Deliverable Report

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OBJECTIVES

Establish culture procedures that identify the on-growing period for the production of grey mullet roe (bottarga) from wild and hatchery juveniles. Additional to the description of the process of oogenesis in captive-reared vs. hatchery-produced grey mullet, in terms of growth and body indices (D7.5), the current deliverable will further evaluate the effect of diet and culture conditions on advanced and spontaneous gonadal development, which is a prerequisite for mass production of the highly prized grey mullet roe (bottarga).

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INTRODUCTION

Mullet, in particular *Mugil cephalus*, which are fished and farmed around the world, have many favorable culture characteristics. They are herbivorous, euryhaline, fast growing, disease resistant and a popular eating fish. In Asia and the Middle East, they are farmed in freshwater ponds and harvested before reaching sexual maturity, which guarantees a superior taste and high market value. In addition to pond culture, wild mullets are intensively fished during their reproductive season where gravid females are in demand due to their roe.
The roe of grey mullet, is known by a variety of names that include bottarga, Sardinian caviar or karasumi and is consumed either as salted individual eggs or as salted and dried whole ovaries or skeins. The catching of mullets and processing of their roe have a long tradition on both the northern and southern coasts of the Mediterranean Sea. In addition, Australia and countries in Asia capture sexually mature fish around the onset of their reproductive season and process the roe into this high cost fishery product for human consumption. The mullet roe production is a simple process involving (i) cleaning the roe sacs of any adhering blood or intestinal tissues after removal from the fish, (ii) coating the cleaned roe with fine salt for 6 – 12 hours, and (iii) pressing the roe to flatten it and drying it in the sun for about one week. At the end of this process, the roe have a yellowish-red color and "chewy" texture that is coated with beeswax and marketed. The unique texture of the dried roe is due to a high content of wax esters that is about 60 – 70% of the lipid content of the product (Lu et al. 1979).

Grey mullets show a group-synchronous ovarian development (Bartulović et al., 2011) and spawning in the Mediterranean occurs in late summer-early autumn (Assem et al., 2008; Bartulović et al., 2011). The size at sexual maturity ranges very widely, with males usually maturing at a standard length (SL) ranging between 25 and 30 cm while females are slightly larger at 27–35 cm SL (Ameur et al., 2003). These size classes are generally regarded as being approximately 3 year old but some studies have given higher and lower ages at 50 % sexual maturity (Bok, 1983; Ameur et al., 2003). The considerable wide range in the above estimates can be possibly ascribed to both differences in the methodology used by the different authors and to the fact that they actually referred to different taxa within the *M. cephalus* species complex (Whitfield et al., 2012).

With the aim to optimize culture protocols for the production of grey mullet roe (bottarga), this study evaluated the effects of fish origin (wild caught and reared in captivity vs. hatchery produced), water salinity (freshwater vs. sea water) and pigment-enriched diet on the roe size and coloration.

MATERIALS AND METHODS

Fish, Experimental System and Trial Procedure

<u>Experiment 1</u> evaluated gonadal development in wild-caught grey mullet that were reared in two different salinity regimes (freshwater vs. seawater). The fish origin was defined as wild caught and reared in captivity or hatchery produced. The wild grey mullet used for this study were caught as fingerlings along the Israeli Mediterranean coast and reared in fresh water earthen ponds for two years. Then, the 2-year old fish (average body weight of 786 ± 33 g) were stocked in round 20 m^3 concrete tanks at densities of 4 kg/m^3 and provided with a continuous supply of freshwater or Red Sea seawater (40-ppt) and maintained at 24-28ºC and natural photoperiod regime throughout the experiment. Fish were fed daily at the rate of 1-1.5% of their body weight using the IRIDA extruded diet (based on the IOLR formula) and contained 35% protein and 7.2% lipid. Following a year of growth, fish (n=11, 15) of the two treatment groups were sampled during the species' natural spawning period (Mid-September). From the sampled fish, the following data were recorded: total length (TL in cm); body weight (BW in g); gonad weight (GW in g); liver weight (LW in g), viscera weight (g), and the weight of body fat (g). The gonado-somatic and hepato-somatic indices were calculated as $GSI = 100xGWxBW^{-1}$ and $HIS= 100xLWxBW^{-1}$, respectively (Table 1). Additionally, to evaluate the ovarian reproductive stage the diameter of the most advanced oocytes were reordered (Table 1).

Table 1. Biometric data, of 3-year old captive grey mullet reared under freshwater (A) and seawater (B) regimes.

A	Sex	Body weight (g)	Total length (cm)	Gonad weight (g)	Oocyte diameter (µm)	Liver weight(g)	viscera (g)	body fat (g)	GSI (%)	HIS (%)
1	F	2252	59.5	86.1	300	24	70.3	12.1	3.8	1.1
2	F	2000	54	13	90	15.7	127.3	60.7	0.7	0.8
3	F	2000	60	34.6	150	21	57.6	11.9	1.7	1.1
4	F	1900	57	285	400	18.9	33.3	6.4	15.0	1.0
5	F	1600	54.5	13	110	11.4	67.6	16.1	0.8	0.7
6	F	1500	53	9.4	100	9.8	85.5	71.3	0.6	0.7
7	F	1060	45.5	24.6	220	11.4	40.5	8.9	2.3	1.1
8	F	1754	56.5	11.5		12.3	74.8	34.4	0.7	0.7
9	M	1570	54.5	1		11.7	61.1	63	0.1	0.7
10	F	2232	56.5	22.8	230	4.6	97.7	54	1.0	0.2
11	F	2150	57	231.6	400	21	51.4	8.9	10.8	1.0
12	M	1056	45.5	0.8		7	37.1	34.3	0.1	0.7
13	F	1096	48	6.6		7	50	10	0.6	0.6
14	F	1094	48.5	4.9		7.1	39.8	7.2	0.4	0.6
15	F	942	46.5	24.5	230	7.9	23.7	2.7	2.6	0.8

B	Sex	Body weight (g)	Total length (cm)	Gonad weight (g)	Oocyte diameter (µm)	Liver weight (g)	viscera (g)	body fat (g)	GSI (%)	HIS (%)
1	F	1030	43.6	3.2	110	8.9	69.8	24.7	0.3	0.9
2	F	1305	44.9	7.5	135	14.1	75.3	38.7	0.6	1.1
3	F	1060	45.9	140.3	550	8.9	26.4	1.7	13.2	0.8
4	F	1805	53.4	11.4	115	20.3	130.5	50.8	0.6	1.1
5	F	1165	44.4	4.9	110	11.2	73.5	36.0	0.4	1.0
6	F	1280	46.8	6.8	110	10.9	85.8	31.8	0.5	0.8
7	M	1295	45.4	0.6					0.0	0.0
8	F	1155	45.2	5.1	110	11.4	74.2	17.2	0.4	1.0
9	F	1215	45.1	6.7	110	12.5	78.5	17.9	0.5	1.0
10	F	1200	46.0	5.4	100	9.6	80.6	23.6	0.4	0.8
11	F	1325	47.2	6.0	100	14.3	93.3	22.9	0.5	1.1

Experiment 2 compared gonadal development in captive-reared and hatchery produced 3-year old grey mullets. The captive reared group was comprised of fish caught from the wild in the Ebro delta (northeastern Spanish coast) at an early stage, transferred to IOLR facility (Eilat, Israel) and reared in captivity for 3 years. The other group were fish produced at the IOLR hatchery and reared in the same facility for 3 years before sampling. Fish in both treatment groups were reared in 20 m^3 tanks supplied with ambient Red Sea seawater (40 ‰) and exposed to natural fluctuations of light and temperature. Fish were fed daily at the rate of 1-1.5% of their body weight using the IRIDA extruded diet (based on the IOLR formula) and contained 35% protein and 7.2% lipid.

Experiment 3 evaluated the effect of a pigment-enriched diet on roe coloration. Three-year old hatchery-produced grey mullet were maintained in outdoor 4 m^3 tanks supplied with ambient Red Sea seawater (40 ‰) which were exposed to natural fluctuations of light and temperature. Fish were fed daily at the rate of 1-1.5% of their body weight using the IRIDA extruded diet (based on the IOLR formula) and contained 35% protein and 7.2% lipid. During early June, concomitant with the onset of gametogenesis, the fish were divided into two groups and fed over 3 months with the IOLR pelleted diet containing either fish oil (FO) or soybean oil (SO) as the main neutral lipid (see D13.3). However, the FO pelleted diet was also supplemented with Marigold petal meal (MgM; 3 mg kg-1 feed) as another carotenoid source, apart from the fish oil and 3% dry Ulva (produced at the IOLR). This meant that the total carotenoid level in the FO+MgM diet was ca.138 mg kg-1 while the SO diet was ca. 99 mg kg-1. During mid-September, coinciding with advanced stages of gametogenesis, 10 to 12 fish were sampled from each dietary group and

the following parameters were measured (**Table 3**); total length (cm), body weight (BW;g), gonadal weight (GW;g), liver weight (LW;g) and viscera weight (g). The gonado-somatic and hepato-somatic indices were calculated as above.

Table 2. Morphometric parameters (i.e., total length, weight of the body, gonads and liver) and body indices (GSI and HSI) of 3-year old captive-reared grey mullet.

#	Sex	Body weight (g)	Total length (cm)	Gonad weight (g)	Liver weight (g)	GSI (%)	HIS (%)
1	F	1330	50.2	21.3	6.9	1.6	0.5
2	F	650	36.7	11.7	9.8	1.8	1.5
3	F	330	32.1	2.3	3.3	0.7	1.0
4	M	370	34.6	0.9	2.8	0.2	0.8
5	M	310	33.1	0.3	2.7	0.1	0.9
6	M	410	35.4	1.1	4.6	0.3	1.1
7	M	820	42.3	0.2	8.7	0.0	1.1
8	M	340	33.8	1.4	2.9	0.4	0.8
9	M	450	34.5	1.2	4.6	0.3	1.0
10	M	270	30.1	0.6	2.6	0.2	0.9
11	F	750	41.4	2.7	7.8	0.4	1.0
12	F	1030	43.2	64.1	7.5	6.2	0.7
13	F	500	37.0	1.2	3.9	0.2	0.8
14	M	900	43.0	1.1	12.8	0.1	1.4
15	M	250	30.4	0.6	3.0	0.2	1.2
16	M	330	33.8	0.4	4.1	0.1	1.2
17	M	370	34.5	1.9	3.5	0.5	1.0
18	M	270	32.5	0.3	3.2	0.1	1.2
19	M	300	33.0	0.7	2.0	0.2	0.7
20	M	290	33.4	0.7	3.1	0.3	1.1

Table 3. Morphometric parameters (i.e., total length, weight of the body, gonads, liver and viscera) and body indices (GSI and HSI) of 3-year old mullet fed a fish oil based diet enriched with Marigold petal meal (FO+MgM) or a vegetable oil based diet (SO).

	sex	Body weight (g)	Total length (cm)	Viscera weight (g)	Liver weight (g)	Gonadal weight (g)	GSI (%)	HIS (%)	Oocyte diameter (µm)
so	F	240	30	18.93	2.56	0.55	0.23	1.06	
	F	390	35	33.73	5.83	1.00	0.26	1.49	
	F	470	35	41.80	6.15	1.55	0.33	1.31	
	F	580	37	18.0	4.68	102.0	17.59	0.81	≥590
	F	1100	46	48.79	10.15	169.29	15.39	0.92	≥600
	F	780	44	34.77	6.84	122.78	15.68	0.88	≥600
	F	525	39	38.94	9.56	51.43	9.80	1.82	≥470
	F	480	37	41.22	6.03	1.27	0.26	1.26	
	M	340	33	38.79	4.76	0.15	0.04	1.40	
	M	580	40	21.36	5.40	15.96	2.75	0.93	
	M	440	36	17.01	6.10	7.87	1.79	1.39	
	M	240	25	24.17	2.15	0.0	0.00	0.89	
	F	1143	48	59.09	11.87	114.0	9.97	1.04	≥500

<table>
<tr><td rowspan="9">FO</td><td>F</td><td>780</td><td>42</td><td>20.65</td><td>4.59</td><td>159.22</td><td>20.41</td><td>0.59</td><td>≥600</td></tr>
<tr><td>F</td><td>530</td><td>37</td><td>29.60</td><td>5.67</td><td>69.49</td><td>13.11</td><td>1.07</td><td>≥500</td></tr>
<tr><td>F</td><td>180</td><td>29</td><td>13.56</td><td>1.50</td><td>0.45</td><td>0.25</td><td>0.83</td><td></td></tr>
<tr><td>F</td><td>370</td><td>24</td><td>31.05</td><td>4.01</td><td>1.08</td><td>0.29</td><td>1.08</td><td></td></tr>
<tr><td>F</td><td>360</td><td>25</td><td>35.06</td><td>3.17</td><td>0.67</td><td>0.19</td><td>0.88</td><td></td></tr>
<tr><td>M</td><td>670</td><td>41</td><td>38.12</td><td>17.96</td><td>9.85</td><td>1.47</td><td>2.68</td><td></td></tr>
<tr><td>M</td><td>230</td><td>29</td><td>19.54</td><td>1.92</td><td>0.03</td><td>0.01</td><td>0.83</td><td></td></tr>
<tr><td>M</td><td>430</td><td>36</td><td>26.16</td><td>5.19</td><td>3.16</td><td>0.73</td><td>1.21</td><td></td></tr>
<tr><td>M</td><td>210</td><td>24</td><td>8.77</td><td>0.9</td><td>0.0</td><td>0.00</td><td>0.43</td><td></td></tr>
</table>

Gonadal histology and reproductive status assessment

For histological evaluations of maturational stage, fixed gonad samples were dehydrated in increasing ethanol concentrations, clarified in xylene and embedded in paraffin wax. Five µm thick sections were cut and stained with hematoxylin-eosin. For the classification of the reproductive status of females, the most advanced oocyte stage was recorded for each specimen, according to the scheme used by Corriero et al. (2007). For the classification of the reproductive status of males, the type of spermatogenic cysts was recorded and the quantity of spermatozoa in the lumen of seminiferous lobules was evaluated subjectively, as in Corriero et al. (2007).
In order to determine oocyte yolk accumulation the largest vitellogenic oocytes, which had a large and centrally located nucleus, were selected. Oocyte diameter (µm) and surface occupied by yolk granules (µm2) were measured from microphotographs taken with a digital camera (DFC 420; Leica, Cambridge, UK) connected to a light microscope (DIAPLAN; Leitz, Wetzlar, Germany), using an image analysis software (Leica Application Suite, version 3.3.0; Cambridge, UK).

Gonad proximate composition, lipid classes and fatty acid profiles

To evaluate gonad biochemical composition, samples of ovaries were removed and kept on dry ice until they were transported to the laboratory, where they were immediately stored at -80ºC until analysis. Dry matter and protein contents were calculated using the methods of analysis of the Association of Official Analytical Chemists (AOAC; 2012). Moisture content was determined in 500 mg samples by thermal drying in an oven at 110ºC, until constant weight. Protein was determined by sample digestion according to the Kjeldahl method. Total lipid (TL) was extracted by sample homogenization in chloroform/methanol (2:1, v/v) according to the method of Folch et al. (1957). The organic solvent was evaporated under a stream of nitrogen and the lipid content was determined gravimetrically (1982) and stored in chloroform/methanol (2:1), containing 0.01% butylated hydroxytoluene (BHT). Analysis of lipid class (LC) composition was performed by one-dimensional double development high performance thin layer chromatography (HPTLC; Merk, Darmstadt, Germany), and methyl acetate/isopropanol/chloroform/ methanol/0.25% (w/v) KCl (5: 5: 5: 2: 1.8, by volume) used as developing solvent system for the polar lipid classes and isohexane/diethyl ether/acetic acid (22.5: 2.5: 0.25, by volume), for the neutral lipid separation. Lipid classes were visualized by charring at 160ºC for 15 min after spraying with 3% (w/v) aqueous cupric acetate containing 8% (v/v) phosphoric acid, and quantified by scanning densitometry using a dual-wavelength flying spot scanner Shimadzu CS-9001PC (Shimadzu, Duisburg, Germany) (Olsen and Henderson, 1989). To determine the fatty acid profiles, TL extracts were subjected to acid-catalysed transmethylation with 1% sulphuric acid (v/v) in methanol. The resultant fatty acid methyl esters (FAME) and dimethyl acetals (DMA) were extracted using isohexane: diethylether (1:1 by volume) and purified by TLC using isohexane/ diethyl ether/acetic acid (90:10:1, by volume) as developing system (Christie, 1982). Fatty acid methyl esters were separated and quantified using a TRACE-GC Ultra gas chromatograph (Thermo Electron Corp., Waltham, MA, USA) equipped with an on-column injector, a flame ionization detector and a fused silica capillary column, Supelcowax TM 10 (30 m x 0.32 mm I. D. x 0.25 µm; Sigma-Aldrich, Madrid, Spain). Helium was used as carrier gas and temperature programming was 50±150ºC at 40ºC min-1 slope, then from 150 to 200ºC at 2ºC min-1, to 214ºC at 1ºC min-1 and, finally, to 230ºC at

40ºC min-1. Individual FAME and DMA were identified by reference to authentic standards, and further confirmation of FAMEs and DMAs identity was carried out by GC-MS (DSQ II; Thermo Electron Corp.).

Carotenoids were obtained only from gonads at advanced gametogenesis and spawning periods, according to the method of Barua et al. (1993), using ethyl acetate/ethanol (10mL, 1:1 v/v), ethyl acetate (5 ml) and hexane (10 ml) as extracting solvents. Afterwards, carotenoids contents were quantified by spectrophotometry at 470 nm.

RESULTS

Salinity effects on roe production in wild-caught and captive-reared grey mullet

Statistical differences in body weight were observed in grey mullet females reared in fresh water (BW=1660 ± 133 g) compared with sea water (1254 ± 68 g) regimes (**Fig. 1**). Nonetheless, in both groups only 10 to 15% of the females had developed gonads, satisfying the criteria for fish roe (bottarga) production in terms of size (GW ≥ 100 g) and maturational stage, i.e., advanced vitellogenesis (**Table 1; Fig. 2**). The vast majority of the females (69% and 90% in freshwater and seawater, respectively) exhibited GSI values lower than 1% (**Fig. 3**). Further regression analyses of the dynamics of GSI values of females in both treatment groups revealed two distinct relationships: (i) a positive correlation with oocyte diameters ($P < 0.0001$; r=0.93), which highlights some important benchmarks for the bottarga production: GSI ≥ 10% and oocyte diameter ≥ 400 µm (**Fig. 4**), and (ii) a negative correlation with the viscera ($P < 0.001$; r=-0.61) and body fat ($P < 0.05$; r=-0.51) weights (**Fig. 5**). Nonetheless, no significant correlation ($P > 0.05$) was obtained between the females' body weight and the respective GSI values (**Fig. 6**).

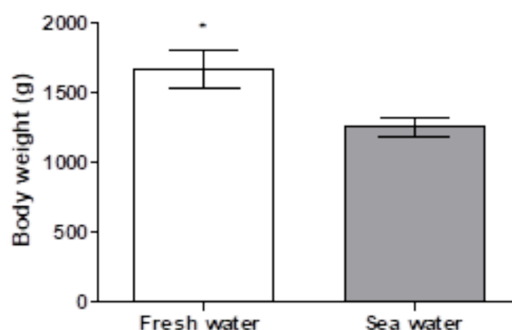

Figure 1. Body weight comparison between 3-year old grey mullet that were caught as fingerlings in the Israeli Mediterranean Sea coast and reared in captivity in fresh or sea water. Asterisk: significant difference compared to the previous month ($P < 0.05$).

Figure 2. Roe derived of 3-year old wild-caught grey mullet that were reared in freshwater (left) and sea water (right) regimes.

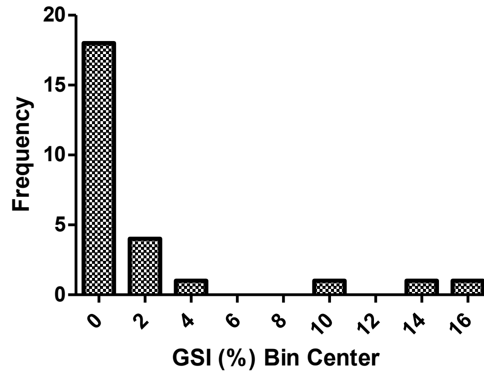

Figure 3. Histogram of the GSI values of 3-year old wild-caught captive-reared grey mullet females.

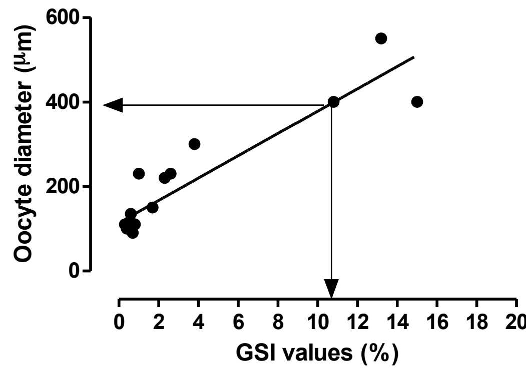

Figure 4. The relationship between oocyte diameter and GSI values in 3-year old wild-caught and captive-reared grey mullet. Arrows point to two important benchmarks for the mullet roe (bottarga) production: GSI ≥ 10% and oocyte diameter ≥ 400 µm.

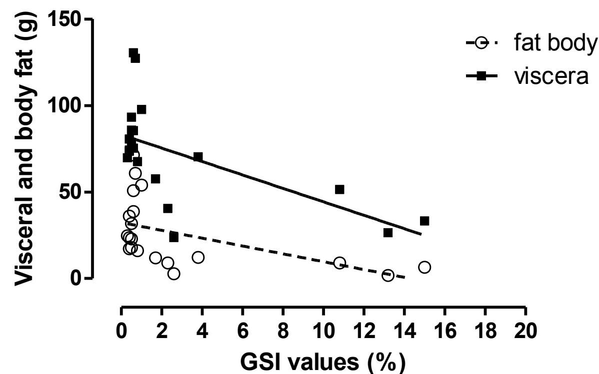

Figure 5. The relationships between GSI values and the weights of fat body and viscera in 3-year old wild-caught and captive-reared grey mullet.

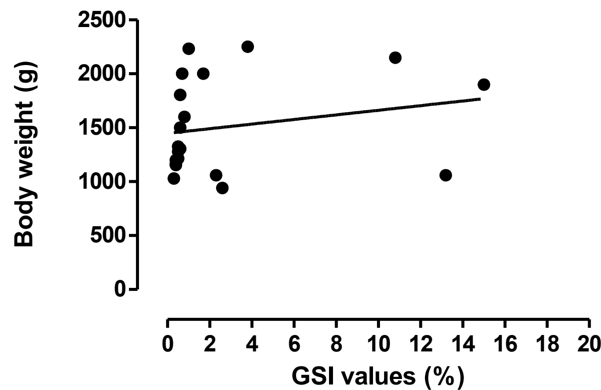

Figure 6. The relationships between GSI values and body weights in 3-year old wild-caught and captive-reared grey mullet.

Gonadal development in hatchery-produced vs. captive-reared grey mullet

Body weights did not vary significantly between hatchery-produced (566 ± 78 g) and captive-reared (765 ± 149 g) females (**Fig. 7A**). However, the GSI values of the hatchery-produced specimens (7.4 ± 2.1 %) were more than 3-fold higher compared with those of captive-reared (1.8 ± 0.92) fish (**Fig. 7B**), although statistical significance was not reached due to the high variance. Moreover, 50% of hatchery-produced females have reached the criteria for bottarga production (GW ≥ 100 g; GSI ≥ 10%) compared to none in the captive-reared group (**Table 2**).

Further comparison of oocyte diameter and oocyte yolk accumulation in the farmed groups (i.e., hatchery-produced and captive-reared) and wild grey mullets caught during their reproductive migration from the Lesina lagoon towards the open sea (see D7.5) indicated that the mean diameter of the largest vitellogenic oocytes of 3-year old hatchery-produced specimens were significantly higher than that of captive-reared fish while both farmed groups had significantly lower oocyte diameter than wild sexually mature grey mullet (**Fig. 8a**). The surface occupied by yolk granules in oocytes of hatchery-produced grey mullet was higher than that of captive-reared specimens and it did not differ from that of wild females (**Fig. 8b**).

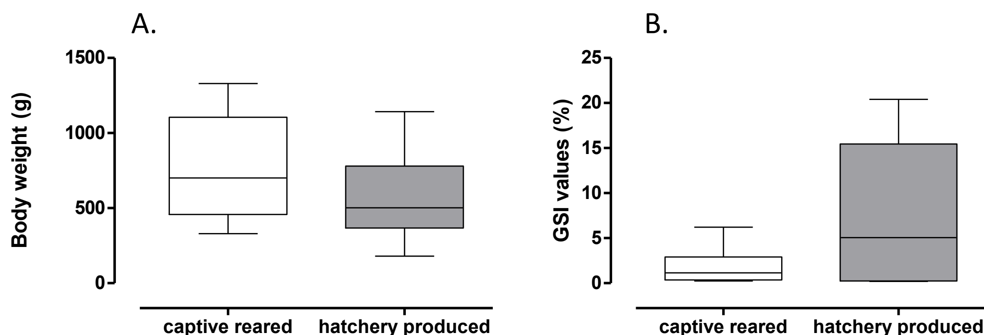

Figure 7. Body weights (A) and GSI values (B) comparison between captive-reared and hatchery-produced 3-year old grey mullet.

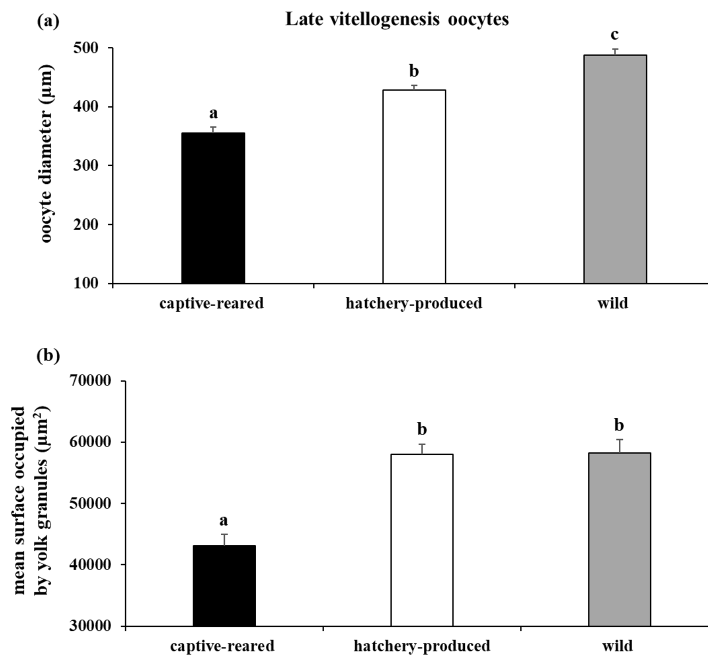

Figure 8. (a) Mean diameter of late vitellogenic oocytes and (b) mean surface occupied by yolk granules in oocytes of age 3 captive-reared and hatchery-produced grey mullets as well as wild adult females. Letters above bars indicate statistically significant differences (P < 0.05).

Dietary effects on gonadal growth and coloration in hatchery-produced grey mullet

No dietary effects on body or gonad weights were observed (**Table 3**). In both treatment groups 50% of the females had fully developed gonads (GSI: 14.56 ± 1.47 %) with oocytes (mean diameter 527 ± 8.4 µm) at the late vitellogenic stage (**Fig. 9B**). The gonads of all other females were undeveloped (GSI: 0.26 ± 0.02%) and showed cortical alveoli as the most advanced oocyte stage (**Fig. 9A**). Nonetheless, a dietary effect could be observed on gonad coloration: bright-yellow vs. pale color in the FO+MgM and SO treatment groups, respectively (**Fig. 10**). Furthermore, the FO+MgM dietary treatment demonstrated significantly (P<0.05) higher carotenoids than the SO diet (**Fig. 11**).

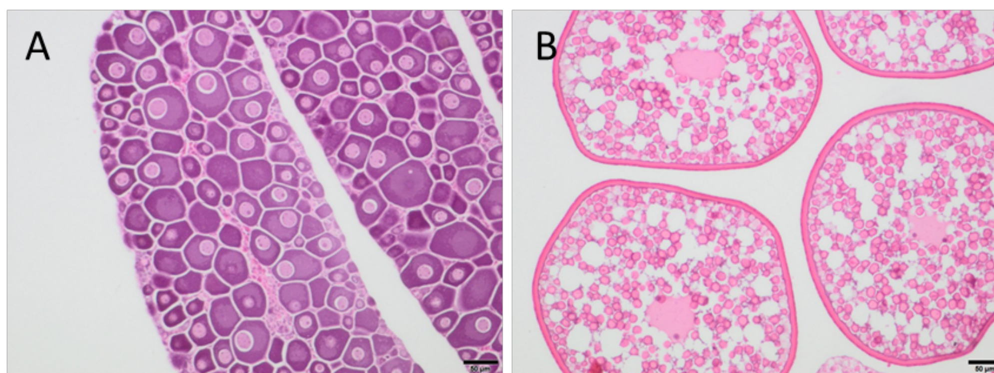

Figure 9. Micrographs of 3-year old hatchery produced grey mullet gonad sections. Ovaries from sexually immature and mature specimens exhibiting oocytes at cortical alveoli (A) and late vitellogenic stage (B), respectively. Haematoxylin-eosin staining. Magnification bars = 50 µm.

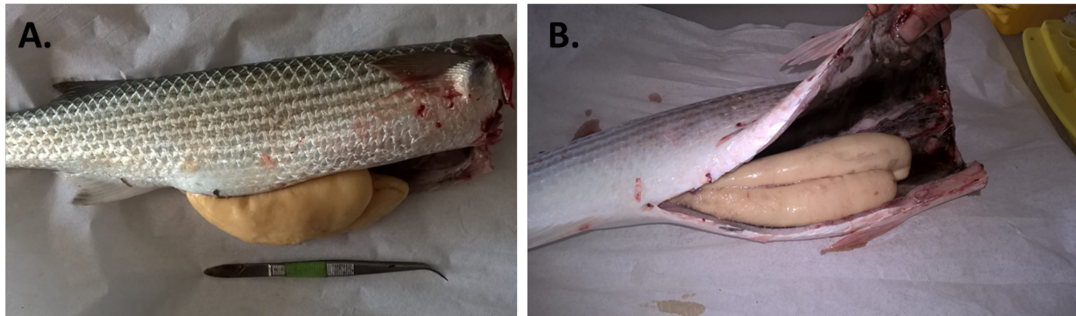

Figure 10. Roe derived from 3-year old hatchery produced grey mullet fed (A) a fish oil based diet (FO) + marigold petal meal (MgM) demonstrating a bright-yellow color and (B) a vegetable oil based diet (SO) showing a lack of color.

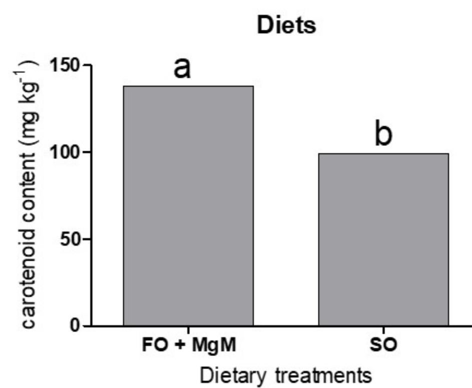

Figure 11. Carotenoid content of the different diets. The fish oil (FO) + marigold petal meal (MgM) and soybean oil (SO) treatments were based on the IOLR formula that were pelleted in a California pelleting mill.

Table 4. Fatty acid content (mg g^{-1}) and main fatty acid composition (% total fatty acids) of gonads from 3-years old mullet fed a fish oil based diet with marigold petal meal (FO+MgM) or a vegetable oil based diet (SO)

	FO+MgM	SO
Total FA	**90.24 ± 4.62**	**81.12 ±14.86**
14:00	0.46 ± 0.02	0.61 ± 0.09
16:00	7.57 ± 0.63	8.24 ± 1.11
18:00	2.36 ± 0.18	2.53 ± 0.17
Total SFA	**10.70 ± 0.75**	**11.87 ± 1.34**
16:11	6.13 ± 0.45	6.45 ± 0.96
18:12	38.41 ± 1.15	37.43 ± 0.32
20:12	7.35 ± 0.84	6.25 ± 1.14
22:12	0.44 ± 0.04	0.33 ± 0.00
Total MUFA	**47.39 ± 1.95**	**47.01 ± 0.48**
18:2n-6	16.61 ± 1.96	16.82 ± 1.42
20:2n-6	0.90 ± 0.06	0.93 ± 0.04
20:4n-6	0.54 ± 0.06	0.55 ± 0.07
Total n-6 PUFA	**19.74 ± 1.87**	**19.91 ± 1.22**
18:3n-3	1.64 ± 0.27	1.50 ± 0.29
18:4n-3	0.22 ± 0.19	0.31 ± 0.08
20:5n-3	1.46 ± 0.15	1.41 ± 0.30
22:5n-3	2.60 ± 0.21	2.64 ± 0.40
22:6n-3	10.91 ± 0.88	10.84 ± 1.85
Total n-3 PUFA	**18.27 ± 1.26**	**18.15 ± 2.89**
DHA/EPA	7.53 ± 0.90	7.75 ± 0.85
ARA/EPA	0.37 ± 0.01	0.39 ± 0.04
n-3/n-6	0.93 ± 0.10	0.92 ± 0.19

Data are means ± SD (n=3). 1 mainly n-7 isomer; 2 mainly n-9 isomer; DHA, docosahexaenoic acid, 22:6n-3; EPA, eicosapentaenoic acid, 20:5n-3; ARA, arachidonic acid, 20:4n-6. nd, not detected.

Table 5. Lipid content (% dry matter) and lipid class composition (% total lipid) of gonads from 3 years old mullet fed a fish oil based diet with marigold petal meal (FO+MgM) or a soybean oil based diet (SO).

	FO+MgM	SO
Total lipid	**30.63±5.98**	**35.87±3.43**
Lipid class		
Lysophosphatidylcholine	0.47±0.15	nd
Sphingomyelin	0.57±0.24	0.65±0.42
Phosphatidylcholine	9.66±0.11	6.80±0.38
PS + PI *	1.25±0.22	0.34±0.12
Phosphatidylglycerol	nd	0.23±0.28
Phosphatidylethanolamine	2.08±0.87	1.78±0.14
Total Polar Lipids	**14.03±0.944**	**9.81±0.67**
Diacylglycerols	nd	1.42±0.35
Cholesterol	10.41±0.30	7.38±0.34
Free fatty acids	2.10±0.67	0.80±0.42
Triacylglycerols	14.54±2.68	11.43±2.16
Wax + Sterol esters	57.10±3.04	68.59±3.00
Unknown	1.82±0.31	0.56±0.23
Total Neutral Lipids	**85.97±0.94**	**90.19±0.67**

Data are means ± SD (n=3). nd, not detected. PS=Phosphatidylserine, PI= Phosphatidylinositol.
* mainly PS

The fatty acid profiles of the female gonads from sexually mature 3-year old hatchery-produced grey mullet fed the FO+MgM or SO diets demonstrated no marked differences between the two groups (**Table 4**). However, the polar lipids, particularly phosphatidylcholine, were lower in the SO gonads compared to the FO+MgM gonads (**Table 5**). Conversely, the FO+MgM gonads were lower in neutral lipids than the SO gonads, particularly wax and sterol esters (**Table 5**).

DISCUSSION AND CONCLUSIONS

The aim of the present work was to assess the effects of fish origin (wild vs. domesticated) and culture conditions on advanced and spontaneous development of gonads comprising the required criteria for the production of high quality bottarga, i.e., minimal size of 100 g, bright yellowish color and chewy texture.

The results of the first experiment indicated that regardless of the water salinity, farmed grey mullet females can proceed through the late stages of vitellogenesis and gain relatively high GSI values (10-20%). These results further attest to the notion that in wild grey mullet the ovary ripening process (i.e., vitellogenesis) occurs in estuarine/brackish waters and then the fish move to sea waters to spawn (Thomson, 1955; Ibáñez and Gutiérrez-Benítez, 2004).

Thus, the ability to undergo vitellogenesis coupled with improved growth performance in freshwater relative to seawater conditions, highlight the relevance of conventional grey mullet farming methods. These

include rearing in freshwater ponds, which contributes not only to fish flesh gain but also for the bottarga production. However, to date farmed mullets are being harvested after 2 years of growth and destined to the fresh fish market, before they reach sexual maturity. Based on our recent results (see D7.5), farmed grey mullet like the Mediterranean wild grey mullet populations, start being reproductive active at the age of 3 years (Bok, 1983; Ameur et al., 2003). Therefore, to facilitate bottarga production the grey mullet grow-out period should be extended to a minimum of 3 years. In this respect, an advanced puberty due to domestication effects and/or a genetic improvement program (Taranger et al., 2010) may increase the farming economic sustainability by accelerating the production cycle of this high-value "mullet caviar".

The fact that relatively low percentages (10-20%) of wild-caught captive reared females have reached late stages of vitellogenesis is in line with previous studies describing the difficulties of Mediterranean grey mullet broodstocks to proceed with gametogenesis in captivity (De Monbrison et al., 1997; Yashouv, 1969). Captive mullet female reproductive dysfunctions were confined to two critical phases, i.e., the early stages of vitellogenesis, and final oocyte maturation and ovulation (De Monbrison et al., 1997). Furthermore, Aizen et al. (2005) reported that some female mullets (approximately 20%) in untreated groups manage to go through the processes of vitellogenesis, while most females do not. It was suggested that low percentages of fully mature females, surrounded by undeveloped females (of the same age and size), typifies a state of social hierarchy, in which the dominant female(s) suppresses sexual maturation of conspecifics. The results of this study further indicate that (i) sexually undeveloped and developed mullet females have comparable body weights and (ii) sexually undeveloped females have remarkably higher energy reservoirs (i.e., visceral and body fat mass) compared with those of the sexually developed ones. These characteristics exclude the incidence of food limitations as a possible cause for the failure of the vast majority of the captive females to develop gonads and support the possible involvement of social hierarchy.

The current study points to favorable effects of captive conditions on mullet females' competence to undergo and complete vitellogenesis. Accordingly, increased abundance (50%) of spontaneously developed gonads (GSI values 10% and above) could be detected among the G2 (second generation) captive-born mullet populations, relative to their wild-born captive-reared counterparts. Additionally, oocytes from the G2 hatchery-produced individuals had the same capacity to uptake vitellogenin and accumulate yolk proteins compared with oocytes of similar size from wild breeders. These results emphasize the potential of hatchery-produced grey mullets to spontaneously develop ovaries up to a condition useful for bottarga production (advanced vitellogenesis).

Mullet's cultured intensively in earthen ponds would be fed formulated feeds to ensure their receiving sufficient nutrients to foster optimal body growth and gonadal development. Natural food (detritus), which develops in the pond, may act as a supplement to the formulated feed provided by the farmer and a rich source of microelements such as vitamins, minerals and pigments. Whether or not the detritus will supply sufficient pigments to generate the characteristic yellowish color of developed ovaries will be dependent on a number of factors, two of which are the composition of the detritus and its abundance relative to the fish population. By supplying sources of natural pigments as part of the formulated feeds to the mullet, we have a greater chance of ensuring that they receive enough to enhance the color of their roe. Indeed, the dietary administration of marigold petal meal (MgM) pigment over 3 months, spanning the entire period of vitellogenesis, markedly contributed to the carotenoid levels in the ovaries giving rise to a bright yellowish roe color. The MgM is a concentrated source of lutein and zeaxanthin (xanthophylls) pigments used to enhance the color of chicken eggs. The xanthophyll content of marigold petal meal (7000 mg/kg of meal) exceeds that of commonly used feed ingredients like yellow corn, corn gluten, alfalfa and algae by a factor which ranges from over 300:1 in the case of yellow corn (22 mg/kg) down to 3.5:1 for dried algae (2000 mg/kg) (North, 1984). Of note, carotenoids are widely present in fish gonads and eggs. They are precursors of vitamin A being involved in reproduction and embryonic development, as well as in the prevention of oxidative stress processes (Miki, 1991; Guerin et al., 2003) and to ensure larval visual function and adequate chromatophore responses. Carotenoids are actively mobilized into the gonads during sexual reproductive activity in aquatic animals. Since fish cannot synthesise either of the vitamins or carotenoids, the maternal dietary content of each prior to oogenesis is an important determinant of reproductive fitness and egg and larval quality. For this reason, the differences of these essential chemical components between

wild mature female gonads and those from eggs produced under different rearing conditions and feeding regimes were investigated in D13.3 in order to collect necessary basic data for the future development of a quality diet for this species.

The basic diets that were used for the pigment-enrichment trial, consisted of the IOLR formulated pelleted diet containing either fish oil (FO) or soybean oil (SO) as the main neutral lipid. Interestingly, despite of the difference of the PUFA in the diets (i.e., 18:2n-6 being higher in the SO diet), homeostatic mechanisms seem to allow the broodstock to maintain a balance resulting in a similar profile of polyunsaturated fatty acids (PUFA) in the gonads, independently of the diet, displaying also a similar pattern to that of the wild counterparts. Higher levels of 18:2n-6 and lower of EPA and DHA are normally present in herbivorous fish compared to carnivorous ones. In addition, the results of this study indicate that in both diets the ovaries described a predominance of neutral lipids (mainly triacylglycerol and wax-esters) over the polar lipids, comprising around 70% of the total lipids in ovaries and eggs. This unique lipid composition, rich in wax esters is considered to play a role as metabolic energy resources for oocyte formation (Zudaire et al., 2014) as well as major contributor to the chewy texture of the dried roe (Lu et al. 1979; Bledsoe et al., 2003).

In conclusion, the present study indicates that: (1) the traditional grey mullet farming procedure in freshwater ponds could be applicable and an advantage for the roe production (2) domestication appears to have a favorable effect on spontaneous development of mullet ovaries up to a condition useful for bottarga production (3) pigment-enriched diets can enhance the roe coloration to meet the criteria for high quality bottarga. However, two stumbling blocks that may restrict the profitability of grey mullet farming for roe production are: (1) an extended grow-out period to a minimum of 3 years and (2) relatively low percentages (20-50%) of females developing ovaries at the appropriate size (≥ 100 g). Future studies therefore, should focus on genetic improvement programs giving rise to advanced sexual maturity and spontaneous ovarian development in captive grey mullet females.

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