogenic
339 oocytes in females that ovulated in 2016 (670 ± 39 µm) was significantly greater (P =
340 0.015) than in females that ovulated in 2015 (590 ± 2 µm). There was no correlation
341 between oocyte diameter and ovulation (latency) time. The eggs from the 13 successful
342 ovulations were stripped in a time series every 2.5 h to evaluate changes in fertilisation
343 success after ovulation. Three types of regressions, sigmoid, Gaussian peak and lineal
344 described the subsequent changes in fertilisation rates over time for each ovulation (Fig.
345 2). There was a high variability in the fertilisation success of ova obtained from 35 to 36
346 h between females (33 ± 43.5 %) while from 38 to 39 h (51 ± 28.0 %) this variability was
347 reduced. The fertilisation success during the period 35 - 36 h that represented the first
348 examination for ovulation was clustered in two groups (bimodal) with poor eggs (<20%
349 fertilisation) and good eggs (>60% fertilisation). The good eggs were obtained from

350 females that had fully ovulated and ova was easily obtained applying little abdominal
351 pressure. The fertilisation rates of the subsequent stripping of ova from these females
352 were described by Sigmoidal functions that represented initial high fertilisation rates that
353 were maintained for at least 2.5 h with a subsequent linear decrease in fertilisation rates.
354 The two inductions in 2015 were also described by the Sigmoidal function. The poor eggs
355 from the 35 - 36 h appeared to be related to incomplete ovulation as all five ova batches
356 were of small volumes as few ova were free in the abdominal captivity. In all cases, in
357 the subsequent examination for ovulation (38 to 39 h), ova were more fluid and
358 fertilisation rates increased before a drastic decrease appeared after 40 h. The fertilisation
359 success of the subsequent examinations after the initial poor ova were described by either
360 a Gaussian peaked curve or a linear regression. The Gaussian peak, described a
361 progression from low to high fertilisation, followed by a decrease in fertilisation success.
362 The linear regressions described a linear increase in fertilisation, which was similar to the
363 increase in fertilisation rates observed in the Gaussian curves. All females induced in
364 2016 exhibited a decrease in fertilisation after 40 h. Although there were differences in
365 the initial time of ovulation both in 2016 and especially between 2016 and 2015, the
366 relationship between fertilisation and time was represented by a Sigmoidal regression (P=
367 <0.0001 and R^2= 0.9175) (Fig. 3). The maximum period of egg viability after ovulation
368 was 3 h when the first revision coincided with ovulation. Therefore, the window to obtain
369 viable eggs extends from ovulation to 3 h after ovulation. After this period, fertilisation
370 decreased drastically.

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

374

375 **3.3. Optimal spermatozoa : egg ratio**

376 Spermatozoa motility for males was 70.71 ± 5.37 % and was used to adjust the dosage of
377 spermatozoa per egg. There was no significant difference in the fertilisation success
378 between the two males used for each female (21 ± 11.2% and 25 ± 14.3%). However,
379 there were significant differences in fertilisation success between different females. The
380 data for the two males used to fertilise eggs from each female was, therefore, combined
381 to obtain two logarithmic regressions with a $R^2 = 0.964$ and $R^2 = 0.937$, respectively. A
382 progressive rise in the fertilisation rates with the increase in spermatozoa : egg ratio with
383 a final stabilisation was observed in both cases. Both females exhibited an increase in
384 fertilisation from a ratio of approximately 2,000 motile spermatozoa : egg until 70,000
385 motile spermatozoa : egg. After 70,000 spermatozoa : egg the regression equations
386 levelled off and further increases in fertilisation rates were low. This indicated that a
387 spermatozoa : egg ratio in excess of 70,000 gave optimal fertilisation. However,
388 comparing means indicated a significant difference for one female from 70,000 to
389 180,000 spermatozoa : egg ratio indicating that a spermatozoa : egg ratio in excess of
390 150,000 may be the most desirable ratio to ensure optimal fertilisation (Fig. 5).

391

392 **4. Discussion**

393 The present study has established a protocol for the *in vitro* fertilisation of meagre with a
394 description of the management of female and male gametes. The window of availability
395 of optimal ova quality extended from when ovulation was complete until 3 h after
396 ovulation. At 18ºC with the described stock, maturity stage, handling and holding
397 conditions, ovulation occurred within 35 to 41 h after GnRHa injection at a dose of 15 µg
398 kg^{-1}, and at 38 - 39 h the majority of fish had completed ovulation and exhibited a peak

399 in ova quality. Sperm could be stripped prior to the expected timing of ovulation of
400 females, diluted in an adapted medium of Leibovitz (1/4) and stored for up to 7 h without
401 affecting spermatozoa motility or velocity. A spermatozoa : egg ratio of 70,000 to
402 150,000 gave the highest fertilisation success with good quality sperm (>60%
403 spermatozoa motility) after the administration of GnRHa, which was shown to enhance
404 spermiation (spermiation stage) and extend the period of time that spermatozoa
405 maintained high levels of motility and velocity before a significant decrease.

406 Meagre is an anadromous species that migrates to estuaries to spawn (Haffray *et al.,*
407 2012). Females have a group-synchronous ovarian development that means that two or
408 more groups of oocytes are present in the ovary during the spawning period (Duncan *et*
409 *al.,* 2012; Duncan *et al.*, 2018) to prepare for different spawning events during the
410 spawning season. This configuration is typical of iteroparous species that spawn two or
411 more times with a separation of a few days or weeks between each spawn. This spawning
412 strategy in the meagre was manipulated with repeated injections of GnRHa to induce
413 weekly spontaneous tank spawning of captive stocks (Mylonas *et al*., 2016; Duncan *et*
414 *al*., 2018). The present study aimed to take this a step further to strip the ovulated ova for
415 *in vitro* fertilisation procedures.

416 Determining the time of ovulation and the period of optimum egg ripeness during which
417 eggs have maximum fertilisation success is important when *in vitro* fertilisation is
418 necessary or required for breeding programs (Samarin *et al.*, 2015). Ovulated ova age
419 over time: exhibiting ripening with improving viability, ripeness with optimal viability
420 and then overripening when the ova lose viability (Bromage, 1995; Mañanós *et al*., 2008;
421 Samarin *et al.*, 2015). Overripening has been described as morphological, physiological,
422 biochemical, histological, cellular and molecular changes in the egg (see review Samarin
423 *et al.*, 2015) and the associated decrease in fertilisation success has been attributed to

424 hypoxia, changes in membrane permeability or a decrease in egg ATP stores. The period
425 of egg ripeness and maximum fertilisation success varies amongst species and with
426 temperature (Bromage, 1995; Samarin *et al.*, 2015). For example, it was reported to be 3
427 - 6 h for Senegalese sole (*Solea senegalensis*) at 16 ºC (Rasines *et al*., 2012), 5 - 15 days
428 over a temperature range of 10 - 17ºC for rainbow trout (*Oncorhynchus mykiss*) (Samarin
429 *et al*., 2008), but only 30 min in white bass (*Morone chrysops*) at 22°C (Mylonas *et al.*,
430 1996). In the present study with meagre, there appeared to be a sequence, starting with
431 egg ripening followed by a window of optimal ripeness and finally overrippening when
432 eggs lost viability, similar to that described for rainbow trout (Bromage, 1995). The
433 ripening period appeared to extend from the initiation of ovulation until ovulation was
434 complete as some females that had incomplete ovulation in the first revision (35 - 36 h)
435 when a low quantity of ova with low fertilisation was obtained, had completed ovulation
436 by the time of the subsequent revision (38 - 39 h). In this revision, greater quantities of
437 ova with higher fertilisation were obtained to indicate that the ova had ripened. Once
438 ovulation was complete, a window of optimal ripeness with high fertilisation rates was
439 maintained for at least 2.5 h as shown in females with high fertilisation in both the initial
440 and the second examination. A sigmoidal regression (R^2= 0.9175) based on all these
441 females (Fig. 3) described a 3 h period of high fertilisation rates before a reduction in
442 fertilisation rates was observed. The rapid decline in fertilisation rates after the window
443 of egg ripeness was indicative of the overripening process that has been described in many
444 species (Bromage, 1995; Samarin *et al.*, 2015).

445 This short 3 h period of ripeness in meagre, highlights the importance of identifying the
446 exact timing of ovulation, in order to time stripping to optimize the acquisition of good
447 quality ova. Identification of the correct time for stripping also leads to a reduction in
448 multiple handling of fish to detect ovulation, which can have severe effects in the egg

449 quality (Zohar and Mylonas, 2001). The timing of ovulation in other finfish species has
450 been shown to be affected by different causes, such as the hormone dose (Rasines *et al*.,
451 2012), the time of day of the hormone administration (Shiraishi *et al*., 2008), the rearing
452 temperature (Samarin *et al*., 2008) and the stage of ovarian development at the time of
453 treatment (Duncan *et al*., 2003). In the present study, under the described holding
454 conditions, ovulation occurred within 35 to 41 h after GnRHa injection. Ovulation was
455 detected at different times (a) in females stripped in different years and (b) in females
456 stripped in the same year. Perhaps the most notable observation was, in 2015, ovulation
457 was detected 39 to 41 h post-injection, while in 2016 ovulation occurred within 35 to 39
458 h after GnRHa injection. The study was performed in both years with the same GnRHa
459 dose, administered at the same time of the day and the same rearing temperature.
460 Therefore, the different stage of gonadal development of females between the two years
461 may be one reason for the different timing of ovulation observed. Females in 2016 that
462 had a higher mean diameter of the largest vitellogenic oocytes (670 ± 39 µm) prior to
463 GnRHa injection ovulated earlier than females that contained oocytes close to 550 µm
464 (2015). The observation that larger oocytes matured and were ovulated earlier agrees with
465 the positive correlation between oocyte diameter and ovulation time observed in the
466 bullseye puffer (*Sphoeroides annulatus*) (Duncan *et al*., 2003). However, there was no
467 correlation between oocyte diameter and time to ovulation in the present study, which
468 may be because a narrow range of oocyte diameter existed among the different eligible
469 females. In 2016, no differences in initial gonadal stage were registered, as females were
470 selected with similar oocyte diameter, and oocyte size does not appear to explain the
471 differences in ovulation responses that were observed. At 35 h post-injection, in some
472 females, the first ova that were obtained by hand-stripping had high fertilisation success
473 which suggests that ovulation was complete before the females were examined. In other

474 females, at this same time, ovulation was incomplete as the number of ova collected was
475 low and those ova from partially ovulated females had low fertilisation. However, at the
476 subsequent examination for ovulation (38 - 39 h post-injection) the highest fertilisation
477 was obtained in almost all females. The females with good egg quality at 35 h maintained
478 high fertilisation and those that gave at first low quantity and quality ova completed
479 ovulation to give ova in greater quantities with higher fertilisation rates. The combination
480 of a 3 h window for the highest ova quality and the poor quality of ova stripped in the
481 early stages of ovulation (at 35 h) that subsequently improved, indicated that females that
482 ovulated close to 35 - 36 h were perhaps best left and stripped at 38 - 39 h. Therefore,
483 altogether the present study has identified that most fish had ovulated and had highest egg
484 quality at 38 - 39 h after GnRHa treatment. However, it is clear that many factors
485 influence the timing of ovulation and future studies with different stocks, husbandry
486 conditions, stages of maturity, etc. must expect deviations from the stripping time in the
487 present study, which can be used as a reference point.

488 Variations in egg quality between females is another factor that can influence fertilisation
489 rates (Springate *et al*., 1984). Fertilisation depends both on ova and sperm quality. Ova
490 quality refers to the eggs developmental competence that means the ability of the ova to
491 be fertilised and to develop into normal embryo. Sperm quality refers to the ability of
492 spermatozoa to fertilise an egg and allow the development of a normal embryo (Migaud
493 *et al.*, 2013). To avoid the effect of sperm quality on fertilisations, sperm from two
494 different males was mixed to fertilise all batches of eggs from different retention times
495 and females. In addition, sperm characteristics were evaluated to ensure the same
496 fertilising ability. Thus, the differences observed may be due to the different quality of
497 ova that has been observed in fish from the same stock (Springate *et al*., 1984).

498 Once the gametes have been obtained from the fish for *in vitro* fertilisation, the length of
499 time spermatozoa and oocytes survive is an important logistic factor to consider (Billard,
500 1988). The viability of ova *in vitro* depends on temperature and has been reported to be
501 species-specific. For instance, in curimata (*Prochilodus marggravii*), fertilisation success
502 of eggs stored at 18°C was drastically reduced when compared with storage at 26°C. The
503 eggs stored at 26°C in curimata lost viability almost completely 2 h after stripping (Rizzo
504 *et al*., 2003) while in kutum (*Rutilus frisii kutum*) at the same temperature viability was
505 lost after 4 h (Samarin *et al*., 2015). According to the results obtained in this study, eggs
506 from meagre have a relatively short period of viability after stripping, during which
507 successful fertilisation can occur while eggs are maintained at hatchery temperature.
508 Therefore, we suggest that meagre eggs should be fertilised within the first 50 min post-
509 stripping to achieve high fertilisation success, as a decrease in fertilisation rates were
510 detected with time with a rapid decrease in viability occurring from 2.5 to 4 h post-
511 stripping.

512 Gamete survival with respect to sperm quality must also be controlled for a proper *in vitro*
513 fertilisation. The experiment conducted in this study showed that when sperm was stored
514 in modified Leibovitz culture medium, the spermatozoa of meagre retained the same
515 initial motility percentage, duration of motility and velocity (which relates to fertilizing
516 capacity) within at least 7 h at 4ºC. A recent study demonstrated the possibility to store
517 meagre sperm at a 1/4 dilution in 0.9% NaCl at 4ºC for up to 10 days (Santos *et al*., 2018)
518 and would improve artificial fertilisation techniques. The values of meagre sperm
519 concentration ($3.21 \pm 1.18\ 10^{10}$ spz mL^{-1}), spermatozoa motility duration (1.71 ± 0.29
520 min) and initial motility (58 ± 12 %) recorded in the present study were similar to other
521 recent values published for meagre (Mylonas *et al*., 2013a; Schiavone *et al*., 2012; Santos
522 *et al*., 2018). Initial velocities recorded here (90.69 ± 5.76 µm/s) were lower, but in the

523 range to those obtained by Santos *et al*. (2018) (140.90 ± 7.75 µm/s) and were a
524 characteristic of fast sperm (Gallego *et al*., 2013). In the present study, the decay in initial
525 motility and velocity values with time after activation was also examined as fertilising
526 ability could be limited to the first seconds after activation. The percentage of motility
527 gradually declined and became significantly lower than initial values after 55 s and
528 exhibited ≥ 50% motility until 43 s after activation. The initial velocity values for meagre
529 declined after 35 s post-activation. Spermatozoa motility is an important aspect in fish
530 breeding and has been directly related to fertilisation rates, but spermatozoa velocities
531 may also serve as a predictor of fertilisation ability. In fact, in some studies the highest
532 coefficients of correlation were found for spermatozoa velocity (Gallego *et al*., 2013).
533 Thus, if spermatozoa velocity is highly correlated with fertilising ability, the period of
534 sperm fertility could be reduced to 35 s in meagre. However, as seen in the present study,
535 GnRHa administration to males maintained initial values of motility percentage (with ≥
536 50% until 62 s) and velocity for a longer period, 75 and 45 seconds after activation,
537 respectively, thus potentially increasing the period of fertilising ability. Therefore, male
538 therapy with GnRHa is recommended because (a) the period in which sperm of good
539 motility and velocity was enhanced or lengthened and (b) spermiation stage -and thus
540 sperm production or availability for stripping- was enhanced, especially towards the end
541 of the season when spermiation stage declined naturally. Mylonas *et al*. (2016) also
542 reported a decline in meagre sperm availability at the end of the spawning season.

543 Assuming both high quality male and female gametes have been obtained and managed
544 correctly, in order to ensure the success of *in vitro* fertilisation protocols, an important
545 aspect is to determine the appropriate spermatozoa : egg ratio for maximum fertilisation
546 success. Usually, an excess of sperm is used in insemination procedures (Gallego *et al*.,
547 2013), but an appropriate combination of the numbers of spermatozoa per oocyte should

548 improve the fertilisation outcome and significantly reduce sperm waste. The spermatozoa
549 : egg ratio is different between species. For example, the optimal spermatozoa : egg ratio
550 can range from 66,000 in European seabass (*Dicentrarchus labrax*) (Fauvel *et al.*, 1999)
551 to 1,000,000 in northern pike (*Esox Lucius*) (Zhang *et al.*, 2011). The present study,
552 showed that maximum fertilisation in meagre occurred within a wide range of
553 spermatozoa : egg ratios. Different characteristics, such as spermatozoa swimming
554 distance (Sanches *et al.*, 2016), micropyle closing time (Suquet *et al.*, 1995), properties
555 in eggs that may attract spermatozoa, as well as egg size are determinants in the
556 spermatozoa : egg ratios among fishes (Bombardelli *et al.*, 2013). Meagre eggs are small,
557 0.90 mm in diameter (Cárdenas, 2010), compared to the 2.32 mm of northern pike (Murry
558 *et al.*, 2008) so a lower spermatozoa : egg ratio should be needed in the case of meagre
559 since at a fixed spermatozoa density per egg the probability of a spermatozoa reaching
560 the micropyle should be higher. However, both meagre and European seabass have
561 similar egg size (Froese and Pauly, 2015), so the other factors mentioned above may have
562 relevance. Therefore, in the case of meagre, with initial velocities values no more than 35
563 s after activation, if velocity is highly correlated with fertilising ability, the period of
564 sperm fertility could be short and a high amount of spermatozoa per egg should be needed.
565 In the present study, both males had good sperm quality (> 60% spermatozoa motility)
566 and the same regression was obtained in the fertilisation rate obtained for each female.
567 This suggests that the wide range found in optimal spermatozoa to egg ratio was due to a
568 difference in the quality of the female gametes, which has been previously observed to be
569 crucial in the fertilisation success achieved in the timing of an ovulation experiment.
570 Bombardelli *et al*. (2013) observed in cascudo-preto (*Rhinelepis aspera*) that more sperm
571 was needed for inseminating batches of ova of lower viability to reach the maximum
572 fertilisation success. However, in the present study, the opposite was found and ova with

573 higher quality required more sperm than ova of poorer quality. This observation would
574 suggest that sperm were not attracted to poor ova and that poor quality batches that have
575 less viable ova need less sperm to achieve maximum rates of fertilisation. Although, in
576 the present study there were only two batches of ova (with duplicate males), this appears
577 to be true as maximum fertilisation of the eggs was approximately double (35% compared
578 to 70% fertilisation) indicating that twice as many ova were available to be fertilised in
579 the good quality batch of ova. Therefore, spermatozoa requirements were double (70,000
580 compared to 180,000) for the good quality batch of ova, as double the number of ova
581 required fertilisation. These arguments would indicate that 150,000 spermatozoa were
582 required per egg of good quality. It appears that the sperm requirement of eggs depends
583 on the quality of ova. Therefore, both sperm quality and ova quality should be taken into
584 account to determine spermatozoa egg ratio making it possible to obtain high fertilisation
585 rates. This also highlights that the use of high-quality gametes, both from males and
586 females, is an essential factor to reach proper fertilisation both for aquaculture and
587 scientific purposes. However, to have high quality viable gametes at the moment of
588 fertilisation to obtain suitable fertilisation rates in a protocol for *in vitro* fertilisation
589 requires attention to many factors including those researched in the present study,
590 reproductive traits, individual variability, timing of taking the gametes from the fish,
591 protocols for ova and sperm storage before fertilisation and characteristics of ova and
592 sperm quality.

593

594 **5. Conclusion**

595 The present study has determined the parameters required for a protocol for *in vitro*
596 fertilisation for meagre. The window of optimal ova quality extended from when
597 ovulation was complete until 3 h after ovulation. Under the described experimental

598 conditions, the optimal time for stripping females during this 3 h window was 38 - 39 h
599 after a single GnRHa injection, which represents a reference time for future studies. Once
600 stripped, meagre ova should be fertilised within the next 50 min. Sperm diluted in an
601 adapted medium of Leibovitz (1/4) can be stored for up to 7 h without affecting
602 spermatozoa motility percentage or velocity. Although sperm quality overall was similar
603 before and after GnRHa treatment, the use of GnRHa is recommended in order to increase
604 sperm availability and increase the period of high spermatozoa motility and velocity after
605 activation. A minimum of 150,000 spermatozoa per egg was optimal to ensure high
606 fertilisation success. This knowledge will aid the aquaculture industry and future research
607 in using *in vitro* fertilisation for meagre selective breeding programs.

608

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620 **7. References**

621 **APROMAR., La acuicultura en España,** 2017. www.apromar.es

622 **Billard, R.,** 1988. Artificial insemination and gamete management in fish. Marine
623 Behaviour and Physiology, 14(1): 3-21.

624 **Bombardelli, R. A., Sanches, E. A., Baggio, D. M., Sykora, R. M., de Souza, B. E.,**
625 **Tessaro, L., Piana, P. A.,** 2013. Effects of the spermatozoa: oocyte ratio, water volume
626 and water temperature on artificial fertilization and sperm activation of cascudo-preto.
627 Revista Brasileira de Zootecnia, 42(1): 1-3.

628 **Bromage, N.,** 1995. Broodstock management and seed quality - general considerations,
629 in: Broodstock Management and Egg and Larval Quality, Bromage, N.R. and Roberts
630 R.J., Eds., Blackwell Science, Oxford, UK, 1.

631 **Brown, R.C., Woolliams, J.A., McAndrew, B.J.,** 2005. Factors influencing effective
632 population size in commercial populations of gilthead seabream, *Sparus aurata*.
633 Aquaculture, 247: 219-225. http://dx.doi.org/10.1016/j.aquaculture.2005.02.002.

634 **Cárdenas, S.,** 2010. Crianza de la corvina (*Argyrosomus regius*). Cuadernos de
635 Acuicultura nº3. Fundación Observatorio Español de Acuicultura. Madrid.

636 **Chavanne, H., Parati, K., Cambuli, C., Capoferri, R., Aguilera Jimenez, C., Galli,**
637 **A.,** 2012. Microsatellites markers to depict the reproductive and genetic patterns of
638 farmed gilthead seabream (*Sparus aurata*): illustration by a case study on mass spawning.
639 Aquaculture Research, 45: 577-590. http://dx.doi.org/10.1111/are.12013.

640 **Colombo, L., Barbaro, A., Libertini, A., Francescon, A., Lombardo, L.,** 1995.
641 Artificial fertilization and induction of triploidy and meiogyongenesis in the european sea
642 bass *Dicentrarchus labrax* L. J. Appl. Ichthyol., 11: 118-125.

643 **Duncan, N. J., Estévez, A., Fernández-Palacios, H., Gairin, I., Hernández-Cruz, C.**
644 **M., Roo, J., Schuchardt, D., Vallés, R.,** 2013a. Aquaculture production of meagre
645 (*Argyrosomus regius*): hatchery techniques, ongrowing and market. In: Allan, G.,
646 Burnell, G. (Eds.), Advances in aquaculture hatchery technology. Woodhead Publishing
647 Limited, Cambridge, UK.

648 **Duncan, N., Estévez, A., Porta, J., Carazo, I., Norambuena, F., Aquilera, C., Gairin,**
649 **I., Bucci, F., Valles, R., Mylonas, C. C.,** 2012. Reproductive development, GnRHa-
650 induced spawning and egg quality of wild meagre (*Argyrosomus regius*) acclimatised to
651 captivity. Fish Physiol Biochem, doi: 10.1007/s10695-012-9615-3.

Duncan, N. J., Mylonas, C.C., Milton, E., Karamanlidis, D., França, M.C., Ibarra-Zatarain, Z., Chiumento, M., Aviles, O., 2018. Paired spawning with male rotation of meagre *Argyrosomus regius* using GnRHa injections, as a method for producing multiple families for breeding selection programs. Aquaculture, 495: 506-512.

Duncan, N.J., Rodriguez M. de O., G.A., Alok, D., Zohar, Y., 2003. Effects of controlled delivery and acute injections of LHRHa on bullseye puffer fish (*Sphoeroides annulatus*) spawning. Aquaculture, 218: 625-635.

Duncan, N.J., Sonesson, A. K., Chavanne, H., 2013b. Principles of finfish broodstock management in aquaculture: control of reproduction and genetic improvement. In: Allan, G., Burnell, G. (Eds.) Advances in aquaculture hatchery technology. Woodhead Publishing Limited, Cambridge, UK.

Estévez, A., Duncan, N., Campoverde, C., Afonso, J.M., Tsigenopoulos, C., Mylonas, C.C., Robaina, L., Izquierdo, M.S., Papandroulakis, N., Andree, K.A., Roque, A., Katharios, P., Chatzifotis, S., Tsertou, M.I., 2015. New advances in meagre (*Argyrosomus regius*) culture: results of the EU Diversify project in 2014 and 2015., in: Aquaculture Europe 15, Rotterdam, Neatherlands, 20-23 October 2015, pp 24-25.

Fatira, E. 2013. Comparative induction of spawning success in meagre (*Argyrosomus regius*) using GnRHa injections or implants. Master Thesis. Biology Department, Univerity of Crete.

Fauvel, C., Boryshpolets, S., Cosson, J., Wilson Leedy, J.G., Labbé, C., Haffray, P., Suquet, M., 2012. Improvement of chilled seabass sperm conservation using a cell culture medium. J. Appl. Ichthyol, 28: 961-966.

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

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

681 **Flett, P. A., Munkittrick, K. R., Van Der Kraak, G., Leatherland, J. F.,** 1996.
682 Overripening as the cause of low survival to hatch in Lake Erie coho salmon
683 (*Oncorhynchus kisutch*) embryos. Can. J. Zool, 74: 851-857.

684 **Froese, R., Pauly, D.,** 2015. FishBase. World Wide Web electronic publication.
685 Available at: www.fishbase.org.

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

689 **Gil, M.** 2013. Recovery of meagre (*Argyrosomus regius*) population in the Balearic
690 coastal ecosystem (Western Mediterranean) (PhD Thesis). Department of Biology,
691 University of the Balearic Islands, Spain.

692 **Haffray, P., Malha, R., Ould Taleb Sidi, M., Prista, N., Hassan, M., Castelnaud, G.,**
693 **Karahan-Nomm, B., Gamsiz, K., Sadek, S., Bruant, J., Balma, P., Bonhomme, F.**
694 2012. Very high genetic fragmentation in a large marine fish, the meagre *Argyrosomus*
695 *regius* (Sciaenidae, Perciformes): impact of reproductive migration, oceanographic
696 barriers and ecological factors. Aquatic Living Resources, 25: 173-183.

697 **Mañanós, E., Duncan, N., Mylonas, C.,** 2008. Reproduction and control of ovulation,
698 spermiation and spawning in cultured fish, in Cabrita, E., Robles, V., Harraez, P (Eds.),
699 Methods in Reproductive Aquaculture. Boca Raton, FL: CRC Press Taylor and Francis
700 Group, pp. 3-80.

701 **Migaud, H., Bell, G., Cabrita, E., McAndrew, B., Davie, A., Bobe, J., Herraez, M.**
702 **P., Carillo, M.** 2013. Gamete quality and broodstock management in temperate fish.
703 Reviews in Aquaculture, 5 (1): 194-223.

704 **Monfort, M. C.,** 2010. Present market situation and prospects of meagre (*Argyrosomus*
705 *regius*), as an emergent species in Mediterranean aquaculture. Studies and Reviews.
706 General Fisheries Commission for the Mediterranean, 89. Rome, FAO. 28pp.

707 **Murry, B. A., Farrell, J. M., Schulz, K. L., Teece, M. A.** 2008. The effect of size and
708 nutrient content on larval performance: implications to protracted spawning in northern
709 pike (*Esox Lucius* Linnaeus). Hydrobiologia, 601 (1): 71-82.

710 **Mylonas, C. C., Duncan, N. J., Asturiano, J. F.,** 2017. Hormonal manipulations for the
711 enhancement of sperm production in cultured fish and evaluation of sperm quality.
712 Aquaculture, 472: 21-44.

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

717 **Mylonas, C. C., Magnus, Y., Gissis, A., Klebanov, Y., Zohar, Y.,** 1996. Aplication of
718 controlled-release, GnRHa-delivery systems in commercial production of white bass X
719 striped bass hybrids (sunshine bass), using captive broodstocks. Aquaculture 140, 265-
720 280.

721 **Mylonas, C. C., Mitrizakis, N., Papadaki, M., Sigelaki, I.,** 2013a. Reproduction of
722 hatchery-produced meagre *Argyrosomus regius* in captivity I. Description of the annual
723 reproductive cycle. Aquaculture, http://dx.doi.org/10.1016/j.aquaculture.2013.09.009.

724 **Mylonas, C. C., Salone, S., Biglino, T., de Mello, P. H., Fakriadis, I., Sigelaki, I.,**
725 **Duncan, N.,** 2016. Enhancement of oogenesis/spermatogenesis in meagre *Argyrosomus*
726 *regius* using a combination of temperature control and GnRHa treatments. Aquaculture,
727 464: 323-330.

728 **Rasines, I., Gómez, M., Martín, I., Rodríguez, C., Mañanós, E., Chereguini, O.,** 2012.
729 Artificial fertilization of Senegalese sole (*Solea senegalensis*): Hormone therapy
730 administration methods, timing of ovulation and viability of eggs retained in the ovarian
731 cavity. Aquaculture, 326-329: 129-135.

732 **Rizzo, E., Godinho, H. P., Sato, Y.,** 2003. Short-term storage of oocytes from the
733 neotropical teleost fish *Prochilodus marggravii*. Theriogenology 60, 1059–1070.

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

736 **Samarin, A. M., Ahmadi, M. R., Azuma, T., Rafiee, G, R., Amiri, B. M., Naghavi,**
737 **M. R.,** 2008. Influence of the time to egg stripping on eyeing and hatching rates in raibow
738 trout *Oncorhynchus mykiss* under cold temperatures. Aquaculture, 278: 195-198.

739 **Samarin, A. M., Gela, D., Bytyutskyy, D., Policar, T.,** 2015. Determination of the best
740 post-ovulatory stripping time for common carp (*Cyprinus carpio* Linnaeus, 1758). *J.*
741 Appl. Ichthyol, 31: 51-55.

742 **Samarin, A. M., Policar, T., Lahnsteiner, F.** 2015. Fish Oocyte Ageing and its Effect
743 on Egg Quality. Reviews in Fisheries Science & Aquaculture, 23(3): 302-314.

744 **Sanches, E. A., Caneppele, D., Okawara, R. Y., Damasceno, D. Z., Bombardelli, R.**
745 **A., Romagosa, E.,** 2016. Inseminating dose and water volume applied to the artificial
746 fertilization of Steindachneridion parahybae (Steindachner, 1877) (Siluriformes:
747 Pimelodidae): Brazilian endangered fish. Neotropical Ichthyology, 14(1).

748 **Sanches, E. A., D. M. Baggio, P. A. Piana, B. E. de Souza & R. A. Bombardelli.,**
749 2011a. Artificial fertilization of oocytes and sperm activation in pacu: effects of the
750 spermatozoa:oocyte ratio, water volume, and in natura semen preservation. Revista
751 Brasileira de Zootecnia, 40: 1-6.

752 **Santos, M., Soares, F., Moreira, M., José, B.** 2018. Evaluation of different extenders
753 for the cold storage of meagre (*Argyrosomus regius*) semen. Aquaculture Research.
754 10.1111/are.13733.

755 **Schiavone, R.; Zilli, L., Storelli, C., Vilella, S.,** 2012. Changes in hormonal profile,
756 gonads and sperm quality of *Argyrosomus regius* (Pisces, Sciaenidae) during the first
757 sexual differenciation and maturation. Theriogenology, 77: 888-898.

758 **Shiraishi, T., Ketkar, S. D., Kitano, H., Nyuji, M., Yamaguchi, Y., Matsuyama, M.,**
759 2008. Time course of final oocyte maturation and ovulation in chub mackerel *Scomber*
760 *japonicus* induced by hCG and GnRHa. Fisheries Science, 74: 764-769.

761 **Springate, J. R. C. Bromage, N. R., Elliott, J. A. K., Hudson, D. L.,** 1984. The timing
762 of ovulayion and stripping and their effects on the rates of fertilization and survival to
763 eying, hatch and swim-up in the rainbow trout (*Salmo gairdneri* R.). Aquaculture, 43:
764 313-322.

765 **Suquet, M., Billard, R., Cosson, J. C.,** 1995. Artificial insemination in turbot
766 (*Scophthalmus maximus*): determination of the optimal sperm to egg ratio and time of
767 gamete contact. Aquaculture, 133: 83-90.

768 **Vallés, R., Estévez, A.,** 2013. Light conditions for larval rearing of meagre (*Argyrosomus*
769 *regius*). Aquaculture, 376-379: 15-19.

770 **Wayman, W. R., Tiersch, T. R. and Thomas, R. G.,** 1998. Refrigerated storage and
771 cryopreservation of sperm of red drum, *Sciaenops ocellatus* L.. Aquaculture Research,
772 29: 267-273.

773 **Wilson-Leedy, J.G., Ingermann, R.L.,** 2007. Development of a novel CASA system
774 based on open source software for characterization of zebrafish sperm motility
775 parameters. Theriogenology, 3: 661-72.

776 **Zhang, J. J., Li, S. Z., Tulake, K., Yan, Q. P. and Li, W. J.,** 2011. The effects of
777 extenders and sperm-egg ratios on fertilizing ability of cryopreserved testicular sperm of
778 northern pike (*Esox lucius* L.). Journal of Applied Ichthyology, 27: 1037–1040.

779 **Zohar, Y., Mylonas, C. C.,** 2001. Endocrine manipulations of spawning in cultured
780 fish: from hormones to genes. Aquaculture, 197: 99-1.

781 **Figure legends**

782 **Table 1.** Sperm quality parameters of sperm samples obtained immediately before and
783 24 hours after a GnRHa injection. Mean (± standard desviation) values of sperm from 5
784 males that were used to fertilise eggs during the weekly fertilisation trials. There were no
785 significant differences in any of the parameters before and after hormonal treatment.

786 **Figure 1.** Mean (± standard deviation, n = 2-5) of meagre (*Argyrosomus regius*) sperm
787 quality parameters. (A) Percentage of motile spermatozoa (%) and (B) Average Path
788 Velocity (VAP) of spermatozoa (µm/s) over time before (back circles) and after (open
789 white circles) GnRHa injection during the experimental period. Different capital and
790 small letters indicate significant differences (P<0.05) over time after spermatozoa
791 activation of samples collected before and after GnRHa injection, respectively. The
792 decline in sperm quality parameters is represented by cubic regressions with the following
793 equations $y = 0.0003x^3 - 0.0517x^2 + 2.362x + 23.7591$ ($R^2 = 0.9933$) and $y = - 0.0001x^3$
794 $+ 0.0131x^2 - 0.6617x + 74.0331$ ($R^2 = 0.9683$) for motility decay before and after GnRHa
795 injection, respectively. The horizontal line indicates the 50% of motility. Regression
796 coefficients for VAP are $R^2 = 0.9951$ and 0.9895.

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

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

811 **Figure 4.** The fertilisation success over time of maintaining meagre (*Argyrosomus regius*) 812 ova in the laboratory at 20ºC before fertilisation. The relationship was described by a 813 quadratic regression ($P<0.05$, $R^2 = 0.927$).

814 **Figure 5.** Mean fertilisation of meagre (*Argyrosomus regius*) eggs fertilised *in vitro* at 815 different spermatozoa : egg ratios. The error bars indicate the standard deviation of mean. 816 Significant differences are indicated by different letters (capital letters for fertilisation of 817 eggs obtained by the combination of female 1 with males 1♂ and 2♂, and lowercase 818 letters for the combination of female 2 and males 1♂ and 2♂) ($P<0.05$).

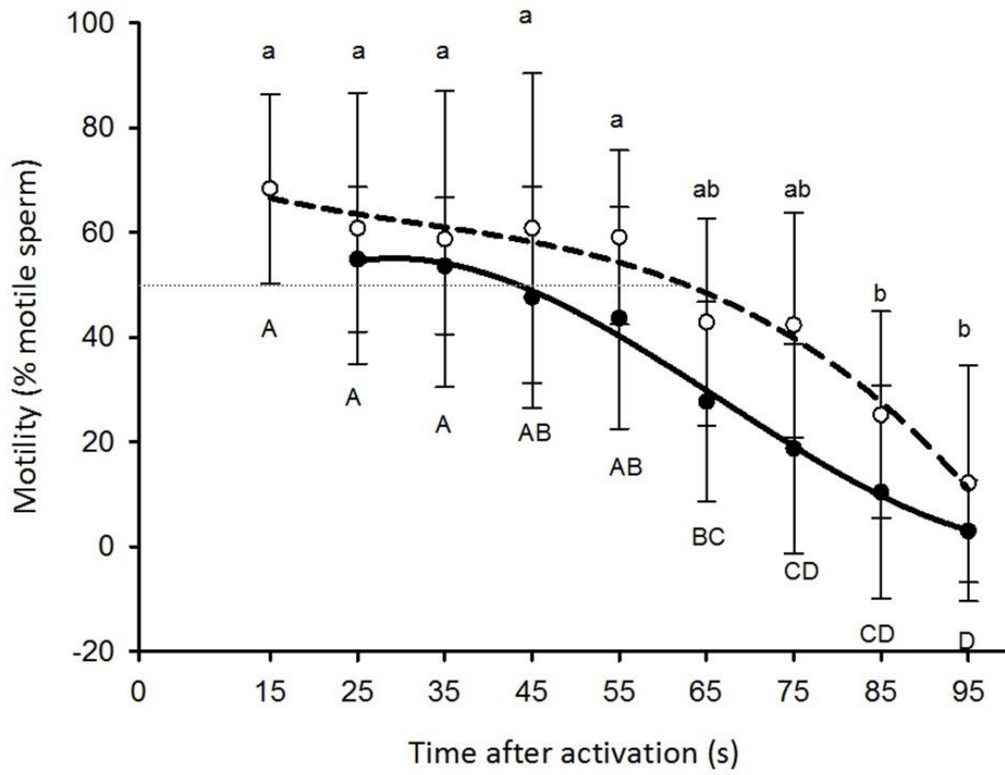
819 Table 1.

Sampling time in relation to GnRHa 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

820

821 Figure 1

822 A



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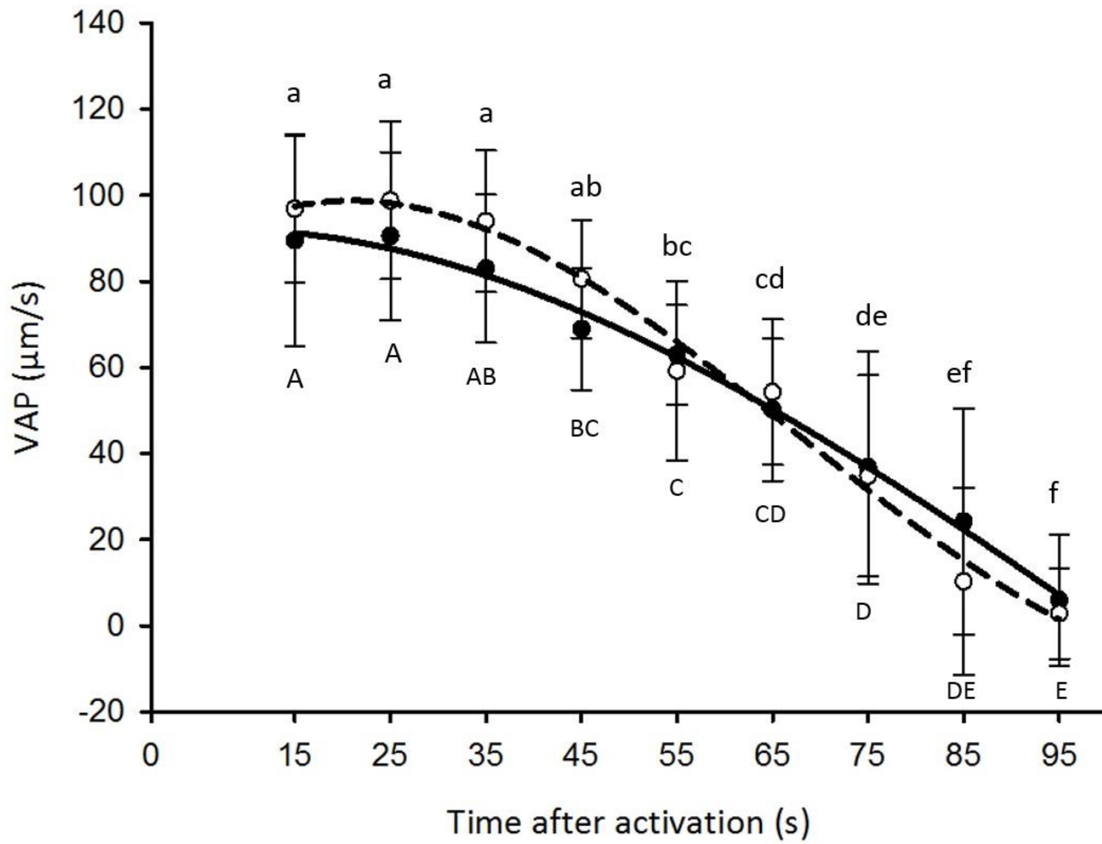
825 B



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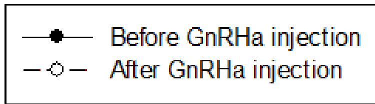

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828 Figure 2.

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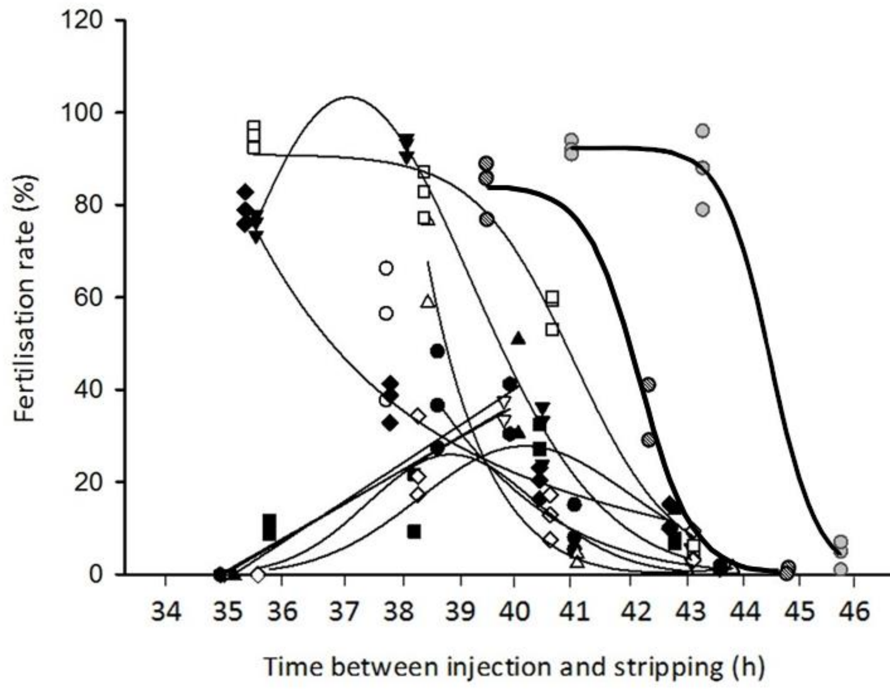

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832 Figure 3.

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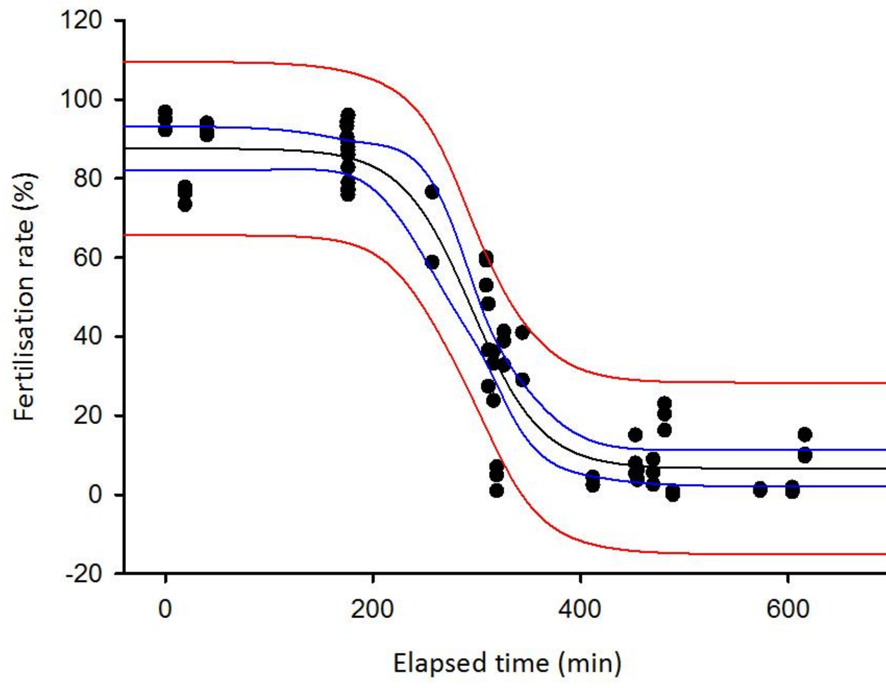

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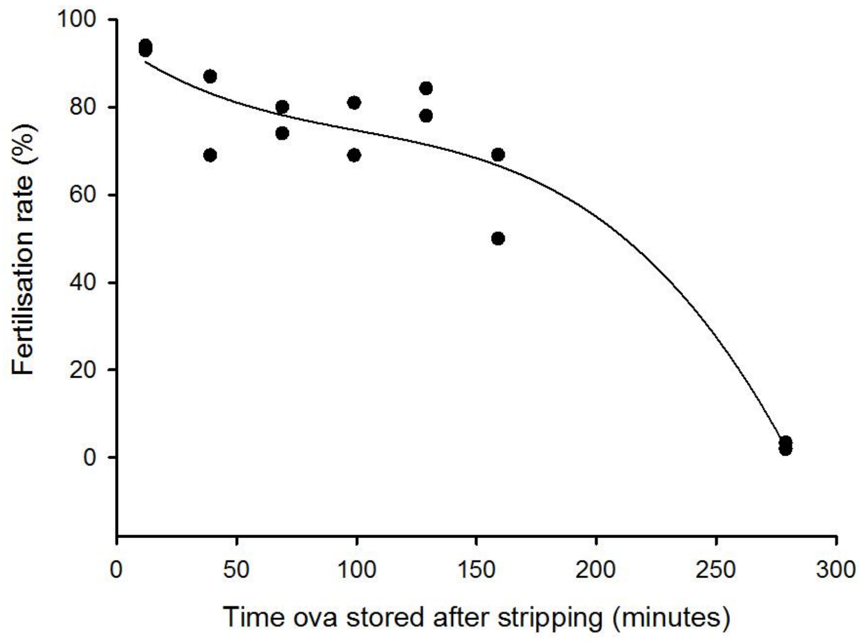
835 Figure 4.



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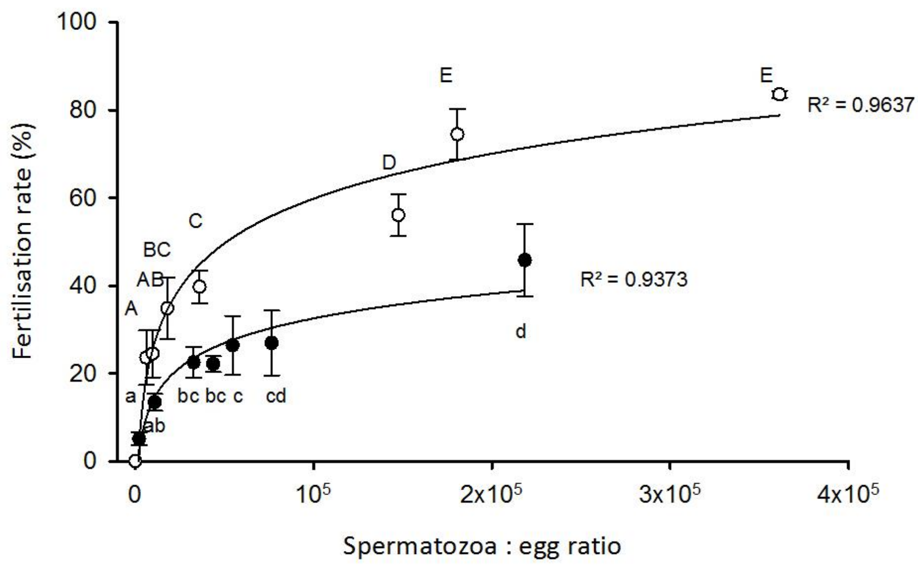
837 Figure 5.



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