Sperm quality in wild-caught and farmed males of the European eel (Anguilla anguilla).

Sperm quality in wild caught and farmed males of the European eel (*Anguilla anguilla*).

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ABSTRACT

Sperm density and performance of wild and farmed male European eels were evaluated to investigate the effect of maintenance in captivity on ejaculate quality. Hormonal stimulation (with human chorionic gonadotropin) lasted for 17 weeks. Different origins of the male European eels partially influenced the quality of the ejaculates. Indeed, wild animals (i.e., collected at a natural site) had greater sperm longevity during the hormonal stimulation, whereas there was no significant effect of the origin on sperm density, spermatocrit, the percentage of motile sperm, and plasma testosterone concentrations. The different origins of the males also affected fatty acid sperm content, with greater unsaturated fatty acid and omega-6 concentrations in wild eels and lesser concentrations of saturated fatty acids and omega-3 fatty acids in farmed eels. Regarding sperm quality of European eels, this is the first study that takes into account the effect of different origins of stimulated males (wild-caught compared with farmed) on sperm quality, and these findings may help to improve the production of high-quality gametes in this endangered species.

Keywords: European eel; Captivity; Sperm; ejaculate Quality; Fatty acids

1. Introduction

The European eel (*Anguilla anguilla*) is a catadromous teleost fish that spends most of its life in freshwater of lakes and rivers until the spawning migration from the European coastal waters to the Sargasso Sea. In the last century, the European eel population has constantly decreased due to predators and anthropogenic pressure (e.g., migration barriers, hydropower turbine–induced mortality, natural habitat degradation, and overfishing; Bevacqua et al., 2015). Eel fisheries and farming activities in Europe totally rely on wild stocks, which dramatically decreased in recent decades (Dekker et al., 2003; Stone, 2003). For these reasons, the European eel is now considered a 'critically endangered' species (IUCN, 2015), and the development of techniques for reproduction in captivity is becoming necessary.

55 During adaptation to the saline environment, eels have with morphological changes as the
56 skin pigmentation turns from yellow to silver and with complex physiological changes that help
57 with adaptation for the long migration period. During silvering, gonadal development is inhibited
58 by complex hormonal control, and it is next released to initiate the reproductive process, but the
59 timing and mechanisms responsible for the cessation of inhibition are still unclear (Tomkiewicz et
60 al., 2011). In captivity, gametogenesis in the European eel can be induced only through hormonal
61 stimulation, and injections of human chorionic gonadotropin (hCG) into males and of a fish
62 pituitary extract into females are commonly used to induce sexual maturation in the European eel,
63 as well as in various other eel species (Tanaka et al., 2001). In addition, injection of 17α, 20β-
64 dihydroxy-4-pregnen-3-one (DHP) for final female maturation and induction of ovulations results
65 in increased fertility and hatching rates (*A. japonica*: Ohta et al., 1996). Fertilized eggs and
66 production of viable embryos and larvae are now feasible (Palstra et al. 2005; Butts et al., 2016;
67 Sørensen et al., 2016).
68 Monitoring of gamete quality is a key issue for efficient artificial reproduction strategies.
69 Indeed, the quality of gametes reliably predicts a gamete's capacity to successfully fertilise or be
70 fertilised and to produce viable offspring (Migaud et al., 2013). In the European eel, there has been
71 reliable spawning techniques and protocols developed (Mordenti et al., 2013), thus, the evaluation
72 of gamete quality is becoming a key step to optimise artificial breeding strategies. Traditionally, the
73 fish farming industry has been more focused on the monitoring of egg and larvae quality, rather
74 than on milt, even though milt quality may also affect the production of healthy offspring
75 (Rurangwa et al., 2004). Nonetheless, recent studies on aquaculture increased the focus on sperm
76 quality (Migaud et al., 2013), and studies on European-eel reproduction are not an exception
77 (Peñaranda et al., 2010; Gallego et al., 2012). Because the progression of gonadal development has
78 not been ascertained in European eels, increasing research interest is focused on assisted
79 reproduction in captivity. Male European eels can be difficult to find (and capture) in nature and
80 still need to be maintained in captivity to hormonally induce gonadal maturation. Thus, there is a

need to determine whether short-term (wild caught) and/or long-term (farmed) captive rearing is affecting reproductive performance of male European eels. Hence, the present study was conducted to compare i) sperm density and performance, ii) sperm fatty acid content, and iii) testosterone profiles between wild-caught and farmed male European eels after hormonal stimulation.

2. Materials and methods

2.1. Fish sampling and maintenance

Thirteen male adult silver eels (Durif et al., 2005) were used. Six individuals (wild) were collected at a natural site near the sluices of the North Adriatic Sea (Val Noghera, Lagoon of Grado, Italy) using a traditional 'lavoriero' (downstream trap). Seven individuals (farmed) were selected on a controlled fish farm (Succi Fish Farming, Goro, Italy). Due to the different origins, the animals of the two groups were subjected to a different feeding regimen until sampling: wild eels were given natural feed, usually made up of Polychaeta, insects, crustaceans, and small fish (see Costa-Dias & Lobón-Cerviá, 2008, Mordenti et al., 2016), whereas farmed eels were fed with an extruded feed for eels (ALTERNA EEL, Skretting; composition: fish meal, fish oil, wheat red dog, wheat gluten, blood meal from poultry, a soya bean protein concentrate, swine haemoglobin, and whey powder).

Both wild and farmed eels were moved to the facilities of the Department of Veterinary Medical Sciences (Cesenatico, Italy). All the animals were weighed, individually marked by inserting fish-tags (FLOY TAG Mod Floy T-Bar Anchor), and maintained in two separate 700 L tanks. Wild and farmed males did not differ in the baseline average weight (mean ± st.dev.: wild, 112.2 g ± 25.8; farmed, 90.9 g ± 16.8; *t* test, t = 1.79, degrees of freedom [df] = 11, p = 0.101). All eels were gradually acclimated during the first 7 days to reach the standard experimental conditions: temperature of 15 ± 0.5°C, salinity of 32 ‰, pH 7.8, and dissolved oxygen 9 ppm. The maintenance water was collected directly from the sea, through a hydraulic pump drawing offshore, and was properly decanted before use. During the experimental period, the eels were maintained in darkness and without feeding according to previously published protocols (Dollerup and Graver, 1985). The

107 animal sampling and manipulation procedures were approved by the Ethical Committee of the
108 Bologna University (n° 19/69/12).
109
110 *2.2. Hormonal stimulation*
111 To induce the ejaculate production, the male eels received a weekly intramuscular injection
112 of 1.0 IU/g hCG (Corulon, 5000 UI, Intervet, Segrate, Milan, Italy) using the previously published
113 protocol (Mordenti et al., 2013). Prior to injection, all the animals were anaesthetised with
114 phenoxyethanol (400 ppm).
115 The hormonal stimulation started in March 2014 and lasted for 17 weeks (experimental
116 timeline in Fig. 1). Most of the male eels started to produce high quality ejaculates starting from the
117 11^{th} week, but all the males produce high quality ejaculates starting from the 13^{th} week; thus, sperm
118 analyses lasted from the 13^{th} to 17^{th} week (Fig. 1).
119
120 *2.3. Ejaculate sampling*
121 Ejaculates were collected 24 hours after the hormone injection (Pérez et al., 2000) with the
122 exception of the 13^{th} week when milt samples of nine males (five wild, four farmed) were sampled
123 48 hours after the hormone injection. The time interval between the injection and ejaculate analyses
124 (24 or 48 hours) was, then, included as a factor in the data analyses (see 2.9. Statistical analyses).
125 Each eel was anaesthetised (phenoxyethanol, 400 ppm), placed in a dry cloth, and the
126 ejaculate was collected by a delicate pressure on the abdomen after accurate cleaning the urogenital
127 area to avoid any contamination by urine or faeces. The ejaculate was collected directly into a 15
128 mL vial with the help of a plastic catheter. To prevent any excessive stress, the males were not
129 stripped to the extent all semen was removed and a maximum amount of 2 mL of an ejaculate was
130 collected. The samples were maintained at 3 to 5 °C until sperm quality analyses, which were
131 performed within 1 hour after collection (Pérez et al., 2000).
132

133 *2.4. Sperm concentration*

134 Sperm concentration was measured by both spermatocrit (i.e., the ratio of packed sperm to
135 the total volume of milt × 100) and sperm density by means of a Neubauer Improved
136 haemocytometer (sperm number per microliter of an ejaculate), and the results of the two methods
137 were then compared according to previously published studies (Sørensen et al., 2013).

138 To measure the spermatocrit, a subsample of the ejaculate was gently mixed by vortexing
139 and was drawn into a microhaematocrit tube (75 mm long; 1.1–1.2 mm diameter), then sealed with
140 wax. Two tubes for each male were collected and centrifuged for 5 minutes at 13000 × *g*. The
141 proportion of sperm was measured on a micrometric scale. The mean of the two measurements per
142 male was used for statistical analyses. Repeatability of the measurement (i.e., the statistical measure
143 of the consistency of repeated measurements, calculated according to Becker, 1984) – determined
144 for each sampling week – was always greater than 0.993.

145 For sperm cell counting, an ejaculate was diluted via a two-step procedure by first diluting
146 the sample 1:5 and then at 1:200, to attain a final dilution of 1:1000. Dilution was conducted with
147 an inactivating extender (3.5 g/l NaCl, 0.11 g/l KCl, 0.39 g/l $CaCl_2$, 1.23 g/l $MgCl_2$, 1.68 g/l
148 $NaHCO_3$, 0.08 g/l glucose, pH 7.7; Fauvel et al., 1999) that maintained the sperm immotile, thus
149 facilitating more precise counting. Sperm cells were counted in a 7 µL sample on the
150 haemocytometer at 400× magnification.

151

152 *2.5. Percentage of motile sperm*

153 This percentage was measured after activation with 2 µL of the stored ejaculate with the
154 addition of 1 mL of UV-sterilised marine water sampled from the storage tanks (see 'Fish sampling
155 and maintenance'). Three microliters of the activated sample were placed on a 12-well multitest
156 slide (MP Biomedicals, Aurora, OH) pre-coated with 1% polyvinyl alcohol (Sigma-Aldrich) to
157 avoid sperm sticking to the glass slide (Wilson-Leedy and Ingermann, 2007) and were covered with
158 a coverslip. The slide was examined at 200× magnification, and the percentage of motile sperm in

159 the total number of sperm in a visual field was manually counted. The measurements were repeated
160 three times for each sample, leading to repeatability (calculated for each week according to Becker,
161 1984) that was always greater than 0.596. All the samples were analysed by the same trained
162 observer to avoid subjective differences in motility evaluation.
163
164 *2.6. Sperm longevity*
165 Sperm longevity, i.e., time leading to cessation of the progressive movement, was estimated
166 as the time from activation until ~90% of sperm within the visual field were no longer motile (not
167 showing head or tail movements) (Gage et al., 2004). To this end, 3 µL of an activated ejaculate
168 was collected and examined on a 12-well multitest slide (MP Biomedicals, Aurora, OH) at 200×
169 magnification. The measurements were repeated three times for each sample, leading to
170 repeatability (calculated for each week, according to Becker, 1984) always higher than 0.793. All
171 the samples were analysed by the same trained observer to avoid subjective differences in motility
172 evaluation.
173
174 *2.7. Sperm fatty acid content*
175 To determine the fatty acid profile, subsamples of an ejaculate (500 µL) were preserved at
176 –20°C until analyses. After thawing, fat was extracted following the method of extraction with
177 methyl-tert-butyl ether (MTBE) (Matyash et al., 2008). After that, an internal standard (13:1 methyl
178 ester) was added to the extract, and direct esterification was performed according to Jenkins (2009).
179 After centrifugation of the esterified sample, the supernatant was injected into Cromatograph
180 Agilent Technologies 7890 A (Agilent Technologies, Santa Clara, CA, USA), with the split at 40
181 mL/min and ratio 160:1. Supelco SP 2560 (Sigma-Aldrich, St. Louis, MO, USA) served as the first
182 capillary column (75 m × 0.18 mm internal diameter, 0.14 µm film thickness) with hydrogen as a
183 carrier at 0.25 mL/min. J&W HP 5ms (Agilent Technologies, Santa Clara, CA, USA) was
184 employed as the second capillary column (3.8 m × 0.5 mm internal diameter, 0.25 µm film

185 thickness) with hydrogen as a carrier at 22 mL/min for 2 min and then 0.18 mL/min to 35 mL/min.
186 The oven temperature was 45 °C, then it was held for 2 min, raised to 170 °C at the rate of 50
187 °C/min, held for 25 min, raised at 240 °C at the rate of 2 °C/min, and held for 16 min, while the
188 injector and the detector temperatures were set to 270 °C and 250 °C, respectively.

189 Fatty acids were identified by comparing the retention time with that of a mixture of 52
190 standard Fatty Acid Methyl Esters (FAMEs; GLC reference standard: 674; Nu-Chek Prep, Inc.,
191 MN, USA) and Menhaden Fish Oil (Supelco, Bellefonte, PA, USA). Amounts of individual
192 FAMEs were expressed as a percentage of the total area of eluted FAMEs. The percentages of
193 saturated fatty acids (SFAs), unsaturated fatty acids (UFAs), monosaturated fatty acids (MUFAs),
194 polyunsaturated fatty acids (PUFAs), and omega-3 and omega-6 fatty acids were also reported.

195

196 *2.8. Testosterone profile*

197 Blood samples were collected from the caudal vein of wild and farmed eels every 2 weeks,
198 from the week preceding the beginning of stimulation until the 18th hormone stimulation (Fig. 1).
199 Blood samples were transferred to heparinised tubes, centrifuged (4000 × *g*, 10 min), and stored at
200 –20 °C until analysis.

201 Testosterone concentrations were analysed in plasma following the method of Gaiani et al.
202 (1984). Briefly, 0.1 mL of plasma was extracted with 5 mL of diethyl ether by mixing for 30 min on
203 a rotary mixer. The tubes were centrifuged at 2000 × *g* for 15 min, and the supernatants were
204 evaporated to dryness under an air-stream suction hood (37 °C). The dry extracts were stored at –20
205 °C.

206 Testosterone concentration was measured by a radioimmunoassay (RIA) validated by
207 Parmeggiani et al. (2015). The dried extracts were dissolved in RIA buffer (1 mL; 74.26 mmol/L
208 Na_2HPO_4, 12.49 mmol/L EDTA Na, 7.69 mmol/L NaN_3), containing 0.1% of BSA (pH 7.5) and
209 were shaken for 10 min. The samples (0.1 mL), 1,2,6,7-3H testosterone (0.1 mL, 30 pg/tube), and
210 rabbit anti-testosterone serum (0.1 mL, 1:50000) were incubated overnight (4 °C); 1 mL of a

charcoal-dextran solution (charcoal 0.25%, dextran 0.02% in phosphate buffer) was then added into the tubes. After 15 min incubation at 4 °C, the tubes were centrifuged (15 min, 3000 × *g*), the supernatant was decanted, and radioactivity was immediately measured on a β-scintillation counter (Packard C1600, PerkinElmer, USA). Cross-reactions of various steroids with the antiserum raised against testosterone were as follows: testosterone (100%), dihydrotestosterone (25.44%), androstenedione (0.6%), 11-ketotestosterone (0.5%), and progesterone and cortisol (<0.0001%). The sensitivity of the assay was 1.65 pg/tube, the intra- and inter-assay coefficients of variation were 6.7% and 11.2%, respectively. To determine the parallelism between hormone standards and an endogenous hormone in eel plasma, a pooled sample containing a high testosterone concentration was serially diluted (1:1–1:8) with RIA buffer. A regression analysis was conducted to determine possible correlation between the two hormone concentrations in the same assay (r^2 = 0.98, $P < 0.01$). The results were expressed in ng/mL. In all the sperm quality analyses, the operator was blinded to the identity of a subject and its group affiliation.

2.9. Statistical analyses

Data were analysed by univariate linear mixed models (LMM; with restricted maximum likelihood estimation) in RStudio, version 1.1.447, using the function 'lme' of the 'nlme' package (Pinheiro et al., 2016). Sperm density, spermatocrit, sperm viability, sperm longevity, the testosterone concentration, and fatty acid content were regarded, alternatively, as dependent variables. Male origin (wild or farmed), sampling week, time from hormone injection to stripping (24 or 48 hours), and the interaction between the origin and week were all considered fixed factors. To account for repeated measurements on individual males, male identity (ID) (nested within origin) was included as a random factor with an estimate of random intercepts for each subject. Because the usual asymptotic inferential results for linear models may not hold in this dataset owing to the limited number of observations, the *P* values of the LMMs were evaluated via a resampling-based strategy (1000 random permutations, see Kherad-Pajouh and Renaud, 2009), by means of the

237 'permmodels' function of the 'predictmeans' package (Luo et al., 2014). At the final step, all non-
238 significant interaction terms were removed from the full models to achieve minimal adequate
239 models (Crawley, 2005). Furthermore, for the sake of simplicity, only results of the latter are
240 reported. In accordance with the permutation strategy applied to the main LMM, pairwise *post hoc*
241 tests were also performed via the resampling-based strategy (1000 random permutations), using the
242 function 'pairwise.perm.t.test' of the package 'RVAideMemoire' (Hervé, 2018). The *P* values were
243 adjusted for multiple testing by the method of Benjamini and Hochberg (1995). The relation
244 between the two measures of sperm concentration (haemocytometer and spermatocrit) was tested
245 through a simple linear regression (lm).
246
247 **3. Results**
248 *3.1. Sperm concentration*
249 The two measurements of sperm concentration, by means of the haemocytometer and
250 spermatocrit, highly correlated in all the experimental weeks (Regression estimate: 0.743; st. error:
251 0.055; *t* value: 13.43, $P < 0.001$, $R^2 = 0.744$, df = 62. Regression function: y= 3.123+0.743x). There
252 was an effect of the sampling week only for spermatocrit values, with lesser values after the 17th
253 week compared to the 13th week ($P = 0.04$). Neither an effect of the male origin nor an interaction
254 between male origin and sampling week was observed (Table 1; Fig. 2 a, b).
255
256 *3.2. Percentage of motile sperm*
257 There was not any difference between wild and farmed eels for the percentage of motile
258 sperm (Table 1). There was only an effect of the sampling week, with a greater percentage of motile
259 sperm after the 17th week (mean ± SE = 61.3% ± 0.4%) compared to the 13th week (mean ± SE =
260 36.2% ± 0.6%).
261
262 *3.3. Sperm longevity*

263 There was an effect on sperm longevity depending on the origin of males (Table 1), with
264 wild caught eels having greater values of sperm longevity. There was an effect of the time interval
265 between hormone injection and sperm quality analyses (Table 1): after the 13th week, sperm
266 longevity was on average less in those ejaculates analysed at 48 instead of 24 hours after hormone
267 injection (mean values in Table S1 in Supplementary Material).
268 There was a marginal effect of the sampling week (P = 0.045, Table 1), irrespective of the
269 origin of males with a trend of an increase in sperm longevity, with a peak at approximately after
270 the 16th week (Fig. 3).
271
272 *3.4. Sperm fatty acid content*
273 There was an effect of the origin of male eels (wild compared with farmed) on sperm SFAs
274 and UFAs was seen (Table 2), with greater concentrations of UFAs and lesser concentrations of
275 SFAs in the wild males compared to farmed eels (Tables 2 and S2). In addition, the percentages of
276 omega-3 and of omega-6 fatty acids were affected by the origin of eels (Table 2), but in the
277 opposite direction for the two fatty acid types: omega-3 concentrations were greater in farmed
278 males, whereas omega-6 concentrations were greater in wild eels (Fig. 4 a, b).
279 There was also an effect of the sampling week on MUFAs, PUFAs, and omega-6 fatty acids
280 (Table 2). The proportion (percentage) of MUFAs was greater during initial sampling week 13
281 when compared to week 16 (13 compared with 16: adjusted P = 0.020). The concentration of
282 PUFAs was greater in week 13 when compared to weeks 15 and 16 (adjusted P = 0.04). The
283 concentration of ω-6 fatty acids was greater in week 13 when compared to final weeks 16 and 17
284 (adjusted P = 0.047). Detailed mean values are reported in Table S2 (Supplementary Material).
285
286 *3.5. Testosterone profile*
287 The mean ± SE of the testosterone concentrations prior to stimulation was 0.38 ± 0.05
288 ng/mL among the farmed males and 0.45 ± 0.15 ng/mL among the wild males. The concentration

289 increased after the first injections of hCG, thus reaching the mean ± SE of 3.43 ± 0.70 ng/mL
290 among the farmed male eels and 4.09 ± 1.43 ng/mL among the wild male eels. This change was
291 followed by a gradual decrease until the end of the experiment. There was no difference in
292 testosterone profiles between wild and farmed male eels, and only an effect of the sampling week,
293 irrespective of the origin of male eels, was observed (LMM: origin, $F_{1,11} = 0.036$, $P = 0.832$; week:
294 $F_{8,86} = 11.350$, $p < 0.01$; interaction origin*week: $F_{8,86} = 0.439$, $p = 0.904$; see Figure S1 in
295 Supplementary Material).
296
297 **4. Discussion**
298 The results of the present study indicate that the different origins of the male European eels
299 partly influence the quality of the ejaculates. Wild caught eels (i.e., caught at a natural site before
300 the hormonal induction) had a greater sperm longevity during hormone stimulation.
301 In the present study, sperm longevity appeared to be the most sensitive sperm variable
302 because there was an effect of the origin of male eels (wild or farmed), by the sampling week, and
303 by the time interval between injection and stripping. Nevertheless, the greatest sperm longevity
304 occurred in wild eels at 16 weeks after the beginning of hormone stimulation, later than the peak in
305 sperm concentration (weeks 13^{th}–14^{th}). This finding suggests that in the present study, sperm
306 longevity had a greater but slower response to hormone stimulation than did sperm concentration.
307 For sperm concentration, there were greater spermatocrit values in wild caught eels only at
308 13 weeks after the beginning of hormonal stimulation. In contrast to another study on the European
309 eel (Pérez et al., 2000), it was highlighted that there was a significant increase in sperm
310 concentration as early as 6 weeks after the beginning of hormone stimulation, a further increase
311 after 8 weeks, and relatively consistent values until the last week of analyses (15^{th}). The
312 experimental regimens of the two studies, however, differ in the dose of hCG administered [which
313 was higher in the study by Pérez et al. (2000): 1.5 compared with 1.0 UI/g in the present study] as
314 well as in the temperature of animal maintenance (20 °C in the previous compared with 15 °C in the

present study). Both of these methodological inconsistencies may be the reasons for the later increase in sperm density in the present study. The hormone dose and injection frequency influence both the duration of spermatogenesis and the spermiation period in the European eel (Tomkiewicz et al., 2011). Furthermore, temperature modulates testis steroidogenesis in the European eel (with an effect on maturation) and there is a delay in spermatogenesis initiation when there are thermal regimens with lesser temperatures imposed (Peñaranda et al., 2016).

As recently demonstrated by Sørensen et al. (2013), in the present study, there was also values for the variables of sperm concentration by means of the haemocytometer and spermatocrit that were highly correlated.

Of note, results of the present study also indicate that the difference in sperm quality between wild caught and farmed male eels was associated with differences in some sperm fatty acids concentrations. The sperm of wild males, which have greater longevity than that of farmed males, also contains greater concentrations of UFAs, according to the results of the present study. The presence of UFAs in cellular membranes is important for maintenance of the properties of the lipid bilayer (Farooqui et al., 2000), and the lipids of the spermatozoan membrane are key determinants of fluidity and flexibility, and thus, of successful fertilisation (Lenzi et al., 1996). In fish, the lipid composition of the sperm membrane is closely related to the functionality of spermatozoa, in particular to sperm viability, sperm motility duration, and sperm cryoresistance (Lahnsteiner et al., 2009). In the rainbow trout *Oncorhynchus mykiss*, sperm UFAs have a positive influence on sperm viability during storage of spermatozoa and on sperm motility after activation (Lahnsteiner et al., 2009). Conversely, the sperm of wild male eels in the present study had lesser amounts of SFAs, and this result seems to be consistent with the findings of Mansour et al. (2011) regarding *Salvelinus alpinus*, where SFA levels were in lesser amounts with high-fertility semen. Furthermore, in the present study there was an effect of the treatment on the percentages of omega-3 and omega-6 fatty acids, with the sperm of farmed males having greater concentrations of omega-3 fatty acids and sperm of wild caught males having a greater concentration of omega-6 fatty acids.

Considering sperm fatty-acid composition of fish spermatozoa is often affected by the diet (Pustowka et al., 2000; Vassallo-Agius et al., 2001; Butts et al., 2015), this opposite effect on omega-3 and omega-6 fatty acids might be due to the difference in diets that the two groups of males consumed during the pre-experimental period. Farmed male eels were fed with artificial fish meal rich in omega-3 fatty acids (ALTERNA EEL, Skretting). This hypothesis is supported by Butts et al. (2015), where it was reported that there were similar percentages of omega-3 and omega-6 fatty acids in the milt of male European eels fed improved commercial diets. Taken together with the previous results on European eels (Pérez et al., 2000), and the present findings seem to support the idea that sperm of greater quality (that of the wild males in the present study) have a lesser percentage of omega-3 fatty acids and a lesser omega-3/omega-6 ratio. Butts et al. (2015) compared the sperm performance of male European eels fed different artificial diets and concluded that lesser concentrations of total omega-3 fatty acids in sperm samples correlate with the least sperm motility and volume. Results of the present study indicate that other dietary components or the prolonged maintenance of farmed males in captivity may be responsible for the enhanced performance on longevity of wild-male sperm. In the 13th week, in which sperm analyses were performed for two different periods after hormone injection (24 or 48 hours later), there was an influence of the time since hormone injection on sperm longevity, with males stripped 24 hours after the injection having greater sperm longevity than those stripped 48 hours after injection. These results are consistent with the findings in other studies on the European eel and confirm that stripping within 24 hours after injection is the best procedure for obtaining sperm of greater quality (Pérez et al., 2000; Asturiano et al., 2006).

As for the plasma testosterone concentration in the present study, as expected (Baeza et al., 2015), there was a significant increase after the beginning of hormone stimulation. Wild and farmed male eels, however, did not manifest a differential response to hormone stimulation in terms of plasma testosterone concentrations in the present study. In fish, gonadotropin stimulation (mainly FSH) induces the secretion of androgens (e.g., testosterone and 11-ketotestosterone) during the

initial stage of male gametogenesis (Nagahama, 1994; Mananos et al., 2008). In a number of species, plasma concentrations of testosterone and 11-ketotestosterone are greater during the later stages of spermatogenesis and rapidly decrease after the onset of spermiation (Nagahama, 1994). As reported in the review by Tokarz et al. (2015) and in the study by Lokman and Young (1998), in male eels, testosterone can be considered a precursor of 11-ketotestosterone, the prevalent androgen in fish. As further support of this hypothesis, Baeza et al. (2015) reported similar trends of testosterone and 11-ketotestosterone concentrations throughout different stages of eel testis development. Accordingly, in the present study, there was an immediate increase in testosterone concentrations in the two groups of animals already at the second injection of hCG: levels ~3 ng/mL at the peak and a subsequent decrease. In the study by Huertas et al. (2006), the peak of testosterone concentrations occurred after the fifth week, with concentrations of ~8 ng/mL. The observed difference between the results of Huertas et al. (2006) and those in the present study might be due to the use of different eel populations as well as to the different procedures for hormonal stimulation.

5. Conclusions

To our knowledge, this is the first study on sperm quality in European eels that takes into account the effect of the different origins of hormonal stimulated males (wild-caught compared with farmed) on sperm quality. Results of the present study indicate that wild-caught males have a greater sperm quality in terms of longevity during hormonal stimulation than do sperm of farmed eels; however, no difference in sperm density, percentage of motile sperm, and plasma testosterone concentrations was detected. Overall, findings in the present study suggest that farmed male eels could still be viable substitutes of wild-caught males to improve the production of European eels, an economically relevant species for which there is a major decrease of wild stocks.

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395 **References**

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Table 1

Results of LMMs (minimal adequate models) on values for sperm variables; Male origin: wild-caught compared with farmed; sampling week: sperm sampling from the 13th week after beginning of hormone stimulation to the 17th week; F-test statistics, numerator degrees of freedom (Num df) and denominator degrees of freedom (Den df) are reported; *P* values are calculated through a permutation-based strategy (1000 permutations), and the values that are different at presented in bold font

	fixed factors	F	Num df	Den df	*p*
Density	male origin	0.847	1	11	0.347
	week	0.932	4	11	0.483
	time from injection	2.171	1	46	0.174
Spermatocrit	male origin	2.011	1	11	0.131
	week	5.000	4	11	**0.002**
	time from injection	3.560	1	46	0.081
% Motile	male origin	1.718	1	11	0.191
	week	6.477	4	11	**0.002**
	time from injection	2.131	1	46	0.147
Longevity	male origin	5.248	1	11	**0.019**
	week	2.644	4	11	**0.045**
	time from injection	6.499	1	46	**0.028**

Table 2

Results of LMMs (simplified minimal adequate models) on fatty acid content of sperm; Origin: wild compared with farmed males; week: sperm sampling from the 13th week after beginning of hormone stimulation to the 17th week; F-test statistics, numerator degrees of freedom (Num df) and denominator degrees of freedom (Den df) are reported; *P* values were calculated via a permutation-based strategy (1000 permutations), and those values that are different are presented in bold

	fixed factors	F	Num df	Den df	*P*
SFA	male origin	3.977	1	11	**0.012**
	week	1.365	4	41	0.278
UFA	male origin	10.818	1	11	**0.001**
	week	0.604	4	41	0.755
MUFA	male origin	3.209	1	11	0.081
	week	5.265	4	41	**0.005**

PUFA	male origin	0.300	1	11	0.660
	week	2.675	4	41	**0.025**
OMEGA 3	male origin	45.160	1	11	**0.001**
	week	0.864	4	41	0.562
OMEGA 6	male origin	80.070	1	11	**0.001**
	week	5.822	4	41	**0.002**

562

563 **Figure legends**

564 **Fig. 1.** Timeline of the experimental procedure

565 **Fig. 2.** Sperm concentrations in wild (■) and farmed (□) male eels during successive sampling

566 weeks; (a) Sperm density in a haemocytometer and (b) according to spermatocrit; Reported data are

567 means ± SE

568 **Fig. 3.** Sperm longevity in wild (■) and farmed (□) male eels during successive sampling weeks;

569 Reported data are means ± SE.**Fig. 4.** Percentages of (a) omega-3 and (b) omega-6 fatty acids in

570 wild (■) and farmed (□) male eels during successive sampling weeks; Reported data are means ±

571 SE

572

573 **Supplementary material**

574 **Fig. S1.** Testosterone concentrations in wild (■) and farmed (□) male eels during successive

575 sampling weeks; Reported results are means ± SE

576 **Table S1**

577 Descriptive statistics for sperm concentration and performance measured in wild and farmed males

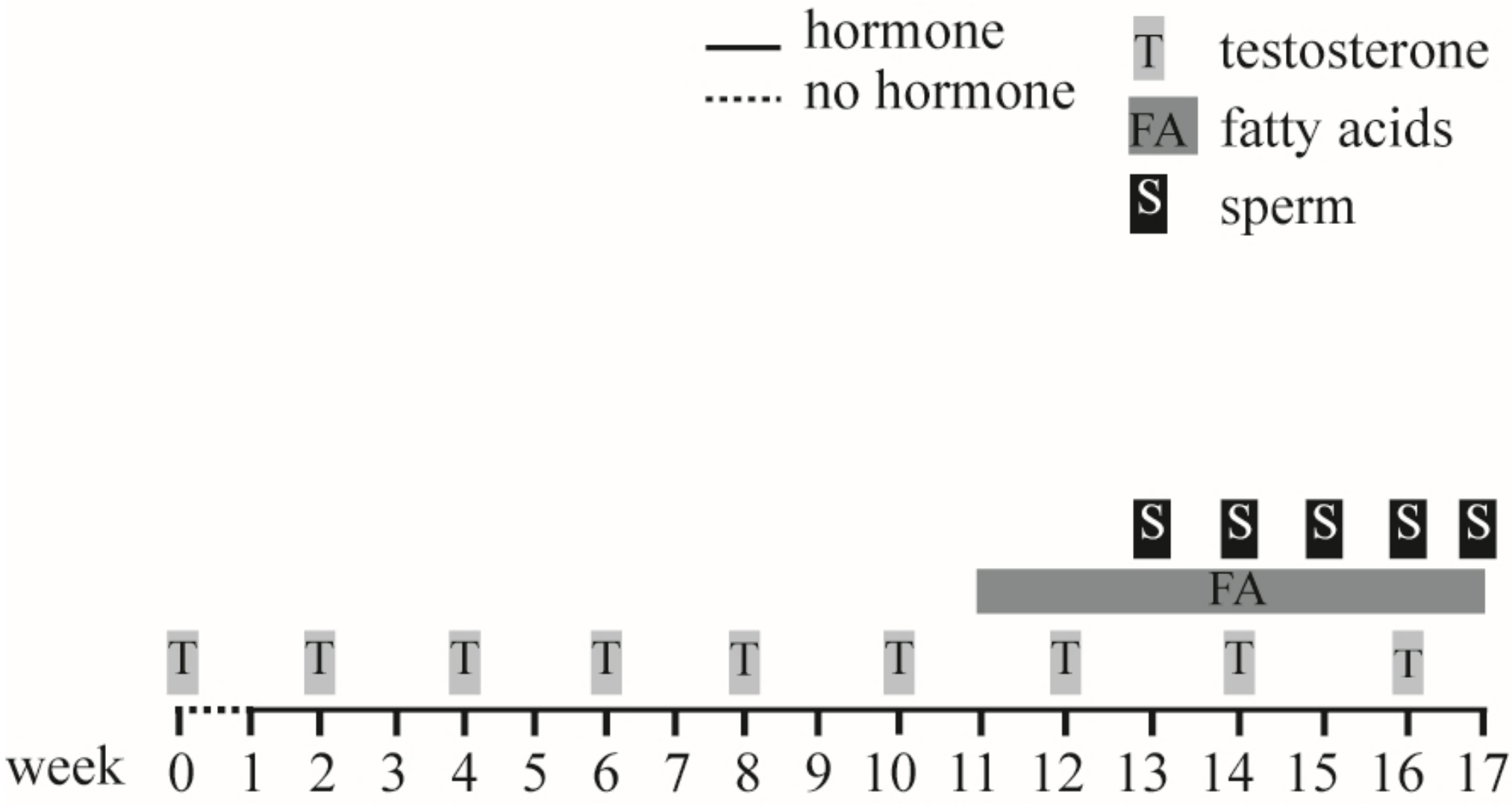
578 in each sampling week

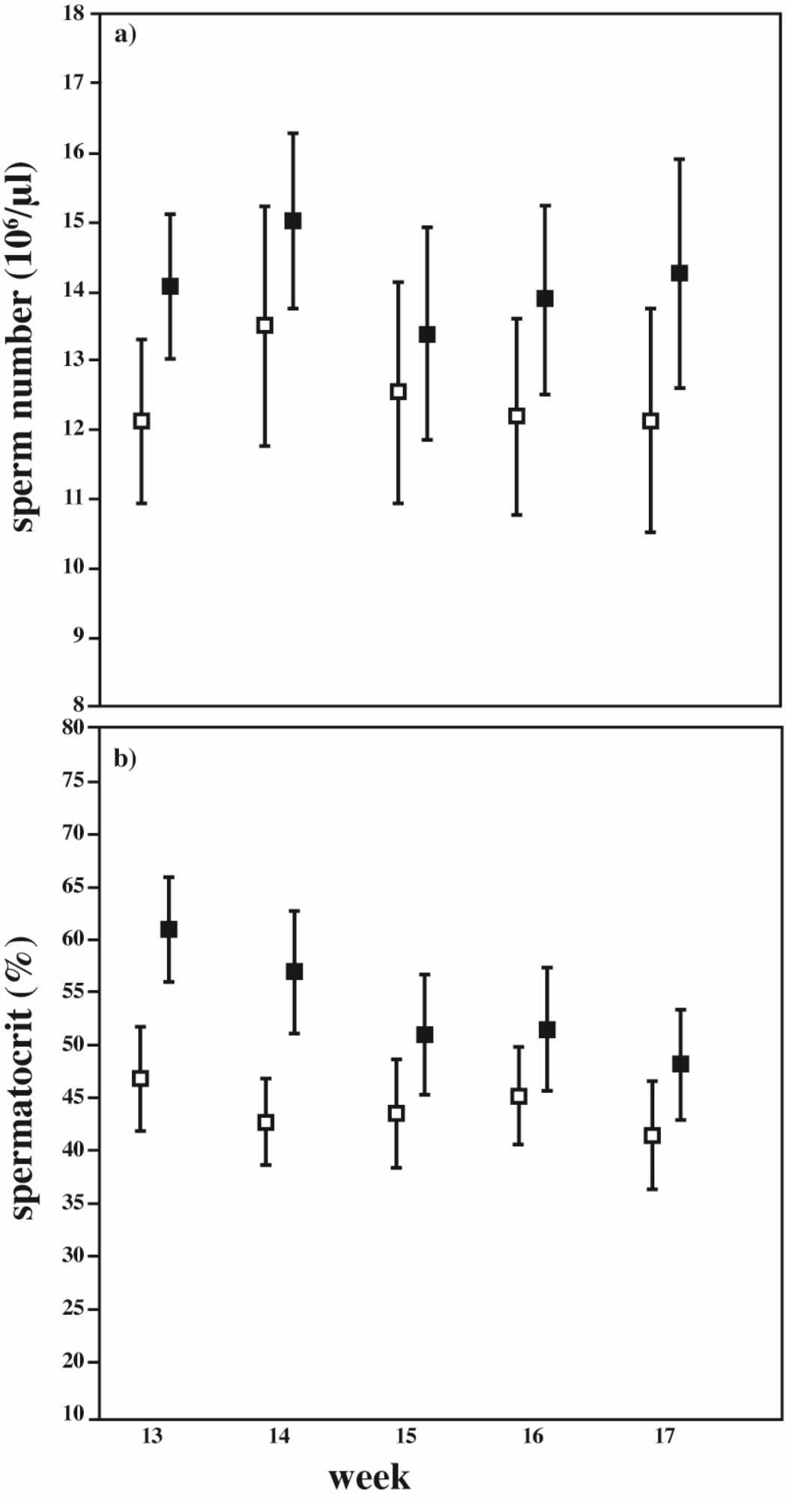
579 **Table S2**

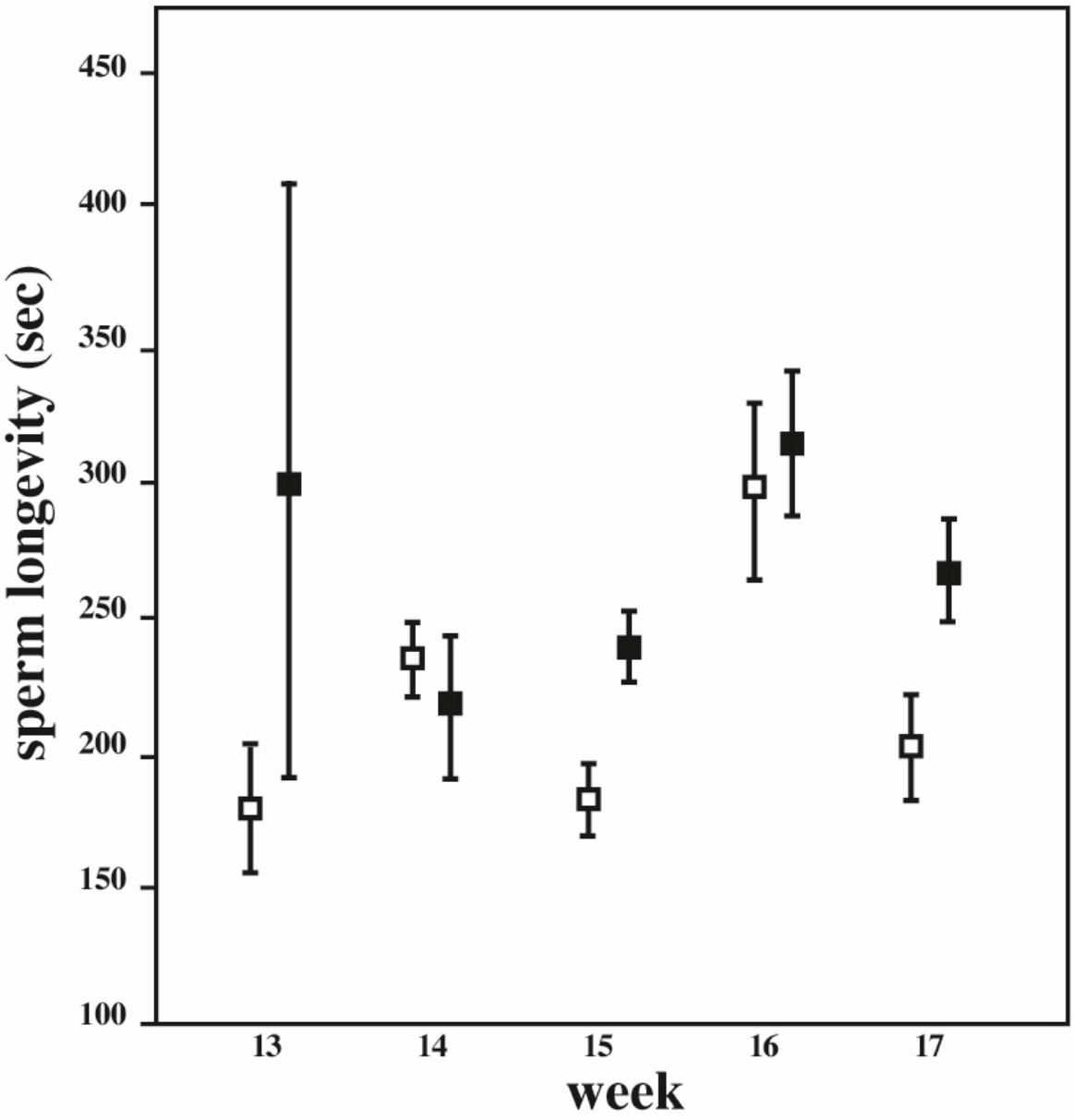
580 Descriptive statistics for the fatty acid content in the sperm of wild and farmed male eels in each

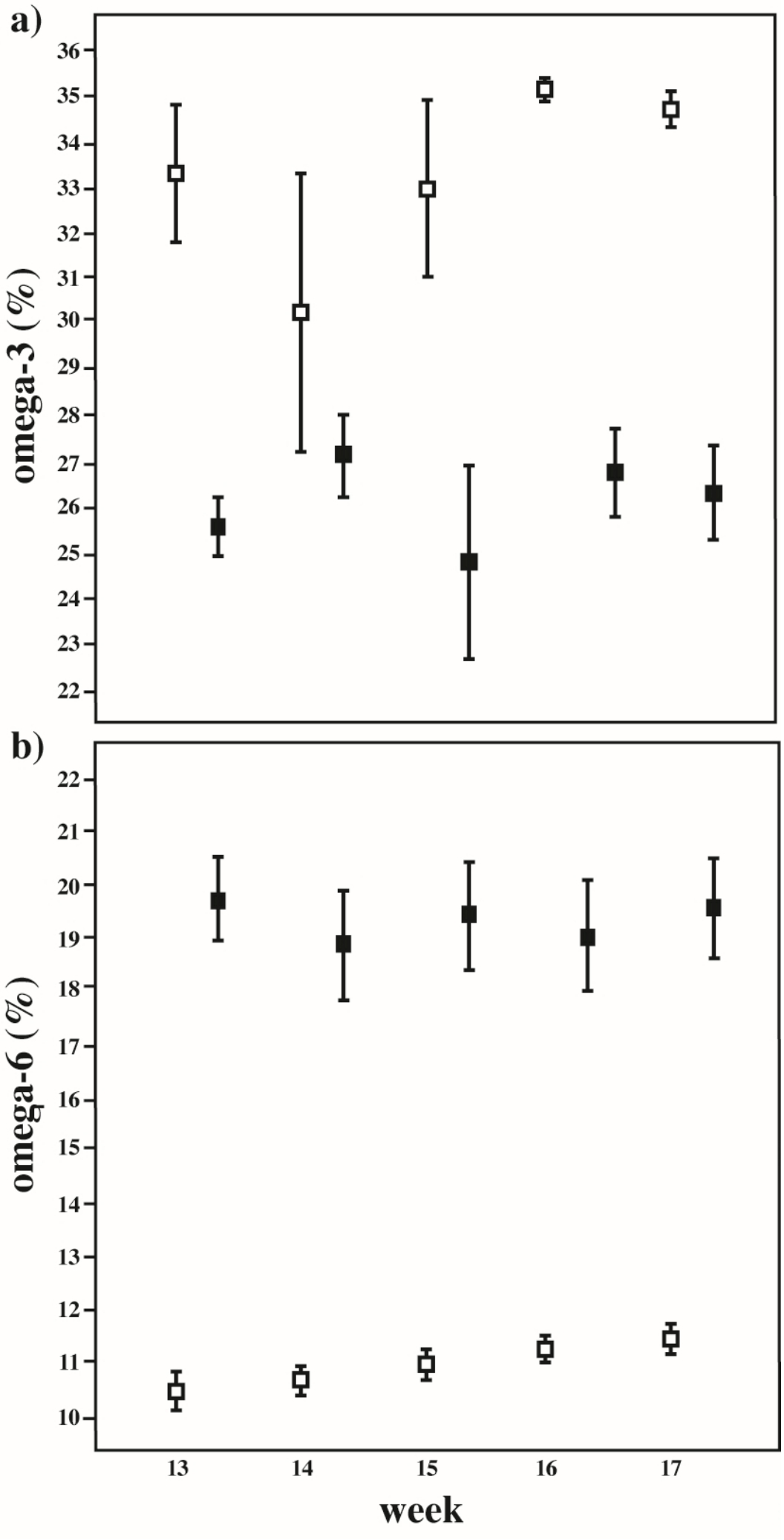
581 sampling week

582


hormone
no hormone
T testosterone
FA fatty acids
S sperm
S S S S S
FA
T T T T T T T T T
week 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17


a)
sperm number (10^6/µl)
18
17
16
15
14
13
12
11
10
9
8
b)
spermatocrit (%)
80
75
70
65
60
55
50
45
40
35
30
25
20
10
13
14
15
16
17
week


sperm longevity (sec)
450
400
350
300
250
200
150
100
13
14
15
16
17
week


a)
omega-3 (%)
36
35
34
33
32
31
30
29
28
27
26
25
24
23
22
b)
omega-6 (%)
22
21
20
19
18
17
16
15
14
13
12
11
10
13
14
15
16
17
week

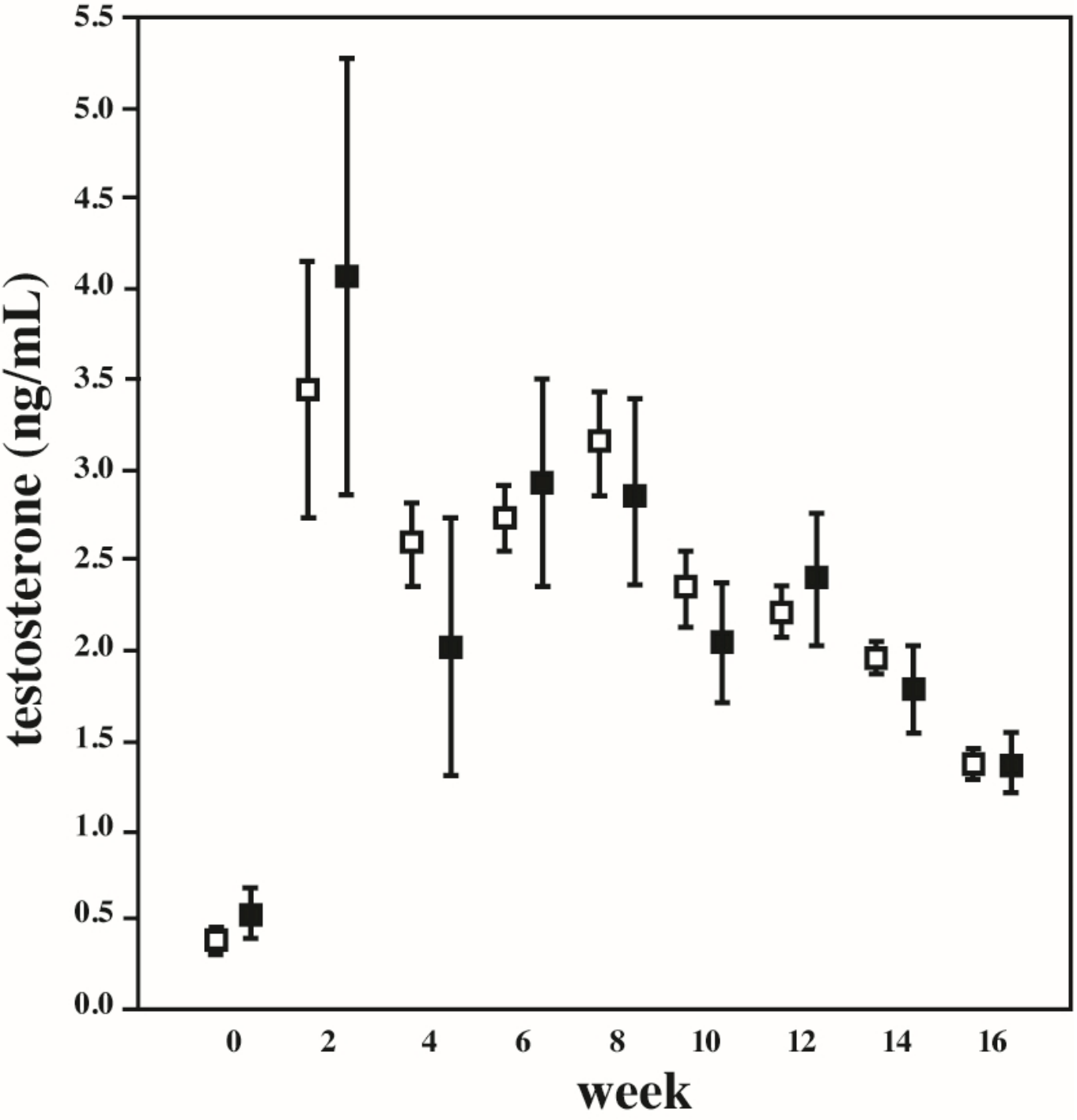

testosterone (ng/mL)
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
14
16
week

Table S1. Descriptive statistics (mean ± SD) and number of samples analysed for sperm density, spermatocrit, % of mobile sperm and sperm longevity, in each experimental week on both wild caught and farmed males.

	Sperm count (10^6/µl)				**Spermatocrit** (%)			
	wild		farmed		wild		farmed	
	mean ± SD	N	mean ± SD	N	mean ± SD	N	mean ± SD	N
week								
13	14.07 ± 2.58	6	12.10 ± 3.14	7	60.68 ± 12.24	6	46.55 ± 13.08	7
14	15.02 ± 3.10	6	13.52 ± 4.14	6	56.69 ± 14.08	6	41.42 ± 11.63	6
15	13.36 ± 3.76	6	12.54 ± 4.24	7	50.73 ± 13.95	6	43.23 ± 13.65	7
16	13.87 ± 3.37	6	12.18 ± 3.76	7	51.25 ± 14.46	6	44.89 ± 12.32	7
17	14.25 ± 4.09	6	12.11 ± 4.26	7	47.89 ± 12.70	6	41.21 ± 13.56	7

	Mobile sperm (%)				**Sperm longevity** (sec)			
	wild		farmed		wild		farmed	
	mean ± SD	N	mean ± SD	N	mean ± SD	N	mean ± SD	N
week								
13	35.60 ± 27.59	5	36.57 ± 16.65	7	293.63 ± 246.59	5	174.64 ± 58.32	7
14	56.00 ± 14.56	6	55.71 ± 18.43	7	213.03 ± 62.67	6	229.05 ± 35.49	7
15	65.50 ± 17.74	6	52.71 ± 11.03	7	233.11 ± 31.87	6	178.38 ± 35.30	7
16	63.33 ± 10.07	6	68.57 ± 17.90	7	308.78 ± 63.13	6	291.00 ± 84.52	7
17	67.83 ± 6.76	6	55.71 v 19.86	7	261.17 ± 46.15	6	196.24 ± 49.95	7

Table S2. Descriptive statistics (mean ± SD) and number of samples analysed in each experimental week, for both wild caught and farmed males, for the % of saturated fatty acids (SFA), unsaturated fatty acids (UFA), monosaturated fatty acids (MUFA), polysaturated fatty acids (PUFA), omega-3 and omega-6 in sperm.

	Sperm fatty acid content (%)							
	SFA				**UFA**			
	wild		farmed		wild		farmed	
	mean ± SD	N	mean ± SD	N	mean ± SD	N	mean ± SD	N
week								
13	26.10 ± 0.84	5	28.95 ± 3.95	6	70.46 ± 0.97	5	67.17 ± 3.96	6
14	25.77 ± 1.25	5	27.71 ± 1.33	6	70.40 ± 1.34	5	68.18 ± 1.22	6
15	26.19 ± 1.41	6	26.84 ± 1.11	7	70.29 ± 1.33	6	68.78 ± 0.94	7
16	26.21 ± 1.12	5	26.47 ± 0.56	6	69.98 ± 1.61	5	69.17 ± 0.45	6
17	27.09 ± 0.80	6	27.05 ± 0.83	6	69.78 ± 1.30	6	69.07 ± 1.41	6
	MUFA				**PUFA**			
	wild		farmed		wild		farmed	
	mean ± SD	N	mean ± SD	N	mean ± SD	N	mean ± SD	N
week								
13	23.02 ± 1.18	5	21.06 ± 1.20	6	47.44 ± 1.61	5	46.11 ± 4.31	6
14	21.86 ± 1.84	5	20.44 ± 1.27	6	48.54 ± 1.21	5	47.74 ± 0.63	6
15	22.02 ± 1.66	6	20.53 ± 0.65	7	48.27 ± 1.60	6	48.25 ± 1.12	7
16	21.30 ± 2.00	5	20.24 ± 0.67	6	48.68 ± 1.49	5	48.93 ± 0.98	6
17	21.45 ± 2.01	6	20.75 ± 0.85	6	48.33 ± 1.22	6	48.32 ± 1.60	6
	OMEGA 3				**OMEGA 6**			
	wild		farmed		wild		farmed	
	mean ± SD	N	mean ± SD	N	mean ± SD	N	mean ± SD	N
week								
13	25.53 ± 1.48	5	33.28 ± 3.74	6	19.75 ± 1.75	5	10.46 ± 0.91	6
14	27.09 ± 2.05	5	30.24 ± 7.49	6	18.85 ± 2.32	5	10.68 ± 0.67	6
15	24.75 ± 5.27	6	32.96 ± 5.12	7	19.42 ± 2.48	6	10.96 ± 0.79	7
16	26.73 ± 2.16	5	35.10 ± 0.40	6	19.05 ± 2.35	5	11.27 ± 0.61	6
17	26.27 ± 2.55	6	34.69 ± 0.98	6	19.54 ± 2.30	6	11.46 ± 0.72	6