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Low sperm to egg ratio required for successful in vitro fertilisation in a pair-spawning teleost, Senegalese sole (*Solea senegalensis*)

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Abstract

Cultured Senegalese sole (*Solea senegalensis*) breeders fail to spawn fertilised eggs and this bottleneck could be solved with the implementation of large-scale *in vitro* fertilisation protocols. However, low production of poor-quality sperm has frustrated the development of *in vitro* fertilisation protocols. Cultured females were induced to ovulate with a 5 µg kg^{-1} single injection of gonadotropin releasing hormone agonist (GnRHa) and good quality eggs (82.6 ± 9.2% fertilisation) were stripped 41:57 ± 1:46 h after the injection. Sperm was collected from cultured males, diluted in modified Leibovitz and used fresh to fertilise the eggs. A non-linear regression, an exponential rise to a maximum ($R = 0.93$, $P < 0.0001$) described the number of motile spermatozoa required to fertilise a viable egg and 1617 motile spermatozoa were sufficient to fertilise 99 ± 12% (± 95% CI) of viable eggs. Similar, spermatozoa egg^{-1} ratios of 592 ± 611 motile spermatozoa egg^{-1} were used in large-scale *in vitro* fertilisations with 190,512 ± 38,471 eggs. The sperm from a single male (145 ± 50 µL or $8.0 \pm 6.8 \times 10^8$ spermatozoa) was used to fertilise the eggs. The mean hatching rate of the large-scale *in vitro* fertilisations was 70 ± 14 % to provide 131,540 ± 34,448 larvae per fertilisation. When unfertilised eggs were stored at room temperature the percentage of viable eggs decreased gradually and indicated the sooner eggs were fertilised after stripping the higher the viability of the eggs. The collection of sperm directly into a syringe containing modified Leibovitz significantly increased the percentage of motile spermatozoa (33.4 ± 12.2 %) compared to dilution in modified Leibovitz immediately after collection (6.6 ± 4.9 %). Senegalese sole have a pair-spawning reproductive behaviour characterised by external gamete fertilisation in close proximity with no sperm competition. The low spermatozoa egg^{-1} ratio required for maximum fertilisation was consistent with this reproductive behaviour and strategy. The provision of a large-scale *in vitro* fertilisation protocol (200 µL of sperm per 100 mL of eggs) will enable the industry to operate sustainably and implement breeding programs to improve production.

Keywords: Flatfish, Teleost, Aquaculture, Gamete, Artificial fertilisation, Assisted reproduction

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1. Introduction

40 Senegalese sole (*Solea senegalensis*) is a promising emerging aquaculture species in Europe. Sole production from land based farms in Spain, Portugal, France and Iceland
42 has increased rapidly to 1,700 t in 2019 (APROMAR, 2019). This increase is driven by good market prices, high market demand and successful culture practices (Morais et al.,
44 2016) that permit cost effective production despite of the need for high levels of investment in culture infrastructure.

46 However, the production cycle is not fully controlled and relies on the capture of wild broodstock that spawn sufficient eggs to achieve targeted aquaculture productions
48 (Anguis and Cañavate, 2005; Martín et al., 2014). The progeny of these wild broodstock and in particular the males exhibit a reproductive behavioural dysfunction and do not
50 participate in the courtship to fertilise eggs (Duncan et al., 2019; Fatsini et al., 2020; Guzmán et al., 2008; Martín et al., 2020). Consequentially, cultured broodstocks that were
52 reared entirely in captivity produce unfertilised eggs (Duncan et al., 2019; Guzmán et al., 2008). Differences between wild and cultured breeders have suggested that the
54 reproductive dysfunction has a bases in a combination of endocrine reproductive control (Guzmán et al., 2011; Riesco et al., 2019), social conditions during rearing (Fatsini et al.,
56 2020; Martín et al., 2020), broodstock nutrition (Norambuena et al., 2013a, 2013b, 2012b, 2012c, 2012a) and olfactory capacity (Fatsini et al., 2017). However, no practical
58 approach has been developed to overcome these reproductive dysfunctions and the low levels of fertilised egg obtained from cultured broodstocks (Fatsini et al., 2020; Guzmán
60 et al., 2011) were insufficient to meet industry needs.

In flatfish culture, *in vitro* fertilisation methods are commonly used to obtain the fertilised
62 eggs required for aquaculture (Mañanós et al., 2008). *In vitro* fertilisation enables aquaculturists to bypass behavioural reproductive dysfunctions of the type observed in
64 cultured male Senegalese sole. However, the application of *in vitro* fertilisation methods for Senegalese sole has been frustrated by the small quantities of poor quality sperm
66 produced by males (Beirão et al., 2011, 2009; Cabrita et al., 2011, 2006; González-López et al., 2020). Rasines et al. (2013, 2012), has described *in vitro* fertilisation procedures
68 for Senegalese sole on an experimental scale. Female sole were induced to ovulate with gonadotropin releasing hormone agonist (GnRHa) and batches of 1 mL of stripped eggs
70 were fertilised with 30 µL of cryopreserved sperm from cultured males. Similarly, Liu et al. (2008) described *in vitro* fertilisation of Senegalese sole eggs on an industrial scale.
72 Again, eggs were obtained from GnRHa induced cultured females and all the eggs from each female were fertilised *in vitro* with the sperm from three to four cultured males.
74 However, few details were given on the amount of sperm or eggs used and the spermatozoa (spz) to egg ratio was not detailed.

76 When sperm is limiting, it is of critical importance to know the spz to egg ratio (amount of spz required to fertilise each egg) to plan *in vitro* fertilisation procedures. Spermatozoa
78 to egg ratios for *in vitro* fertilisation of fish eggs show considerable variation ranging from $\times 10^3$ to $\times 10^6$ (Beirão et al., 2019). However, it would appear that flatfish have
80 lower spz requirements as winter flounder (*Pseudopleuronectes americanus*) required 3.4 $\times 10^4$ spz egg^{-1} (Butts et al., 2012) and turbot (*Scophthalmus maximus*) required just 3000
82 to 6000 spz egg^{-1} (Chereguini et al., 1999; Suquet et al., 1995). Many flatfish species have reproductive strategies and behaviours to spawn as a pair (Carazo et al., 2016; Gibson et
84 al., 2014). Monogamous fish species (classified as species that spawn in a pair) were shown to have smaller testes compared to polyandrous species (group spawning of males
86 with a female) (Baker et al., 2020). Monogamy or pair-spawning will also reduce sperm

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competition (sperm from two or more males compete to fertilise the eggs) and decreasing
88 sperm competition has been related to smaller testes and lower sperm production (Parker and Pizzari, 2010; Stockley et al., 1997). Senegalese sole spawn as pairs (Carazo et al.,
90 2016; Duncan et al., 2019) and spawning pairs show a degree fidelity during and between spawning seasons (Fatsini et al., 2020; Martín et al., 2014). Therefore, pair-spawning and
92 low sperm competition would appear to explain the small testes size (García-López et al., 2005; González-López et al., 2020) and low sperm production reported for Senegalese
94 sole (Beirão et al., 2011, 2009; Cabrita et al., 2011, 2006; González-López et al., 2020). In addition, a preliminary study found that Senegalese sole achieved a high percentage of
96 fertilisation with a low spz to egg ratio (Marrero-Alemán et al., 2019) and indicated that *in vitro* fertilisation with the low numbers of sperm may be a viable solution to the
98 industries problem to control the reproduction.

The present study, aimed to determine the spz to egg ratio required for *in vitro* fertilisation
100 in Senegalese sole. The first aim was to determine the sperm to egg ratio on an experimental scale and then use similar ratios for commercial large-scale *in vitro*
102 fertilisations as a proof-of-concept. Additional aims were, to determine the viability of ovulated eggs stored at room temperature and to improve sperm collection methods.

104 **2. Methods**

2.1 Experimental animals

106 All Senegalese sole broodstock used were cultured fish that had been hatched and reared entirely in captivity. Females used had an average weight of 1.53 ± 0.28 kg and males
108 had a weight of 1.05 ± 0.25 kg. Fish were maintained in 10,000 L tanks in IRTA Sant Carles de la Rápita (Catalonia, Spain). Prior to experiments, fish were held in surface sea
110 water (~35 ppt, >5 mg.L^{-1} O2) and a controlled natural temperature cycle (9-20 ºC) using recirculation systems (IRTAmar®). Tanks were covered with shade netting and
112 photoperiod was natural with natural light. The fish were fed four days a week with either unfrozen polychaetes and mussels (0.75 % of biomass) or 5 mm pelleted Broodfeedlean
114 broodstock diet (0.55% of biomass) (Sparos, Olhão, Portugal). During experiments conducted from April to June, fish were held in the same conditions with the exceptions
116 that water temperature was maintained at a constant 16 ± 1 ºC and fish were not feed 24 hours before any manipulation.

118 The fish were handled (routine husbandry and experimentation) in accordance with European regulations on animal welfare (Federation of Laboratory Animal Science
120 Associations, FELASA, http://www.felasa.eu/). For all handling and sampling, fish were anesthetised with 60 mg L^{-1} tricaine methanesulfonate (MS-222; Sigma-Aldrich, Spain).

122 **2.2 Gametes**

Eggs were obtained by inducing ovulation. Ovarian biopsies were taken from females
124 with swollen ovaries and the diameter of 20 oocytes were measured (x40 Carl Zeiss Axiostar microscope). Females were selected that had mean oocyte diameter ≥ 600 µm
126 (Marrero-Alemán et al., 2019). The females were administered 5 µg kg^{-1} of GnRHa (Sigma code L4513, Sigma, Spain) (Agulleiro et al., 2006) between 18:00 to 19:00 h. The
128 females were held with constant temperature (16 ± 1 ºC) and total darkness until ovulation. Females were checked for ovulation every 2-3 hours starting from 40 h after
130 the administration of GnRHa (Marrero-Alemán et al., 2019; Rasines et al., 2013, 2012) and all eggs were stripped from the ovulated females. Percentage fertilisation when spz
132 were in excess was used to indicate egg quality.

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Sperm was obtained from males by repeatedly, gently massaging the testes and applying
134 pressure along the full length of the sperm ducts to the urogenital pore. All sperm with
urine contamination was collected in a 1 mL syringe. González-López et al. (2020),
136 demonstrated that avoiding urine contamination was almost impossible and that high
numbers of motile spz were obtained stripping sperm mixed with urine. The volume
138 collected was measured with the syringe to an accuracy of 10 µL and the sperm was
transferred to a 1.5 mL Eppendorf and immediately diluted with modified Leibovitz
140 (González-López et al., 2020) using the dilution required for the experiment (see below).
The sperm motility was initially observed (x 100 Carl Zeiss Axiostar microscope) by
142 activating 1 µL of diluted sperm with 19 µL of clean seawater. Sperm samples with low
or no motility were rejected. All sperm samples were stored over ice or at 4ºC
144 (refrigerated) until analysis or used to fertilise eggs.

The spz concentration (spz mL^{-1}) was measured using a Thoma cell counting chamber. A
146 10 µL sample of sperm was diluted 1:500 in 10% formalin and 10 µL of the dilution was
pipetted into the counting chamber. After 10 minutes for spz to sediment, the chamber
148 was observed using a microscope (x100 magnification with Olympus BH microscope),
photographed (IC Capture software and GigE digital camera model: DMK 22BUC03
150 Monochrome, The Imaginsource, Bremen, Germany) and the number of spz counted
(ImageJ software, http://imagej.nih.gov/ij/).

152 Sperm motility parameters were determined as described by González-López et al.
(2020). Spermatozoa were activated by mixing 1 µL of diluted sperm (1:4 with modified
154 Leibovitz) with 20 µL of seawater with 30% bovine serum albumin (BSA, Sigma, Spain).
One µL of activated sperm was pipetted into an ISAS R2C10 counting chamber (Proiser
156 *R+D, S.L.* Paterna, Spain) previously mounted and focused on the microscope (200x
magnification Olympus BH). Video recording was initiated when spz were activated and
158 tracks were recorded (IC Capture software and GigE digital camera) until motion ceased.
Videos (AVI format) of spz tracks from 15 to 17 s (unless otherwise stated) after
160 activation were converted into image sequences (jpeg format using Virtual Dub 1.10.4
software http://www.virtualdub.org/). The image sequences were analysed using ImageJ
162 software with the computer-assisted sperm analysis (CASA) plugin (ImageJ
http://rsb.info.nih.gov/ij/plugins/) using the settings: brightness and contrast, -10 to
164 15/224 to 238; threshold, 0/198 to 202; minimum sperm size (pixels), 10; maximum
sperm size (pixels), 400; minimum track length (frames), 10; maximum sperm velocity
166 between frames (pixels), 30; frame rate, 30; microns/1000 pixels, 303; Print motion, 1;
the additional settings were not modified. The parameters, percentage of motile spz (%
168 motility), Curvilinear Velocity (VCL, µm/s) and Average Path Velocity (VAP, µm/s)
were recorded. All sperm samples were analysed in triplicate.

170 The experiments (unless otherwise stated) aimed to use gametes (eggs and sperm) as soon
as possible after collection to avoid possible loses of viability due to storage. Researchers
172 worked as two groups to strip eggs and sperm at the same time and complete *in vitro*
fertilisations soon afterwards.

174 **2.3 Spermatozoa to egg ratio experiment**

Five different females and five different males were used during this experiment. When
176 an ovulated female was encountered, sperm was collected and checked to find a male
with ≥ 300 µL of sperm that exhibited motility. The sperm was serially diluted with
178 modified Leibovitz (González-López et al., 2020) to achieve eight dilutions: 1:4; 1:19;
1:79; 1:319; 1:959; 1:2879; 1:5759; 1:11519. A sample of the first dilution (1:4) was used

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180 to determine the spz concentration and percentage motility. The spz concentration in the dilution 1:4 was used to calculate the spz concentration in each dilution and spz motility
182 to calculate the concentration of motile spz. The eggs and diluted sperm were used to make three triplicate fertilisations for each serial dilution. Fertilisations were made in 100
184 mL beakers by pipetting in close sequence, 0.5 mL of eggs, 20 µL of diluted sperm and 5 mL clean seawater. A 1 mL pipette with a cut tip was used to pipette eggs and a 100 µL
186 pipette with a cut tip was used to pipette diluted sperm. The eggs, sperm and seawater were gently mixed by rocking and swirling the beaker. After 3-5 minutes, the volume of
188 seawater was topped up to 100 mL. The beakers of fertilised eggs were transferred to a 16 ºC incubator. After 24 hours the eggs from each beaker were concentrated in a sieve
190 and placed in a 10 mL Bogorov camber and ≥ 50 eggs were randomly examined using a binocular microscope (Nikon C-DSS230) to determine the number of developing eggs.
192 In addition, the number of eggs in three 0.5 mL samples was counted for each female.

2.4 Proof-of-concept experiment

194 Seven different females and seven different males were used during this experiment. The experiment aimed to make large-scale *in vitro* fertilisations using spz egg^{-1} ratios from
196 the previous experiment to fertilise the number of eggs (> 100,000 eggs) that would be required in a commercial fish farming scenario. When an ovulated female was
198 encountered, sperm was collected and checked to find a male with ≥ 150 µL of motile sperm. The sperm was immediately diluted 1:4 in modified Leibovitz and a sample of 50
200 µL of diluted sperm was taken to determine spz concentration and percentage motility (CASA). All the eggs were stripped from the female into a clean, dry 1 L jug and the
202 volume of eggs was measured with an accuracy of 10 mL. Three samples of 0.5 mL of eggs were taken and counted. The remaining sperm obtained from the male was added to
204 the eggs followed by a volume of seawater that was equal to the volume of eggs. The eggs, sperm and seawater were gently swirled to mix the contents. After 2-3 minutes the
206 jug was topped up to 1 L with seawater. The eggs were then divided into two or three parts and each part was placed in a 30 L incubator with the same conditions as the
208 broodstock holding tanks. The number of eggs in each incubator was estimated by mixing the incubator homogenously and taking three 100 mL samples and counting the eggs in
210 each sample. The eggs were left two days to hatch and the number of hatched larvae in each incubator was estimated as above for the eggs. The hatch rate was calculated from
212 the number of eggs stocked and number of larvae hatched and the mean was calculated for the replica incubators used for each female - male pair.

214 **2.5 Egg viability experiment**

Three different females and three different males were used during this experiment. When
216 an ovulated female was encountered, males were checked to find a male with ≥ 100 µL of motile sperm. The sperm was immediately diluted 1:4 in Leibovitz. All the eggs were
218 stripped from the female into a clean, dry 1 L jug. The eggs were covered and stored at room temperature inside a building (out of sun light). As soon as possible after the
220 gametes were stripped the first fertilisation was completed as previously described. The time the eggs were stripped and the time the first fertilisation was made was recorded.
222 Further fertilisations were completed at 30 to 60 minute intervals. Fertilisations were completed in duplicate or triplicate using 0.5 mL of eggs and 20 µL of diluted sperm that
224 ensured an excess of motile spz per egg. As described above, the beakers of fertilised eggs were transferred to a 16 ºC incubator and after 24 hours the percentage of developing
226 eggs was determined for each fertilisation.

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2.6 Sperm collection experiment

228 Thirteen males were used for this experiment. Sperm from each male was collected as previously described, with the exception that the sperm was collected either into an empty
230 clean syringe (100 µL of sperm) or into a syringe that contained modified Leibovitz (to give a 1:4 dilution, 50 µL of sperm collected into 200 µL of Leibovitz). Both collection
232 methods were used for each male and the sequence of collection was alternated. For seven animals sperm was first collected into a clean syringe and then into a syringe that
234 contained modified Leibovitz and for six animals the reverse, first directly into modified Leibovitz and then a clean syringe. The 100 µL of sperm collected into a clean syringe
236 was immediately divided into 50 µL that was diluted in 200 µL of Leibovitz (1:4 dilution) and 50 µL that was kept as undiluted sperm as a control. The time of collection and
238 dilution after collection was recorded. The sperm motility parameters were analysed (CASA) for the samples collected by these three methods: collected directly into
240 Leibovitz, collected before dilution in Leibovitz and undiluted sperm. Sperm motility parameters were analysed 30 s after activation for 2 s. The parameters were measured at
242 the time of collection (0 h), six and 24 h after collection.

2.7 Data analysis and statistics

244 All means are with one standard deviation unless otherwise stated. For the spermatozoa egg^{-1} ratio experiment, the percentage of viable eggs fertilised was calculated by dividing
246 the actual fertilisation rate by the mean fertilisation rate when sperm was in excess. The number of motile sperm was calculated by multiplying the volume of diluted sperm added
248 by the spermatozoa concentration and the percentage motility. To examine the effect that gamete quality amongst the five pairs of fish had on fertilization, a linear regression was
250 applied to percentage of motile sperm (sperm quality) against number of sperm (motile and immotile) required per viable egg and to percentage of viable eggs (egg quality)
252 against number of motile sperm required per egg. To describe the variation of percentage of viable eggs fertilised in relation to the number of motile spz per egg, a non-linear
254 regression based on an equation for an exponential rise to a maximum with double, five parameters was applied to the data. For the egg viability experiment, the percentage of
256 viable eggs fertilised was calculated as above. To describe the variation of percentage of viable eggs fertilised in relation to time the eggs were stored at room temperature a non-
258 linear regression based on an equation for a four parameter logistic curve was applied to the data.

260 The data set for percentage motility from the sperm collection experiment was not normally distributed with a high percentage of zeros and skewed positively to a few
262 higher values. The data set could not be transformed to normality. The data was analysed twice. In one analysis, the data from the time point zero was ranked and analysed with a
264 two-way ANOVA with the independent variables order of collection (1st or 2nd) and collection treatment (collected directly into Leibovitz, diluted in Leibovitz and undiluted
266 sperm). In a second analysis the data set was scored into samples with or without motility and a Chi squared analysis was make to compare expected proportions of samples with
268 motility with actual proportions with motility between sperm collection treatments (collected directly into Leibovitz, diluted in Leibovitz and undiluted sperm) and time of
270 storage (0 h, 6 h and 24 h). The Marascuillo procedure (Prins, 2012) was used to make a multiple comparison between proportions of individual treatments and time points. All
272 the samples with motility were then separated (i.e. all the zeros were excluded) into a smaller data set that was transformed to normality with the Logit transformation. The
274 transformed data was compared with a one way ANOVA followed by Holm-Sidak

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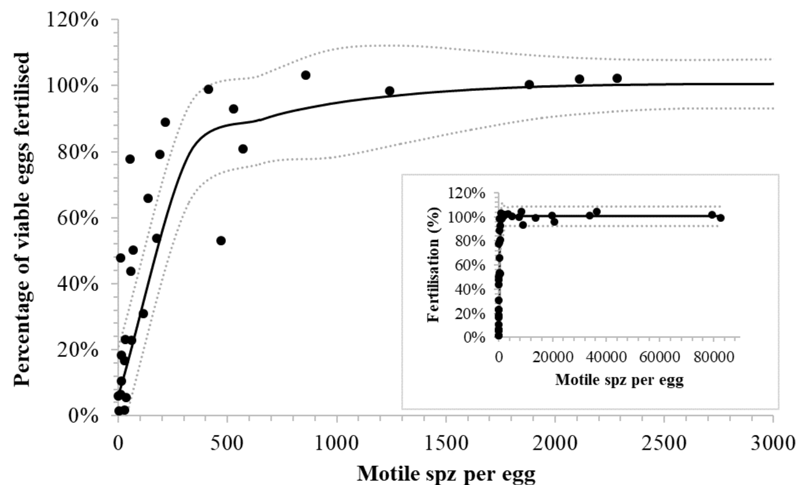
pairwise multiple comparison to compare mean motility for each treatment at each time
276 point. A P<0.05 was used to indicate significant differences. All statistical comparisons
and regressions were made using Sigma Plot 12 (Systat Software, Inc., San Jose, CA
278 95110. USA) except for the Marascuillo procedure that was completed with an Excel
(Microsoft) worksheet written by the authors.

280 **3.0 Results**

A total of 46 males were checked to obtain 28 (60.9 %) males with the required sperm
282 quantity and quality for the experiments. All males were only used once. A total of 20
females were selected by ovarian swelling and oocyte diameter determined. Five females
284 were rejected as the ovaries contained ovulated ova and two as the ovaries had solid cysts.
A total of 13 females had oocytes ≥ 600 μm and were induced with GnRHa. Five females
286 were not used as four females did not ovulate and one female had low quality eggs (0%
fertilisation). Eight (62%) females ovulated good quality eggs that were used in the
288 different experiments. The eggs from some females were used for more than one
experiment. The mean latency time from injection with GnRHa to the ovulation was
290 41:57 ± 1:46 h and mean fertilisation was 82.6 ± 9.2%. The mean fecundity was 130,789
± 36,723 eggs fish^{-1} or 87,174 ± 24,378 egg kg^{-1} of female body weight. The maximum
292 time difference between stripping eggs and sperm was 30 minutes, therefore, either sperm
or eggs were stored for 30 min or less before fertilisation experiments were initiated.

294 **3.1 Spermatozoa to egg ratio experiment**

The percentage of viable eggs fertilised in relation to number of motile spz per egg
296 showed a rapid increase from zero that was represented by a non-linear regression based
on an equation for an exponential rise to a maximum with double, five parameters (R =
298 0.93, P < 0.0001) (Fig. 1). The non-linear regression described that only 326 motile spz
per egg fertilised 79 ± 15% (± 95% CI - confidence interval) of viable eggs, that 649
300 motile sperm fertilised 90 ± 13% (± 95% CI) of viable eggs and 1617 motile spz fertilised
99 ± 12% (± 95% CI) of viable eggs.



302

Figure 1. The percentage of viable eggs fertilised in relation to the number of motile spermatozoa
304 **(spz) per viable egg for Senegalese sole (*Solea senegalensis*).** The insert figure shows the entire data set
up to over 80,000 spz per egg and the large figure shows a close up of the data up to 3,000 motile spz per
306 egg. The continuous line shows a non-linear regression based on an equation for an exponential rise to a
maximum with double, five parameters (R = 0.93, P < 0.0001) that represents the variation in percentage
308 of viable eggs fertilised in relation to number of motile sperm per egg. The dotted lines indicate 95%
confidence intervals for the non-linear regression.

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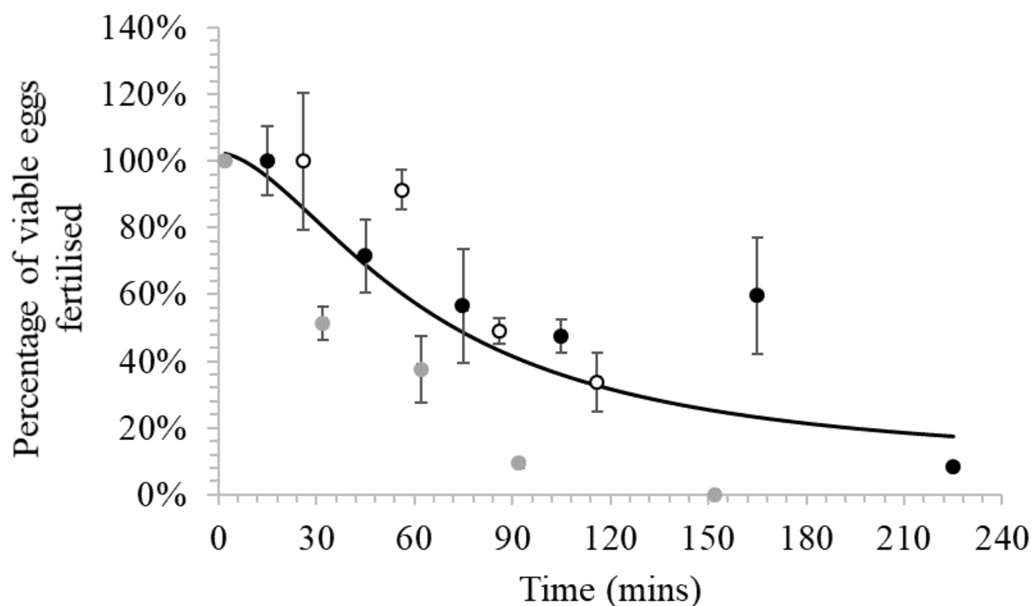
310 Amongst the five pairs, the percentage motility of the spz was correlated to the number of spz (motile and non-motile) required to fertilise a viable egg (R^2 = 0.83, P = 0.021).
312 However, there was no correlation between percentage of viable eggs and number of motile sperm required to fertilise each egg (R^2 = 0.03, P = 0.37). Caution is required in
314 the interpretation as n was low (n = 5) and the statistical power (at α = 0.05) of the tests was 0.66 and 0.13, respectively for percentage motility and viable eggs.

316 **3.2 Proof-of-concept experiment**

The proof-of-concept large-scale *in vitro* fertilisations (n = 7) gave a mean percentage
318 hatch of 70 ± 14 % to produce a mean of 131,540 ± 34,448 larvae per fertilisation (Table 1). The sperm from a single selected cultured male with a volume of 145 ± 50 µL and
320 total spz count of $8 \pm 6.8 \times 10^8$ was sufficient to fertilise large numbers of eggs (190,512 ± 38,471) and produce large numbers of larvae (131,540 ± 34,448). The mean number of
322 spz per egg used for the commercial fertilisations was 2,981 ± 2,932 spz egg^{-1} or 592 ± 611 motile spz egg^{-1}.

324 **3.3 Egg viability experiment**

The percentage of viable eggs fertilised decreased gradually from after being stripped
326 (Fig. 2). The non-linear regression based on an equation for a four parameter logistic curve (R = 0.80, P = 0.008) represented the variation in percentage of viable eggs
328 fertilised in relation to the time eggs from the three females were stored at room temperature. The non-linear regression indicated that after 30 min, the percentage
330 fertilisation had decreased from 100% to 81 ± 26% (± 95% CI), after an hour to 57 ± 20% (± 95% CI) and after two hours 32 ± 19% (± 95% CI) fertilisation of viable eggs.



332

Figure 2. The percentage of viable eggs fertilised in relation to time eggs were stored at room
334 **temperature for three Senegalese sole (*Solea senegalensis*) females.** Different dots represent different females. The line shows a non-linear regression based on an equation for a four parameter logistic curve (R
336 = 0.08, P = 0.008) that represents the variation in percentage of viable eggs fertilised in relation to time the eggs from the three females were stored.

338 **Table 1. Data from seven commercial scale *in vitro* fertilisations made for Senegalese sole (*Solea senegalensis*).** Latency time from application of GnRHa (5 µg kg $^{-1}$) to stripping of ovulated eggs, volume of eggs used, total number of eggs used, volume of sperm used (sperm from single male before dilution), total number of spermatozoa (spz)
340 added, number of spz per egg added, percentage motility of spz, number of motile spz per egg, mean percentage hatch in incubators and total number of larvae produced.

Female	Latency time	Vol. eggs (mL)	Total nº eggs	Vol. sperm (µL)	Total nº Spz. x10^8	Nº spz. per egg	Percentage spz motility	Total nº motile spz. per egg	Percentage hatch ± SD	Total nº larvae
1	41:20	120	161,280	140	14.0	8,681	10.4	905	82 ± 16	132,415
2	43:15	150	252,450	140	3.3	129	21.9	28	79 ± 14	198,530
3	40:45	75	144,250	100	6.9	4,802	38.1	1830	78 ± 24	112,600
4	42:00	110	181,940	120	1.5	824	30.0	247	58 ± 10	104,625
5	39:30	100	198,467	160	3.5	1,747	22.2	388	77 ± 7	153,245
6	43:40	100	167,500	120	4.9	2,925	8.3	242	71 ± 11	117,500
7	44:40	150	227,700	120	4.0	1,757	28.8	507	44 ± 9	101,865
Mean	**41:57**	**115**	**190,512**	**145**	**8.0**	**2,981**	**22.8**	**592**	**70**	**131,540**
± SD	01:46	27.5	38,471	50	6.8	2,933	10.7	611	14	34,448

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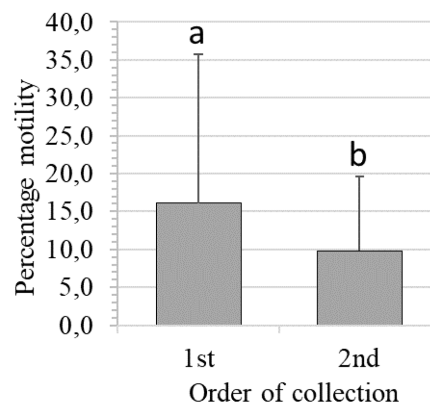
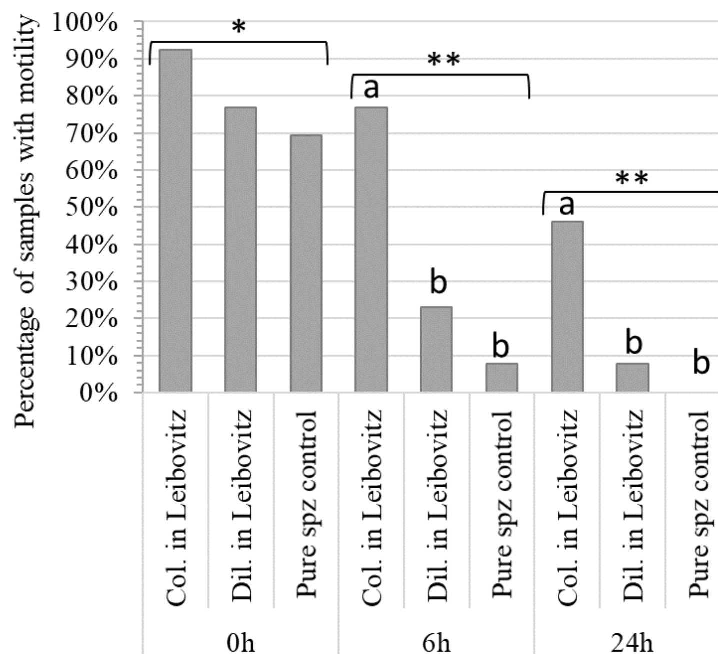
342 ### 3.4 Sperm extraction experiment

The order of collection of the sperm appeared to have a significant effect and the first part
344 of the sperm collected had significantly (P = 0.04) higher motility than the second part
(Fig. 3). However, caution is required in the interpretation as there was considerable
346 variation and the statistical power of the test was low (at α = 0.05, power = 0.44). The
percentage of samples that had motility decreased significantly (P < 0.001) with time (Fig.
348 4). At the time of collection (t = 0) there were no differences in the percentage of samples
with motility, but after six and 24 h of storage at 4ºC the samples collected directly into
350 modified Leibovitz had significantly (P < 0.05) more samples with motility.



352 **Figure 3. Mean percentage motility (± 1 standard deviation) of sperm samples collected first or second from each Senegalese sole (*Solea senegalensis*) male.** Different letters indicate a significant
354 difference (P = 0.04, α = 0.05, power = 0.44).



356 **Figure 4. The percentage of sperm samples from Senegalese sole (*Solea senegalensis*) (n = 13) that had sperm motility for different time points 0 h after collection and after six (6 h) and 24 hours (24
358 h) of storage at 4ºC.** Three sperm collection methods were tested, collected directly into modified Leibovitz (Col. in Leibovitz), diluted in modified Leibovitz (Dil. in Leibovitz) after collection and undiluted
360 sperm (control). Different letters indicate a significant difference (P < 0.05) within a time point and different number of asterisk indicate significant difference (P < 0.05) between time points.

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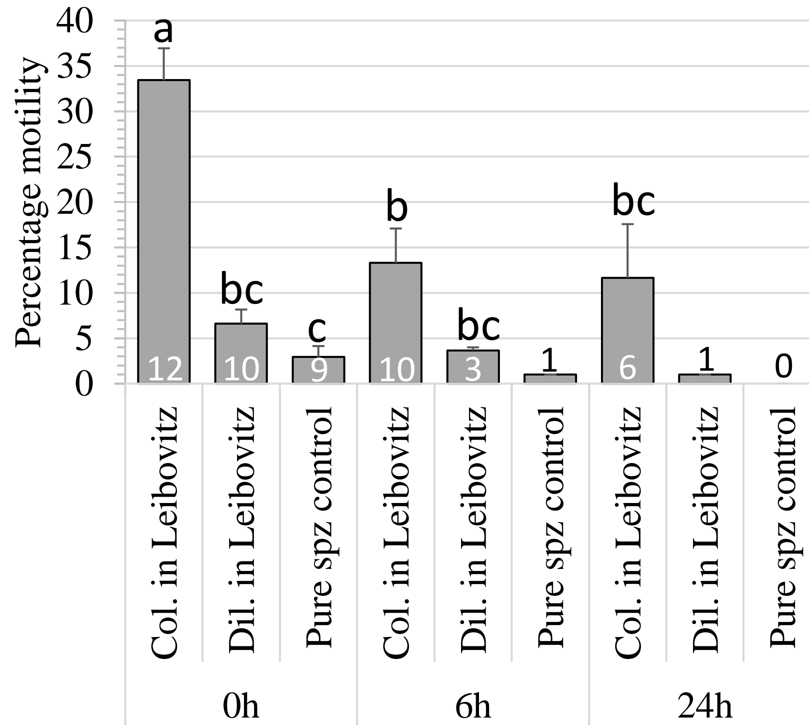
362 The percentage motility was significantly higher ($P < 0.05$) in samples collected directly into modified Leibovitz at t = 0 compared to other time points (t = 6 and 24) and other
364 collection methods, diluted in modified Leibovitz and undiluted (control), at all time points (t = 0, 6 and 24) (Fig. 5). The motility of samples collected directly into Leibovitz
366 and stored for six hours was significantly ($P < 0.05$) higher than undiluted sperm samples at collection (t = 0). The time difference in mixing sperm with Leibovitz was 4 ± 2
368 minutes, between samples collected directly into Leibovitz and the sperm diluted in Leibovitz after collection.



370

Figure 5. Mean percentage motility (± 1 standard error of the mean) of sperm samples collected from
372 **Senegalese sole (Solea senegalensis) using three collection methods, collected directly into modified Leibovitz (Col. in Leibovitz), diluted in modified Leibovitz (Dil. in Leibovitz) after collection and**
374 **undiluted sperm (control).** The motility of samples was tested at different time points 0 h after collection and after six (6 h) and 24 hours (24 h) of storage at 4°C. Different letters indicate a significant difference
376 ($P < 0.05$) amongst collection methods and time points. The "n" of each mean is at the bottom of each column. Means with less than n = 3 were not included in the statistical test.

378

4.0 Discussion

4.1 Low spermatozoa to egg ratio in Senegalese sole

380 The present study demonstrated that low numbers of spermatozoa egg^{-1} ensured high levels of fertilisation in Senegalese sole. An exponential rise to a maximum ($R = 0.93$, P
382 < 0.0001) described the number of motile spz required to fertilise a viable egg, the relationship rose steeply from 0 to 90% fertilisation and from approximately 500 motile
384 spz plateaued at 90 to 100 % fertilisation. A total of 1617 motile spz per egg were sufficient to fertilise 99 ± 12% (± 95% CI) of viable eggs. A low mean spz egg^{-1} ratio also
386 gave high rates of hatching in the proof-of-concept trials with large-scale *in vitro* fertilisations. A mean of 190,512 ± 38,471 eggs from a single female were fertilised with
388 a mean volume of sperm of 145 ± 50 µL from a single male. This volume of sperm

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provided a mean of 592 ± 611 motile spz per viable egg, which was sufficient to achieve
390 70 ± 14 % hatch and produce 131,540 ± 34,448 larvae per *in vitro* fertilisation. The spz egg^{-1} ratios for Senegalese sole were at the lower end of ratios required for fish. Studies
392 have shown that different fish species require a wide range of spz egg^{-1} ratios to achieve high rates of fertilisation (Beirão et al., 2019). These ratios ranged from 3000 spz egg^{-1}
394 for turbot (Chereguini et al., 1999) to 1×10^5 spz egg^{-1} for Atlantic cod (*Gadus morhua*) (Butts et al., 2009). Large-scale *in vitro* fertilisations protocols used in a hatchery
396 generally refer to the volume of sperm needed to fertilise eggs, for example in rainbow trout 1 mL of sperm was recommended for 10,000 eggs (Bromage, 1992) and for Atlantic
398 halibut (*Hippoglossus hippoglossus*) 1 mL of sperm for 1 L of eggs (Brown, 2010). For Senegalese sole, the present study has shown that a conservative estimation for hatcheries
400 based on the experiments and the 95% standard deviations, would be 200 µL of sperm (≈ 2.5×10^8 motile spz) to fertilise 100 mL of eggs (≈ 150,000 eggs).

402 **4.2 Improved *in vitro* protocol**

An aspect that complicates *in vitro* fertilisation protocols is ensuring good gamete quality
404 for fertilisation. Generally, the sperm is obtained first and stored until eggs are obtained (Mylonas et al., 2017). Considering that Senegalese sole sperm has poor quality
406 (González-López et al., 2020) and eggs have a short period of viability (Rasines et al., 2013, 2012), the storage of eggs and collection methods of sperm were examined in the
408 present study to improve the *in vitro* fertilisation protocol. In addition, the present study ensured sperm or eggs were not stored for longer than 30 minutes before fertilisation to
410 limit the effect of gamete quality deterioration. This proved to be necessary as the decline in quality of stripped Senegalese sole eggs stored at room temperature was gradual and
412 continuous. Egg quality appeared to decline during the first 30 min of storage with no plateau period of good egg quality, which indicated the sooner eggs were fertilised after
414 stripping the higher the viability of the eggs. This decline in egg quality after ovulation and stripping has been described in a wide range of species as the overripening process,
416 where eggs age and in association with morphological and biochemical changes lose viability and fertilisation rates decline (Mañanós et al., 2008; Ramos-Júdez et al., 2019;
418 Samarin et al., 2011). A rapid decline in egg quality has been observed in other species, but the decline was initiated after a period of good egg quality of 1 h in curimata
420 (*Prochilodus marggravii*) (Rizzo et al., 2003) and 50 min in meagre (*Argyrosomus regius*) (Ramos-Júdez et al., 2019). There is considerable variation across species and
422 some species have very different egg storage capacities, for example eggs from a Cyprinidae species kutum (*Rutilus frisii*) (Samarin et al., 2011) maintained good egg
424 quality during eight hours of storage and salmonid eggs can be stored successfully for 4-5 days (Bromage, 1992).

426 To improve sperm quality different methods of sperm collection were compared. The collection of sperm directly into modified Leibovitz significantly increased motility at the
428 time of collection and the storage capacity in terms of motility and number of samples with motility. Senegalese sole sperm is difficult or impossible to collect without urine
430 contamination (González-López et al., 2020). The urine has negative effects on the sperm quality, which as in other species appeared to change osmolality and pH, which
432 prematurely activated spz (Cejko et al., 2010; González-López et al., 2020; Linhart et al., 2003; Perchec Poupard et al., 1998). The collection into modified Leibovitz mitigated the
434 negative effect of urine contamination to improve the sperm quality (González-López et al., 2020). Other studies on species where urine contamination was difficult to avoid have
436 focused on improved collection methods that reduce urine contamination with the use of

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catheters that were inserted into the sperm duct (Babiak et al., 2006; Sarosiek et al., 2016)
438 or used a collecting pipette in combination with vacuum aspiration (Gallego et al., 2013a). The very small volumes and small diameter of the urogenital pore in Senegalese sole
440 make these kinds of approaches difficult or impossible. Small volumes of contaminated sperm obtained from some species of birds were collected directly into syringes with
442 extender to maintain sperm quality (Personal communication, Dr. Ignacio Giménez Nebot, Rara Avis Biotec S.L., Valencia, Spain). Therefore, in the present study, this
444 approach was taken to reduce the time that spz were in contact with urine contamination before dilution in the Leibovitz extender. A significant improvement in sperm quality was
446 obtained by collecting the sperm directly into a syringe containing modified Leibovitz, which reduced by 4 ± 2 minutes the time before sperm was diluted with Leibovitz.

448 **4.3 Sustainable Senegalese sole aquaculture**

The success of massive *in vitro* fertilisations, combined with indications of egg viability
450 during storage and improved methods for collecting and managing sperm, provided a protocol that can be used on an industrial scale to obtain eggs from cultured breeders for
452 hatchery production. At present, the Senegalese sole aquaculture industry relies on obtaining eggs from wild adult breeders captured in the commercial fishery and there is
454 no sustainable fishery for Senegalese sole (https://fisheries.msc.org). Therefore, obtaining viable eggs from cultured breeders has been a bottleneck that makes the
456 industry unsustainable and unable to implement breeding programs. Breeding programs are an essential part of an aquaculture business plan that enable companies to improve
458 growth and product quality. However, ideally, reproduction must be controlled to enable the selection and production of viable gametes from any animal that has the desired
460 production traits. In the present study, 61% of the males checked for sperm had the required quantity and quality needed for the experiments and *in vitro* fertilisations. This
462 availability of males, has already be improved as therapies with recombinant gonadotropins exist that both increase sperm production and quality (Chauvigné et al.,
464 2018, 2017). These recombinant gonadotropin therapies, significantly increased sperm production by up to seven times and significantly increased the sperm quality parameters,
466 percentage motility, progressivity and velocity of spz.

In the present study, females were initially selected by ovarian swelling and it is unclear
468 what percentage of females would be available for selection over a reproductive season. Of the females that were GnRHa-induced, 62% ovulated good quality eggs. Therefore,
470 studies are needed to identify the number of females available for GnRHa induction and to improve the success rate of GnRHa inductions. The present study used a GnRHa dose
472 of 5 µg kg^{-1} to induce the ovulation of eggs compared to 25 µg kg^{-1} used by Rasines et al. (2013, 2012). These studies had similar holding conditions and temperature (16 ºC) and
474 obtained very similar timing of ovulation with means close to 42 h (range of 39 to 44 h). Egg quality appeared to be higher in the present study, but differences in methods and
476 particular sperm storage and usage make comparisons inappropriate. Different doses of GnRHa have been compared to induce spontaneous liberations of eggs in Senegalese sole.
478 Agulleiro et al. (2006), tested the injection of GnRHa doses of one, five and 30 µg kg^{-1}. The dose of 5 µg kg^{-1} produced the most eggs and the dose of 30 µg kg^{-1} produced no
480 liberations of eggs. Guzman et al. (2009) compared injections of 5 and 25 µg kg^{-1} of GnRHa, but found no differences in number of eggs released between GnRHa injected
482 and untreated control fish. However, the number of oocytes advancing to hydration in the females treated with 5 µg kg^{-1} appeared to be higher than in females treated with 25 µg
484 kg^{-1} and controls. The present study, combined with studies on GnRHa induced

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spontaneous liberations of eggs, would indicate that the lower dose of 5 µg kg^{-1} of GnRHa
486 provided similar ovulation timing and egg quality as 25 µg kg^{-1} and perhaps suggest that
better results may be obtained with lower doses.

488 **4.4 Gamete quality and fertilisation**

Logically, the spz egg^{-1} ratio required is related to the success of individual sperm to
490 fertilise an egg. The success of spz will depend on factors of the fertilisation environment
and gamete quality / characteristics that hinder or aid the spz to reach the micropyle of
492 the egg. The environment used for *in vitro* fertilisation has been shown to affect the spz
egg^{-1} ratio. For example, the volume or space provided for fertilisation affected the spz
494 egg^{-1} ratio, as larger volumes increased the space to be travelled to fertilise the egg and
increased the number of spz required (Bombardelli et al., 2013; Chereguini et al., 1999;
496 Sanches et al., 2016). Therefore, variation in the fertilisation environment complicates
the comparison of different studies within and amongst species. However, as in the
498 present study the fertilisation environment can be standardised between tests and
replicated to ensure results can be compared.

500 Gamete characteristics and / or quality vary amongst species and individuals. Variations
in gamete quality have been shown to affect the spz egg^{-1} ratio. Obviously, percentage
502 sperm motility affects the ratio (Gallego et al., 2013b; Moccia and Munkittrick, 1987),
but velocity has also been shown to affect the ratio in walleye (*Sander vitreus*)
504 (Casselman et al., 2006) and pufferfish (*Takifugu niphobles*) (Gallego et al., 2013b).
Sperm with higher percentage motility (Gallego et al., 2013b; Moccia and Munkittrick,
506 1987) and spermatozoa with higher mean swimming speeds had lower spz egg^{-1} ratios to
fertilise a high percentage of eggs (Casselman et al., 2006; Gallego et al., 2013b). In the
508 present study, sperm percentage motility was related positively with spz egg^{-1} ratio (R^2 =
0.83, P = 0.021). Senegalese sole sperm has variable and generally poor quality with low
510 percentage motility (González-López et al., 2020). In the present study as in other studies
(Ramos-Júdez et al., 2019), the effect that percentage motility has on the spz egg^{-1} ratio
512 was removed by examining the relationship between the number of motile sperm and
eggs fertilised. Many studies simply express total number of sperm (including immotile
514 sperm) in the spz egg^{-1} ratio, however, this is inaccurate and should be stated with the
percentage motility of the sperm used. Although the practice of using total spz is accepted
516 in the literature it should only be applied to species that have little variation in motility
and preferably high levels (close to 100%) of motility amongst individuals.

518 In the present study, the quality of eggs did not appear to be related to the spz egg^{-1} ratio.
However, the low variation in egg quality (82.6 ± 9.2%) and n (n=5) may have reduced
520 the possibility to determine a relationship. Previous studies are contradictory indicating
that eggs with higher quality required more (Ramos-Júdez et al., 2019) and less
522 (Bombardelli et al., 2013) spz than low quality eggs. It would appear probable that both
the quality and the characteristics of the unfertilised egg are implicated in the fertilisation
524 success. Good quality eggs would have more eggs to be fertilised and low quality eggs
would have an environment with more space to encounter viable eggs amongst the
526 inviable eggs. In addition, fish eggs of some species have been shown to have no
mechanisms to attract spz whilst other species eggs have chemical and physical properties
528 that guide the spz to the micropyle (Yanagimachi et al., 2017). The ability to attract spz
to the eggs would in theory reduce the spz egg^{-1} ratio. These observations would suggest
530 number of motile spz per viable egg should be used to ensure egg viability does not affect
the spz egg^{-1} ratio. However, further work is required to determine the effect of egg
532 quality on the spz egg^{-1} ratio.

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4.5 Why do Senegalese sole have a low spz egg^{-1} ratio?

534 Senegalese sole spawn as a female and male pair with no involvement of other individuals (Carazo et al., 2016) and spawning pairs show a degree fidelity during and between
536 spawning seasons (Fatsini et al., 2020; Martín et al., 2014). Therefore, Senegalese sole fertilisation does not involve sperm competition as all the sperm originates from a single
538 male and does not compete with sperm from other males. In addition, the two sexes swim in synchrony with the genital pores held close together (Carazo et al., 2016). The male
540 urogenital duct is slightly raised and the female oviduct forms a kind of well when eggs are being stripped (personal observations), which together with the closeness of the fish
542 during gamete liberation (Carazo et al., 2016) suggest that the male and female place the spz next to the eggs in very close proximity. Other studies have demonstrated that these
544 behavioural and reproductive strategies are related to low spz egg^{-1} ratios or low sperm production. Reducing the space of the fertilisation environment has been shown to reduce
546 the spz egg ratios^{-1} required (Bombardelli et al., 2013). Different species have very varied strategies and behaviours that will alter the space of the fertilisation environment, which
548 can range from mass spawning in aggregations in open water (Domeier and Colin, 1997; Ibarra-Zatarain and Duncan, 2015) to spawning between two fish in an enclosed space or
550 in very close proximity (Carazo et al., 2016; Tatarenkov et al., 2006). The spawning behaviour and number of individuals involved will influence the degree of sperm
552 competition that gametes must negotiate to achieve fertilisation. Sperm competition has been shown to influence fertilisation success and the number of spz that a species
554 produces (Parker and Pizzari, 2010; Stockley et al., 1997). Monogamy, and the absence of sperm competition was demonstrated to reduce testes size across different taxa and
556 monogamous fish species defined as spawning in a pair had significantly smaller testes compared to polyandrous species (group spawning of males with a female) (Baker et al.,
558 2020). Therefore, as has been suggested for fresh water fish (Kholodnyy et al., 2020), reproductive strategies and behaviour appear to be linked to gamete requirements to
560 achieve high rates of fertilisation. Consequentially, the reproductive strategy and behaviour of Senegalese sole support the described low spz production (García-López et
562 al., 2005; González-López et al., 2020) as well as the low spz egg^{-1} ratio for fertilisation (present study). It can be hypothesised that spz egg^{-1} ratio is related to reproductive
564 strategies and sperm production, however, more work is required across a wide range of species to determine the existence of a relationship.

566
4.6 Conclusion

In conclusion, Senegalese sole require a low spz egg^{-1} ratio to achieve high percentages
568 of fertilisation both on an experimental scale and in proof-of-concept large-scale *in vitro* fertilisations. The low spz egg^{-1} ratio required to fertilise all viable eggs was consistent
570 with the reproductive behaviour and strategies of the species. The protocol (200 µL of sperm per 100 mL of eggs) described in the present study will enable the Senegalese sole
572 aquaculture industry to operate sustainably and establish breeding programs.

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