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Sperm motility in European hake, *Merluccius merluccius*, and characterization of its spermatozoa concentration and volume, spermatocrit, osmolality and pH

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Abstract:

Due to stock declines there is a growing interest in farming of European hake *Merluccius merluccius*. Thus, knowledge of its sperm biology is of importance not only for purposes of broodstock management, but also for the development of sperm preservation techniques. Hake sperm were collected from mature males caught during the summer–early autumn waters off western Norway and during the winter–early spring in the Bay of Biscay (France). Sperm quality characteristics were assessed after storage at 4 °C for 25 ± 14 h. Average (± SD) values for Norwegian and French samples respectively were (i) sperm volume: 3.9 ± 4.0 and 2.6 ± 4.0 ml; (ii) spermatozoa concentration: 6.6 ± 3.2 and 13.9 ± 5.1 × 10^9 spermatozoa/ml; (iii) spermatocrit: 80.2 ± 3.3 and 81.8 ± 10.7%; and (iv) total number of spermatozoa: 23.5 ± 30.0 and 35.1 ± 36.2 × 10^9. Average osmolality and pH (± SD) of French samples were 349 ± 28 mOsmol/kg and 7.6 ± 0.1, respectively. Activation by transfer into full sea water (100 SW) or 10% ovarian fluid in sea water (10 OF) occurred synchronously for virtually all spermatozoa and the percent sperm motile decreased with post activation time. When transferred into 50% sea water diluted with distilled water (50 SW) only a few spermatozoa were activated initially but subsequently reached a maximum percentage of motility followed by a decline. Hake sperm motility declined rapidly to 50% of motility 70 s after activation with 100 SW. Sperm were motile for longer when activated with 50 SW (1570 ± 295 s) or 10 OF (718 ± 71 s) compared to 100 SW (317 ± 121 s). Undiluted hake sperm stored at 4 °C up to 10 days retained 10% motility when activated with 100 SW. When cryopreserved, the motility recovery index of the cells at thawing ranged from 0 to 76.4%. These results describe for the first time the sperm traits of European hake following successful cryopreservation, and also show the importance of activation medium on sperm motility.

Keywords: Cryopreservation; *Merluccius merluccius*; Osmolality; pH; Sperm motility; Spermatocrit

Key-words: Cryopreservation, *Merluccius merluccius*, osmolality, pH, sperm motility, spermatocrit.

1. Introduction

The reproductive biology of European hake (*Merluccius merluccius*) has been studied extensively in the field, but mainly in the context of fecundity regulation and fisheries analysis, and concentrating primarily on females (Murua and Motos, 2006). European hake is a highly important commercial species throughout its geographical range, especially in Spain and Italy.

Because European hake catches have been decreasing since the 1960's (FAO, 2006), the commercial viability of aquaculture production and the interest in hake as a potential aquaculture species has recently increased (Quémener et al., 2002; Kjesbu et al., 2006). Some researchers have stated that the hake is one of the most promising new species for marine aquaculture (Engelsen et al., 2004). Quémener et al. (2002) highlighted its fast growth rate as factor contributing to the high potential value for aquaculture, as well as its excellent flesh quality which gives it a very high market value when sold fresh.

For successful domestication of hake, a better understanding of its reproductive biology, including sperm biology, is needed for purposes of broodstock management and also for the development of sperm storage techniques, including cryopreservation. Such information is presently lacking, except for spermatozoa ultrastructure which was studied by Medina et al. (2003), and spermatozoa motility characteristics studied by Cosson et al. (2008a). Male reproductive biology has not received much attention primarily because hake is sensitive to handling, difficult to keep alive after capture

(Hickling, 1933; Belloc, 1935), and therefore has only rarely been kept in captivity (Bjelland and Skiftesvik, 2006). To date, only two captive broodstocks have been established, the oldest is in Brekke, Norway (R. Salte, Norwegian University of Life Sciences, pers. comm.), and a more recent one in Vigo, Spain (F.J. Sanchez, Instituto Español de Oceanografia, Vigo, Spain, pers. comm.).

Male fertilization potential is dependent on sperm quality (Trippel, 2003). The most common factors employed in studying sperm biology are the structure and motility parameters of the spermatozoa, the biochemistry of the spermatozoa and seminal plasma, and the metabolism of spermatozoa (respiration and energetics of motility) (Billard and Cosson, 1992; Billard et al., 1995). The sensitivity of sperm to storage and cryopreservation is important information for broodstock management programs. This can be assessed by studying the activation, motility, and fertility of stored sperm (Billard and Cosson, 1992; Billard et al., 1995).

The peak spawning time of hake is in March in waters south of the Bay of Biscay (France), and occurs progressively later at higher latitudes (Casey and Pereiro, 1995). The spawning time along the west coast of Norway is expected to occur during late summer; Kvenseth et al. (1996) showed records of ripening hake in August in these waters. Male hake from both the northern (Norway) and middle (Bay of Biscay) sections of their geographic range were collected as these regions are located in proximity to aquaculture research facilities and the associated industry.

In the present study, sperm production characteristics (volume, spermatozoa concentration, spermatocrit and total number of spermatozoa), biochemistry of the total sperm (osmolality and pH), motility characteristics of the spermatozoa after activation including survival after storage at 4ºC and freezability for cryopreservation were studied in European hake. Collecting sperm from mature males from the same area in different

time periods would in the future be of interest to evaluate seasonal changes in sperm quality.

2. Materials and methods

2.1. Fish and sperm collection

Adult wild European hake were sampled during the spawning season from two geographically separated Atlantic populations, one from Western Norway (61° 34'N, 5° 56'E), and the other from the Bay of Biscay, France (47° 44'N, 4° 2'W). Fish were caught by gillnets (13.5 cm mesh size) set overnight at depths of between 30-180 m over sandy sea bottom. Each morning sperm were collected from live or recently dead fish (few hours) which were retrieved from the gillnets. Less than 10% of the mature males were discarded according to these criteria. Sperm were collected from 19 sexually mature males from mid-August until mid-October in 2005 and 2006 off Western Norway (main spawning season for Norwegian hake) and from 45 sexually mature males from March to early April in 2006 and 2007 in the Bay of Biscay (end of spawning season for French hake). Sperm were manually stripped: the fish genital pore was rinsed with fresh water and dried quickly off before sperm were collected in graduated syringes. Special care was taken to collect all the available sperm from each fish and not to contaminate the sperm sample with blood, urine or seawater. Any potentially contaminated sperm samples were discarded. Syringes were stored in individual labeled sealed plastic bags to avoid cross-contamination and samples were stored in a polystyrene rack above crushed ice which filled the bottom of a closed polystyrene box. The ice was changed approximately every 6 h to maintain a constant temperature of 4ºC until the samples were brought back to the laboratories within 7 to 49 h post gamete collection.

122

123 *2.2. Fish indices*

124 Stripped fish (61 out of 64 collected) were transported to the laboratories to be dissected
125 within 10 to 52 h of sampling. There, fish were measured for total length (TL in cm),
126 total weight (TW in g), gonadosomatic index (GSI in %), hepatosomatic index (HSI in
127 %) and condition factor (K). The following formulas were used to calculate these
128 variables:

129 $$GSI = (\text{Gonad weight} / \text{Total weight}) * 100$$

130 $$HSI = (\text{Liver weight} / \text{Total weight}) * 100$$

131 $$K = (\text{Total weight} / (\text{Total length})^3) * 100$$

132

133 *2.3. Sperm production indices*

134 Total sperm volume was estimated with 0.1 ml precision. Spermatozoa
135 concentration was estimated by counting spermatozoa in a Fuchs-Rosenthal chamber
136 (Glaswarenfabrik Karl Hecht GmbH&Co, Hecht Assist, Sondheim, Germany) or in a
137 Malassez chamber (Preciss, Strasbourg, France) at 400 X magnification. Samples were
138 diluted from 1:500 to 1:1000, depending on sperm concentration, in a non-activating
139 medium (NAM) described for sea bass (*Dicentrarchus labrax*) sperm (Fauvel et al.,
140 1998). After several minutes to allow sedimentation of cells, heads were counted.
141 Sperm counts were duplicated using separate subsamples, and spermatozoa
142 concentrations estimated based on the average of the counts. Spermatozoa concentration
143 was expressed as the number of spermatozoa per ml of sperm. The total number of
144 spermatozoa for each male was estimated from the total sperm volume multiplied by the
145 spermatozoa concentration. Spermatocrit values were measured by collecting duplicate
146 subsamples of freshly stripped sperm in 75 mm heparinized capillary tubes (KEBO

147 Laboratories AB, Stockholm, Sweden). After proper sealing, the samples were spun for
148 5 minutes at 10.000 rpm (250 g) in a Hettich Haematocrit centrifuge (Andreas Hettich
149 GmbH & Co., Tuttingen, Germany). The spermatocrit value was calculated as the
150 percentage of spermatozoa volume relative to total sperm volume. Osmotic pressure
151 (mOsmol / kg) and pH of the total sperm were measured respectively using an
152 Advanced Micro Osmometer (13/13DR-Autocal, Hermann Roebling MESSTECHNIK,
153 Berlin, Germany) and a pH-meter (Minisis 8000, Tacussel Electronic, Villeurbanne,
154 France) with a micro-electrode (XC 161, Radiometer Analytical, Copenhagen,
155 Denmark).
156
157 *2.4. Measurement of sperm motility characteristics*
158 Sperm motility was evaluated by the same reader on every collected sample as
159 soon as they were transported to the laboratories which were set up with similar
160 conditions in both locations and equipped with the same material. Norwegian sperm
161 (Nw-sperm) motility was first evaluated within 18 ± 8h (mean ±SD) after gamete
162 collection and French (Fr) within 22 ± 13h. Sperm motility was assessed using a two
163 step dilution procedure. Undiluted sperm as collected from sperm ducts were diluted
164 with NAM (dilution 1:20). No movement of sperm was observed and 0.3 µl of this
165 diluted sperm were added to a 20 µm-depth Leja sperm counting chamber (Leja
166 Products BV, Nieuw-Vennep, the Netherlands) followed by 4.5 µl of the activating-
167 medium (AM see below). Sperm and diluents were kept in the polystyrene box as
168 described above with the bottom filled with crushed ice (4°C) when preparing the
169 mixture. The different AMs tested were: 100 % filtrated sea water (100 SW) (filter and
170 UV filter, Unik filtersystem AS, Os, Norway), 50 % filtrated sea water diluted with
171 distilled water (50 SW), and 10 % ovarian fluid diluted with SW (10 OF). The

172 respective osmolalities (mOsmol / kg) of these three different AMs, measured using an
173 Advanced Micro Osmometer (13/13DR-Autocal, Hermann Roebling MESSTECHNIK,
174 Berlin, Germany), were: 998, 498 and 936 respectively. Bovine Serum Albumin (BSA
175 0.1 %, Sigma -Aldrich Inc., Sigma ref A9647, St-Louis, MO 63103 USA) was added to
176 both the NAM and AM to prevent spermatozoa sticking to glass surfaces (Cosson et al.,
177 2003). Following sperm activation at room temperature in both locations (22°C), video
178 recordings were made using a Sony CCD black and white camera, mounted on an
179 Olympus negative-phase contrast microscope 200 X, linked to a video monitor. By
180 simple visual observation of the video recordings, the same trained reader throughout
181 the study estimated the percent sperm motile every 15 s after activation until all
182 spermatozoa ceased activity (in replicates for each sperm sample tested). Efficient
183 mixing was obtained within 5 s and therefore, video-recordings and percent sperm
184 motile observations started with a 5-10 s lag phase. Vibrating or stationary spermatozoa
185 (non progressive) were considered as non-motile. The total motility duration was
186 defined as the time from the activation until 10 % of spermatozoa were still motile.
187 Changes in the percent sperm motile as a function of time post activation (p.a.)
188 with the different AM were plotted. Storage time of sperm ($\leq$ 22 h) did not significantly
189 affect the percent sperm motile as a covariate after sperm activation in 100 SW
190 (intercept: $P > 0.05$, $F = 2.36$, $df = 5$; slope: $P > 0.05$; $F = 1.75$; $df = 3$; ANCOVA).
191 Only sperm with storage time of 10 h were kept for sperm activation in 50 SW. Sperm
192 samples activated in 10 OF were all tested 10 h after gamete collection. Therefore, for
193 evaluating the percent sperm motile as a function of the time p.a., data collected from
194 sperm samples with storage periods lower than 30 h were used for activation in 100 SW
195 and with storage periods of 10 h for sperm activation in 50 SW and 10 OF.
196

197 *2.4.1. Sperm storage at 4°C*

198 As soon as sperm samples were brought back to the laboratories
199 (Høyteknologisenteret, HIB, Bergen, Norway and Ifremer Brest, France) they were
200 stored in refrigerators at 4°C. The effect of storage at 4°C on percent sperm motile was
201 assessed on sperm samples activated by dilution in 100 SW. These measurements were
202 performed approximately every 24 h after stripping until the sperm sample did not show
203 any cell movement. To plot percent sperm motile versus sperm storage period at 4°C,
204 only the percent sperm motile recorded at 15 s after activation were considered. The
205 sperm storage period was calculated as the time between stripping and assessment of
206 sperm motility. The storage suitability at 4°C of a sperm sample was defined as the
207 storage period at which 10 % of spermatozoa were still motile.

208

209 *2.4.2. Cryopreservation*

210 The time period between sperm stripping and cryopreservation was calculated
211 for each sperm sample (n = 1 Nw + 21 Fr) and ranged from 21 to 54 h. Hake sperm
212 samples were frozen after dilution (1:2) in Mounib's Modified Medium extender,
213 supplemented with 10 % BSA and 10 % of dimethyl sulfoxide DMSO (Sigma -Aldrich
214 Inc., Sigma ref D2438, St-Louis, MO 63103, USA) used as a cryoprotectant according
215 to the method of Dreanno et al. (1997) used for turbot (*Psetta maxima*). This protocol
216 used for turbot was also shown to be successful for cryopreserving sea bass sperm
217 (Fauvel et al., 1998). Hake sperm samples (one volume) were mixed with freezing
218 extender (two volumes), corresponding to a final DMSO concentration of 0.94 M. No
219 equilibration time was allowed. The diluted sperm sample was back aspirated into
220 CBS^{TM} 0.3 ml straws (Cryo Bio System, I.M.V. Technologies Group, L'Aigle, France)
221 using a micropipette (in triplicate for each male) and immediately sealed with the

222 SYMS sealing system (Cryo Bio System, I.M.V. Technologies Group, L'Aigle, France).
223 Freezing was achieved using a polystyrene box covered with a lid (external
224 measurements L x W x H = 33 x 25 x 30 cm), half filled with liquid nitrogen (LN). The
225 straws were placed inside and floated in nitrogen vapour, 6.5 cm above the surface, on a
226 raft (Styrofoam and plastic grid). After 15 min (temperature of the straws decreased
227 from 21 to - 60ºC; unpublished data), straws were deep plunged into LN (-196ºC) and
228 stored submerged until thawing. For thawing, straws were immersed for five seconds in
229 a 35°C water bath. After drying, straws were cut open to carefully release the volume
230 into an Eppendorf tube. Separate 2.5 µl aliquots of this frozen-thawed sperm were then
231 immediately activated with 397.5 µl of 100 SW.
232
233 *2.5. Data analyses*
234 Statistical analyses were performed using the software SigmaStat 3.1. Data were
235 presented as means ± SD. Significant differences in sperm characteristics between
236 Norwegian and French samples were examined using a Student's t-test when data were
237 distributed normally and variances were not significantly different or a Mann-Whitney
238 Rank Sum test if one of these two previous conditions or both were invalid. No
239 significant size effect (TL) on sperm characteristics was found ($P > 0.05$; t-test),
240 therefore analyses could be conducted on all sizes pooled. Relationships between sperm
241 production indices and characteristics of individual fish were investigated with Pearson
242 correlations for Norwegian and French samples, separately.
243 Tested sperm samples ranging from 60 to 92.5 % at 15 s p.a. were used for
244 sperm motility analyses. Percent sperm motile were regressed on time p.a. The
245 coefficient of determination (R^2) calculated by ordinary least-squares regression,
246 allowed us to define the regression line approximating at best the real data points (i.e.

247 the regression with the highest R^2 was selected). The effect of 100 SW was tested on
248 both Nw and Fr sperm samples (50 SW and 10 OF were tested only on Nw-sperm
249 samples). ANCOVA with interaction and Ln time transformed data were used to
250 compare regressions fitted separately to Nw and Fr data: since no significant difference
251 was observed in motility after activation (intercept: $P > 0.05$, $F = 3.50$, $df = 1$; slope:
252 $P > 0.05$; $F = 1.58$; $df = 1$; ANCOVA), a general regression was fitted to the pooled
253 data of both groups (Nw and Fr samples). Data were Ln transformed to satisfy the
254 homogeneity of variances assumption for the errors and to linearize the fit as much as
255 possible.

256 The origin of sperm samples as well as the storage times between collection and
257 first measurements for Nw and Fr samples were not significantly different ($P > 0.05$,
258 Mann-Whitney Rank Sum Test). Changes in percent sperm motile as a function of
259 sperm storage period at 4ºC for sperm activated with 100 SW were compared between
260 Nw and Fr samples at 15 s p.a. Because no significant differences were observed
261 between both groups (intercept: $P > 0.05$, $F = 0.02$, $df = 1$; slope: $P > 0.05$; $F = 1.06$;
262 $df = 1$; ANCOVA) a general regression was fitted to the pooled data and the regression
263 with the highest R^2 selected.

264 A repeated measures ANOVA was conducted to test the difference between
265 percent of fresh compared to thawed sperm. Normality and equal variance were not
266 achieved. Therefore, the motility data (%) were arcsine transformed data prior to
267 analysis using the following formula: ASIN x {√(percent sperm motile / 100)}. A mean
268 motility recovery index was calculated: motility recovery index = (% motility thawed
269 sperm / % motility fresh sperm) * 100.

270

271 **3. Results**

3.1. Fish and sperm production characteristics

The Norwegian hake were significantly longer, heavier, and had a higher reproductive investment (GSI) compared to French hake (Table 1). GSIs estimated for Nw-hake caught from mid-August until mid-September varied around 4.8 % (with a decline from 7.5 to 2.9 %) while GSIs estimated on Fr-hake caught at the end of March, early April were stable at ~ 1.1 % (Table 1). Spermatozoa concentration was twice as high in the samples from Fr compared to Nw-fish samples (Table 1). Sperm production indices from Nw and Fr-stripped hake were estimated and showed high variations for all variables except for pH (Table 1).

3.2. Fish characteristics vs sperm production indices

No significant correlations were observed between TL, TW, K or HSI and any of the sperm characteristics. Likewise, no significant correlation was observed between Nw-spermatocrit and Nw-spermatozoa concentration while a significant positive correlation was observed between Fr-spermatocrit and Fr-spermatozoa concentration ($r = 0.539$, $n = 18$; $P < 0.05$). A significant positive correlation was observed between Fr-spermatozoa concentration and Fr-GSI ($r = 0.831$, $n = 6$; $P < 0.05$). A significant negative correlation appeared between Fr-osmolality and Fr-pH ($r = -0.786$, $n = 9$; $P < 0.05$).

3.3. Measurement of sperm motility characteristics

3.3.1. Sperm motility versus time post activation

Activation by transfer into 100 SW or 10 OF occurred synchronously for virtually all spermatozoa. The percent sperm motile (y) was highest immediately after activation, and then declined with time p.a. (x) (Fig. 1). Significant correlations were

297 observed between percent sperm motile and time p.a. when sperm were activated with
298 100 SW: $y = -26.5 \ln(x) + 162.7$; $R^2 = 0.875$; $P < 0.001$ and when sperm were activated
299 with 10 OF: $y = -0.09 x + 81.91$; $R^2 = 0.873$; $P < 0.001$. In contrast, following activation
300 with 50 SW only a few spermatozoa were activated immediately, with maximum
301 percent sperm motile being attained at 608 ± 174 s p.a., after which the percentage of
302 motile cells again gradually declined over time (Fig. 1). A significant correlation was
303 observed between percent sperm motile and time p.a. when sperm were activated with
304 50 SW: $y = -2 \exp(-05 x^2) + 0.030 x + 27.02$; $R^2 = 0.213$; $P < 0.01$.

305 Taken together, there were clear differences in the decrease of motile sperm
306 depending upon whether sperm samples were activated with 50 SW, 10 OF or 100 SW
307 (Fig. 1): spermatozoa showed longer total motility durations when activated with 50 SW
308 (1570 ± 295 s) or 10 OF (718 ± 71 s) compared to activation by 100 SW (317 ± 121 s).
309 According to our regressions, the time p.a. at which 50 % of motility is reached was
310 more than five times longer when sperm were activated with 10 OF (355 s) than when
311 sperm were activated with 100 SW (70 s).

312

313 *3.3.2. Sperm storage at 4°C*

314 The percentage of motility observed when sperm were transferred into 100 SW
315 decreased with sperm storage period (Fig. 2). Undiluted sperm could be stored at 4°C
316 for 10 days (237 h) and still retain 10% of motility. According to our observations, after
317 2 days (48 h) at 4°C the percent sperm motile ranged from 0 to 90 %. After
318 approximately 9 days (220 h), percent sperm motile values of 50 and 65 % were still
319 observed in two individuals. The decrease in percent sperm motile with storage period
320 varied highly among individuals.

321

322 *3.3.3. Cryopreservation*

323 For every sperm sample tested, fresh sperm always showed higher motility percentages
324 compared to thawed sperm. However, for 4 out of 22 sperm samples (1 Nw and 3 Fr)
325 the effect of cryopreservation on sperm motility was not significant ($P > 0.05$, repeated
326 measures ANOVA). Hake sperm showed a mean motility recovery index of
327 15.6 ± 17.0 % for Fr-sperm samples (n = 21) and the only Nw-sample tested showed a
328 motility recovery index of 76.4 %.

329

330 **4. Discussion**

331 Basic biological features of European hake sperm are presented in this study. It was
332 necessary to collect hake sperm samples from wild individuals because no hake
333 spawning broodstock was available at the time of the study. The Fr-sampling was
334 carried out in March - April which was late in the season according to Lucio et al.
335 (2000) who found the peak spawning season for hake spanning from February to March
336 in the Bay of Biscay. The Fr-hake collected in the present study showed low GSI (from
337 0.95 to 1.24 %), and probably reflects the fact that these males were sampled towards
338 the end of their spawning season, concomitant with a decrease in testes weight. These
339 males had most likely completed most of their spawning activity, and as a result their
340 testes only contained residual sperm. In contrast, higher GSI values were observed in
341 Nw-hake (7.5 %), indicating fully mature individuals at the beginning of their spawning
342 season.

343

344 *4.1. Hake sperm production indices*

345 In comparison to other marine species, the sperm volume found in this study (3.9 and
346 2.6 ml respectively in Nw and Fr samples) was low compared to cod (*Gadus morhua*)

(Tuset et al., 2008) and high compared to turbot (0.2 ml, Chauvaud et al., 1995). Similarly, spermatozoa concentration and total number of spermatozoa recorded for hake were lower than values observed in cod (Trippel, 2003; Stockley et al., 1997) but higher than values found in turbot (0.7 to 11 x 10^9 spermatozoa / ml, Chauvaud et al., 1995). Hake exhibited spermatocrit values close to those reported in cod (Trippel, 2003). The somewhat thicker sperm in Fr-samples is consistent with the fact that Fr-sperm were collected at the end of the spawning season (Rakitin et al., 1999). The average osmolality calculated for Fr-hake sperm samples (349 ± 28 mOsmol / kg) was close to values reported previously in cod (360 - 380 mOsmol / kg) by Hwang and Idler (1969) and Litvak and Trippel (1998) as well as Rouxel et al. (2008) (332 - 407 mOsmol / kg). The seminal fluid pH measured in Fr-hake sperm samples (pH 7.6) was lower than values measured in cod (pH 7.9 - 8.4; Hwang and Idler, 1969; Litvak and Trippel, 1998; Rouxel et al., 2008). Hence, sperm production of hake was lower than cod and higher than turbot.

The significant relationship found between Fr-spermatocrit and Fr-spermatozoa concentration suggests the potential utility of spermatocrit as a simple and rapid estimator of spermatozoa concentration in Fr-hake. However, this result needs more investigation as this relationship was not apparent for the Nw-data in the present study. These types of relationships have previously been reported in cod (Rakitin et al., 1999) and in haddock (*Melanogrammus aeglefinus*) (Rideout et al., 2004).

4.2. Hake sperm motility

When activated with 100 SW at room temperature (22ºC), all hake spermatozoa ceased any activity after 465 s. Under similar recording conditions the duration of sperm motility in cod is 700 - 800 s (Cosson et al., 2008a). Furthermore, the percent sperm

motility of hake declined by 50% at 70 s post activation. In cod, 95% of the cells were still motile after 50 s (Cosson et al., 2008b). When hake sperm were activated with 100 SW or 50 SW, it was shown that total motility duration is inversely related to AM osmolality. This observation is supported by earlier findings of Billard (1978) and Lahnsteiner and Patzner (1998), who showed durations of sperm motility in a number of marine species were longer in partly diluted sea water compared to full-strength sea water, probably because it leads to a less harmful osmotic environment for sperm (extreme osmotic situations could provoke local membrane distortions leading to flagellar blebs or coils) (Cosson et al., 2008c). When activated with 10 OF, spermatozoa also showed a longer total motility duration compared to 100 SW. The presence of OF in the spawning medium can have significant positive effect on teleost spermatozoa motility and fertility (Litvak and Trippel, 1998; Elofsson et al., 2003). By prolonging gamete contact times during the fertilization process one may significantly increase egg fertilization success as suggested by Butts et al. (2009). The implications of these findings are relevant to aquaculture practices. “Wet fertilization” is commonly practiced for many marine species as it allows the mixing of sperm first with an AM, thus avoiding having to pour sperm on eggs within seconds (Trippel and Morgan, 1994).

4.3. Sperm storage

Hake sperm viability was maintained for 10 days at 4°C. These results were comparable to observations in cod sperm by De Graaf and Berlinsky (2004), where undiluted sperm stored at 4°C still showed 11.0 ± 0.7% of motile cells after 10 days. However, after 48 h at 4ºC the percent sperm motile for hake ranged from 0 to 90 %. Sperm samples showing less than 40 % motility after 48 h at 4ºC all originated from France. Rouxel et al. (2008) observed higher storage potential of cod sperm when collected during the

mid-period of the spawning season, as compared to sperm collected at the beginning or end of the spawning season. Our French data corroborate this finding: a low storage potential was highlighted for the sperm collected at the end of the spawning period (Fr-hake).

Generally, DMSO is the most commonly used and most successful cryoprotectant for sperm cryopreservation of marine fish (Leung and Jamieson, 1991; Stoss, 1983; Suquet et al., 2000). The effect of DMSO is concentration dependent, with a concentration between 5 and 20 % commonly used (Suquet et al., 2000). In agreement with this, our results showed that DMSO at 10 % could be used as cryoprotectant for hake sperm. The techniques established by Dreanno et al. (1997) for turbot sperm cryopreservation appear to be suitable for European hake, as the mean motility recovery index for Fr-samples was 15.6 ± 17.0 % and the motility recovery index for the Nw-sample was 76.4 %. The mean value for Fr-samples was low compared to the results found in cod by De Graaf and Berlinsky (2004) who achieved a motility recovery index of 66.0 ± 2.1 %. However, the motility recovery index appears to be related to the time of sperm collection. For example, according to Rideout et al. (2004), cod sperm collected at the end of the spawning season showed a reduced post-thaw motility compared with those collected two weeks after the earliest part of the spawning season.

In conclusion, this study provides original data on European hake sperm quality which should be beneficial for the future development of any aquaculture program of this species.

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567

568 Table 1. Fish and sperm characteristics of European hake from two areas:
569 Statistically significant differences between Norwegian (Nw) and French (Fr) samples
570 are indicated with * P<0.05, ** P<0.01, and *** P<0.001. Osmolality and pH were
571 measured for sperm samples from Fr-hake only. GSI values are presented by date of
572 collection.

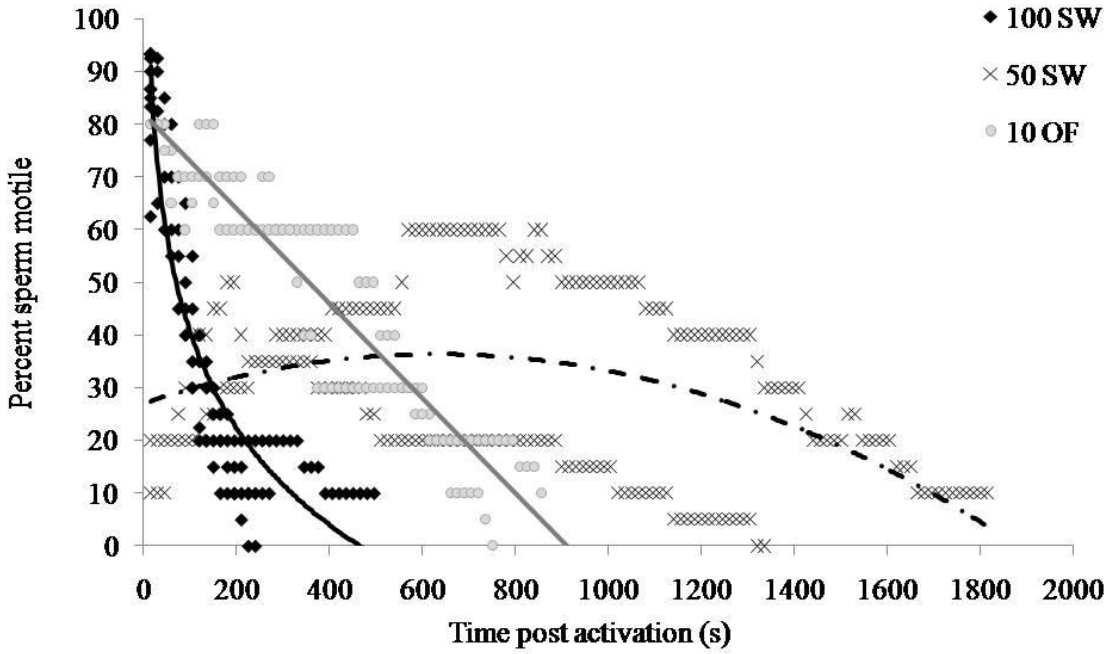
Variables		Origin	*N*	Mean	S.D.	Significance
Sampled fish characteristics						
TL (cm)		Nw	19	68.5	8.5	***
		Fr	42	40.1	9.5	
TW (cm)		Nw	16	2498	822	***
		Fr	42	490	351	
K		Nw	16	0.69	0.05	n.s
		Fr	42	1.24	3.98	
GSI (%)	collection date	Nw	10	4.80	2.94	
	17.08.05			7.52	2.28	
	22.08.06			5.31		
	01.09.05			4.35	3.40	***
	12-14.09.06			2.94	1.87	
		Fr	18	1.10	0.51	
	20-21.03.06			1.24	0.55	
	04-05.04.06			0.95	0.44	
HSI (%)		Nw	16	2.62	1.04	n.s
		Fr	17	1.89	0.93	
Sperm production indices						
Sperm volume (ml)		Nw	19	3.9	4.0	n.s
		Fr	45	2.6	4.0	
Spz conc. ($x10^9$ spz / ml)		Nw	5	6.6	3.2	**
		Fr	29	13.9	5.1	
Spermatocrit (%)		Nw	5	80.2	3.3	n.s
		Fr	19	81.8	10.7	
Tot. no of spermatozoa ($x10^9$spz)		Nw	5	23.5	30.0	n.s
		Fr	29	35.1	36.2	
Osmolality (mOsmol/ kg)		Fr	13	349	28	
pH		Fr	9	7.6	0.1	

573

574 **Figures legends**

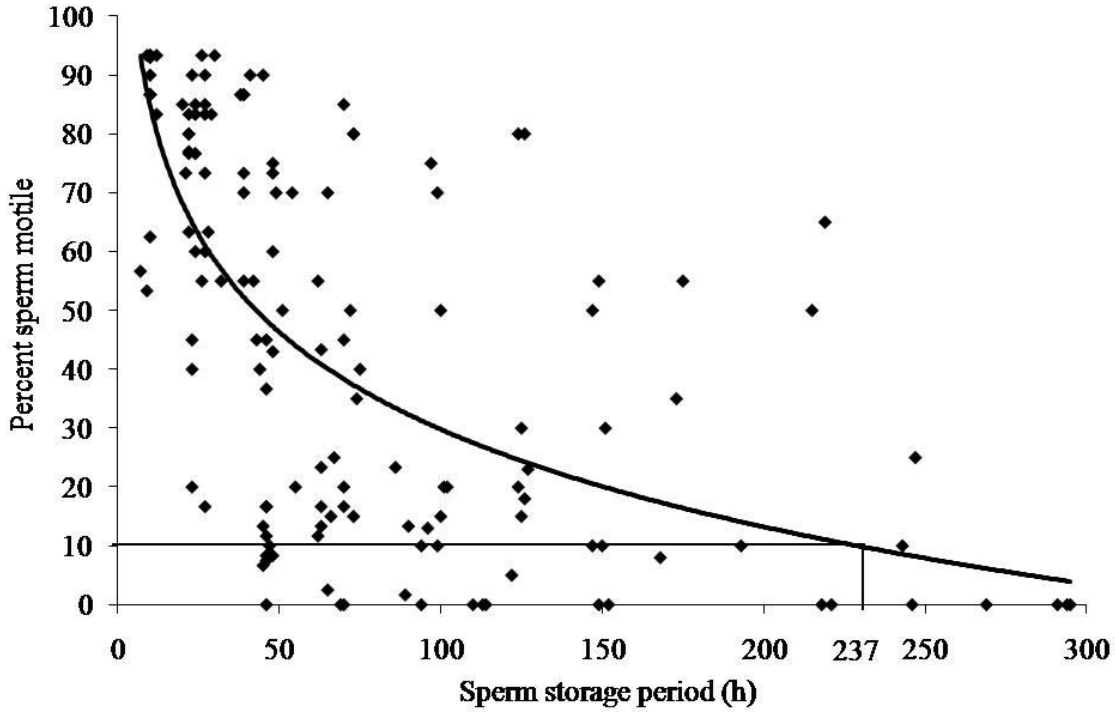
575 Fig. 1. Changes in the percent sperm motile with time post activation of European hake:
576 Sperm stored for periods ≤ 22 h were activated by dilution in 100 % sea water (100 SW)
577 (plain black signs and black regression line) (N = 3 Norwegian hake + 11 French hake),
578 50 % SW diluted with distilled water (50 SW) (cross and dashed regression line)
579 (N = 2 Norwegian hake) or 10 % ovarian fluid in SW (10 OF) (plain grey signs and grey
580 regression line) (N = 2 Norwegian hake).
581
582 Fig. 2. Changes in percent sperm motile of European hake with duration of sperm
583 storage at 4°C. Sperm (N = 5 Norwegian hake + 29 French hake) were activated with
584 100 % sea water (100 SW). A regression was fitted to the entire data set:
585 $y = -23.9 \ln(x) + 139.8$; $R^2 = 0.426$; $P < 0.001$
586

587 **Figures**



588
589 Fig. 1.

590



591
592 Fig. 2.