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Dietary inclusion of *Tenebrio molitor* larvae meal: effects on growth performance and final quality traits of blackspot sea bream (*Pagellus bogaraveo*)

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1 **Running Title: *Tenebrio molitor* dietary inclusion and blackspot sea bream growth and quality**

2

3 **Dietary inclusion of *Tenebrio molitor* larvae meal: effects on growth performance and final**
4 **quality traits of blackspot sea bream (*Pagellus bogaraveo*)**

5

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21

22 **Abstract**

23 This study evaluated the effects of diets containing *Tenebrio molitor* (TM) larvae meal in partial
24 substitution of fishmeal (FM) on growth performances, marketable, physical and chemical traits of

25 wild-caught blackspot sea bream, a valuable finfish species potentially candidate for Mediterranean
26 aquaculture. One hundred thirty fish were randomly divided into three groups with three replicates
27 each. Fish were fed three diets presenting increasing levels of TM in FM substitution for 131 days:
28 TM0, TM25 and TM50 with 0%, 25% and 50% of fishmeal replacement respectively. Daily intake
29 ratio, feed conversion ratio and specific growth rate were not affected by different diets, like
30 slaughter traits and carcass yield. No significant differences were detected for some fillet quality
31 parameters, such as water holding capacity and texture characteristics (hardness, cohesiveness,
32 resilience, gumminess and adhesiveness), whilst pH value was found lower in TM50 than in TM0
33 and TM25 specimens. Different diets did not affect the colour of the skin dorsal region, unlike the
34 skin ventral region where significant variations in colour were observed, as lightness and hue were
35 lower while redness was higher in TM50 group than in the other two groups. Regarding colour of
36 fillet epaxial region, yellowness and chroma were higher when TM was added in the diets; instead
37 hue resulted higher in fish fed diet containing FM as exclusive source of protein. In the fillet hypaxial
38 region, the colour presented yellowness and chroma values lower in TM0 and TM25 groups than in
39 TM50 group; whilst this last showed the lowest value for hue. Fillets proximate composition was not
40 affected by the diet, unlike the fatty acids profile. Σ n3FA, especially EPA, was higher in fish fed TM0
41 diet. On the contrary Σ n6, especially linoleic acid, significantly increased with TM inclusion in the
42 diets. The Σ n3/ Σ n6 FA ratio was linearly (TM0>TM25>TM50) reduced by TM inclusion in the diet and
43 TM50 specimens had the highest (*i.e.* the worst) values for Atherogenicity and Thrombogenicity
44 Indexes. Since no detrimental effects on growth performance were found, the use of *Tenebrio*
45 *molitor* meal as alternative protein source in blackspot sea bream diet seems to be encouraging, but
46 the effects on fillet quality should be considered.

47

48 **Key words:** *Tenebrio molitor*, insect meal, blackspot sea bream, growth performance, quality traits.

49

50 **1.Introduction**

51 In Italy, aquaculture industry has undergone a significant growth and relies on the production of few
52 traditional finfish species like rainbow trout (*Oncorhynchus mykiss*), European sea bass
53 (*Dicentrarchus labrax*) and gilthead sea bream (*Sparus aurata*). Over the past 20 years, there has
54 been increased interest to develop new and emerging aquaculture species, as possible
55 opportunities for further diversification of Italian aquaculture industry, avoiding market saturation
56 and reducing competition among producers in the Mediterranean area (Parisi et al., 2014).
57 Blackspot sea bream, *Pagellus bogaraveo* (Brünnich, 1768), could represent an alternative to the
58 most commonly farmed marine fish species in Italy and in other Mediterranean countries, due to its
59 high nutritive and commercial value and the quality of its firm and flavorful flesh (Rincón et al.,
60 2016). At the beginning of the 1990s, the early studies on blackspot sea bream rearing were carried
61 out in Spain (Martinez-Tapia et al., 1990; Peleteiro et al., 1994) and nowadays this country has
62 almost an exclusive monopoly in breeding *P. bogaraveo* on large scale, with until now limited
63 productions of about 200 tons year⁻¹ (FAO, 2012). Furthermore, this finfish species has shown a
64 good adaptation to captivity conditions, giving positive feedback with regard to reproduction, larval
65 rearing, pre-fattening and fattening. However, the feeding trials performed on blackspot sea bream
66 are currently scarce and, due to the difficulty to purchase alive farmed subjects, many of them have
67 been conducted on wild-caught specimens (Silva et al., 2006; Ozorio et al., 2009), characterized by
68 a very wide genetic variability.

69 The nutrient requirements of *P. bogaraveo* are still largely unknown. Some researchers found that
70 40% crude protein in the diet was the necessary level to obtain a better combination between
71 specific growth rate and feed conversion ratio (FCR = 2.1); moreover, the protein daily intake for
72 body maintenance was estimated to be 4.3 g kg⁻¹day⁻¹ for sizes between 20 and 60 g (Silva et

73 al.,2006). Since blackspot sea bream is an omnivorous species predominantly carnivorous, fishmeal
74 is the favorite ingredient to satisfy its high protein requirements. Unfortunately, the production and
75 use of this conventional protein source is no longer sustainable, both economically and
76 environmentally, for the fast growing aquaculture industry (Boyd, 2015). Some feeding trials on
77 farmed blackspot sea bream were carried out to test the use of alternative protein and lipid sources
78 as a replacement for fishmeal and fish oil respectively in practical diets (Palmegiano et al., 2007). By
79 preliminary results, the substitution of these ingredients with vegetable sources did not have
80 adverse effects on growth performance and fish quality (Maricchiolo et al., 2007; Palmegiano et al.,
81 2007; Dapra` et al., 2009). In the recent years, there has been increased attention to insects,
82 especially to their larval stage, as an encouraging candidate for the replacement of fishmeal and
83 other conventional protein sources in aquafeeds (Henry et al., 2015). FAO (2013) endorsed insects
84 for their sustainability, as they require little land or energy to be produced, they grow and reproduce
85 easily, and are very efficient in the bioconversion of organic streams (van Huis, 2013). During this
86 process, they accumulate high levels of proteins and lipids.

87 Moreover, they are part of the natural diet of both freshwater and marine fish. Consequently, the
88 interest of researchers in the use of insect meal as ingredient for aquafeeds has rapidly grown and
89 recently their use was approved by the European Commission.

90 Some feeding and growth trials were carried out on various fish species, to evaluate the effect of
91 different insect meals inclusion in partial or complete fishmeal substitution in aquafeed (Nandeesh
92 et al., 1990; Fasakin et al., 2003; Alegbeleye et al., 2012). Not only the performance results obtained
93 in these trials but also the sensory and the consumer evaluations (Sealey et al., 2011; Lock et al.,
94 2016; Borgogno et al., 2017) highlighted the possibility of insect meals inclusion in aquafeeds.
95 However, the use of diets containing insect meals, as new potential protein source, still has not been
96 investigated in feeding of farmed blackspot sea bream.

97 *Tenebrio molitor* (mealworm), belonging to the *Tenebrionidae* family, is one of the most promising
98 candidate as innovative protein source for fishmeal substitution in fish feeds. Larval and pupal
99 stages of *Tenebrio molitor* are rich in protein and lipid and are easy to breed and feed (Ghaly and
100 Alkoaik, 2009). Various studies were performed using mealworm in aquafeeds and encouraging
101 results were observed on growth performance and nutrient utilization in *Clarias gariepinus* (Ng et
102 al., 2001), *Sparus aurata* juveniles (Piccolo et al., 2017) and *Ameiurus melas* fingerlings (Roncarati
103 et al., 2015).

104 The aim of this research, therefore, was to evaluate the effect of two different dietary inclusion
105 levels of *Tenebrio molitor* larvae meal as a partial replacement for fish meal on growth performance
106 and quality traits of blackspot sea bream, in comparison with a diet containing fish meal as exclusive
107 source of protein.

108

109 **2. Materials and Methods**

110 The experimental protocol was designed according to the guidelines of the current European
111 Directive (2010/63/EU) on the protection of animals utilised for scientific purposes.

112 *T. molitor* larvae, utilised to produce the full-fat meal (TM; Gaobeidian Shannon Biology CO., Ltd.,
113 Shannong, China) and employed in this trial, were grown on wheat bran substrate (environmental
114 temperature: 22°C; relative humidity: 65%), then separated from the substrate by sieving, oven
115 dried and milled.

116

117 *2.1 Diet formulation*

118 Three experimental diets were formulated (Table 1) to meet the requirements of blackspot sea
119 bream, as reported in previous research (Silva et al., 2006). The diets were isoenergetic (about 23.5
120 MJ kg⁻¹ gross energy), isoproteic (about 45.9% crude protein) and isolipidic (about 20% ether

121 extract) and were prepared at the Department of Veterinary Medicine and Animal Production of
122 Federico II University (Naples, Italy). A control diet (TM0), in which fishmeal (FM) was the sole
123 protein source, was formulated. FM was partially replaced with full fat *Tenebrio molitor* larvae meal
124 at 25 and 50% (as fed basis) in the other two diets (TM25 and TM50, respectively). In order to keep
125 the diets isoenergetic, the quantities of the other ingredients used in the formulation (starch and
126 fish oil) were modified. In particular, since the used TM contained high fat levels, the fish oil content
127 was reduced by 27 and 45% with increasing percentage of TM inclusion in TM25 and TM50 diets,
128 respectively. For the diets preparation, all dietary ingredients were ground, well mixed and pelleted
129 at 3.5 mm of diameter in a commercial meat grinder. After dried for 48 hours at 40 °C, pellets were
130 screened and stored at 4°C in the dark until used.

131 The diets ingredients, the chemical composition of the full-fat *Tenebrio molitor* larvae meal (TM)
132 and of the diets (TM0, TM25 and TM50), their amino acid and fatty acid profiles are reported in the
133 Tables 1, 2 and 3, respectively.

134

135 *2.2 Fish feeding trial*

136 The feeding trial was conducted at the IAMC facilities of Messina headquarters of CNR (Messina,
137 Italy) on wild-caught blackspot sea bream specimens. Fish were caught during the summer 2014
138 and held in tanks for about 4 months for acclimation to captive conditions. During that time, fish
139 were first fed defrosted fish and then gradually adapted to a commercial diet (44% protein, 18.5%
140 fat; Vita Mare of Veronesi Company, VR, Italy). In February 2014, a total of 129 mixed-sex blackspot
141 sea bream (*Pagellus bogaraveo*) specimens were individually weighed and randomly distributed into
142 nine cylindrical tanks (350 L), in an open circuit system, with intake and discharge of water from and
143 towards the sea. Because the specimens had non-uniform sizes, as derived from fishing activity, fish
144 were divided by weight classes in 9 groups (tanks): 3 small (average weight: 110.67±/ 1.1 g; 14

145 individuals per tank; initial average stocking density 4.5 g L⁻¹), 3 medium (average weight: 159.69 +/-
146 7.8 g; 15 individuals per tank; initial average stocking density 6.8 g L⁻¹) and 3 large (average weight:
147 246.36 +/- 5.6 g; 14 individuals per tank; initial average stocking density 9.8 g L⁻¹). The water
148 parameters were measured daily. The water temperature increased over the weeks from 14 °C in
149 the month of February to 20-21 °C in the month of June, following the normal seasonal increase of
150 temperatures in waters of Messina Straiton which overlooks the IAMC-CNR plants. The dissolved
151 oxygen (DO) ranged between 5.5 and 7.8 mg L⁻¹. After stocking, fish were fed the control diet and
152 adapted over 3 days to the experimental conditions.

153 Each diet was assigned in triplicate to the experimental groups (tanks) according to a completely
154 random design (one tank with small fish, one tank with medium fish and one tank with large fish for
155 each diet). The feeds were offered to blackspot sea bream over 131 days and the fish were fed by
156 hand to visual satiety and *ad libitum* (*i.e.* until the first feed item was refused), twice a day (at 9:00
157 and 16:00 h). The feeds were distributed over the whole water surface of the tanks in order to be
158 accessible simultaneously for all the fish. The feed consumption was recorded weekly, weighing the
159 residues in the containers.

160 During this period, fish were weighed (per tank) every 4 weeks, after a 24-h fast and under light
161 sedation (Tricaine methanesulfonate-MS222, Sigma-Aldrich, Milano, Italy; 50 mg L⁻¹) to reduce the
162 stress. Tanks were inspected daily for mortality.

163

164 2.3 Growth performance

165 All fish of each tank-group were individually weighed at the groups constitution and successively
166 group weighed at the beginning and at the end of the trial and the following performance indexes
167 were calculated for the fish of each treatment (diet):

- 168 • DIR (daily intake rate, %) = ((feed intake (g)/mean weight (g))/no. days) × 100;

- 169 • FCR (feed conversion ratio) = [total feed supplied (g DM) / weight gain (g)];
- 170 • SGR (specific growth rate, %/day)=100 ×[ln (final body weight) – ln(initial body weight)]
- 171 /number of feeding days.

172 At the end of the trial, a subsample of 24 specimens (n. 8 fish per diet from tanks containing large

173 individuals) were randomly sacrificed through an overdose of anaesthetic (Tricaine

174 methanesulfonate-MS222, Sigma-Aldrich, Milano, Italy; 250 mg L⁻¹). After the slaughtering, sampled

175 fish were transported, in dry ice to the Laboratories of the Department of Agri-Food Production and

176 Environmental Sciences (DISPAA), University of Florence (Firenze, Italy) and immediately stored at

177 – 80 °C until analyses. Before the analyses, all whole fish were defrozen, subjected to morphometric

178 measurements and then filleted; the fillets obtained were analysed for physical and chemical

179 characteristics.

180

181 *2.4 Morphometric and product properties*

182 After measurements of the individual body weight, each fish (n= 24) underwent the following

183 measurements by an orthometric meter: total length and intestinal length. Then, liver, visceral fat

184 and viscera (digestive system, liver and visceral fat) were separated and weighed. The eviscerated

185 fish were also weighed and subsequently filleted; the fillets (right and left) and the skin from right

186 fillet were subsequently weighed. Afterwards, from each residual of filleting process head, fins and

187 frame were removed and separately weighed. These data were utilized to calculate the dressed

188 yield (DY), with skin fillet yield and without skin fillet yield (FY); head, fins, frame and total waste

189 incidences; condition factor (CF) and relative intestinal length (RIL) as follows:

- 190 • DY (%) = [eviscerated weight (g) / body weight (g)] × 100;
- 191 • with skin FY (%) = [right fillet weight (g) + left fillet weight (g) / body weight (g)] × 100;

- 192 • without skin FY (%) = [(right fillet weight (g) + left fillet weight (g) – (right skin weight (g) ×
193 2) / body weight (g)] × 100;
- 194 • Head (%) = [head weight (g) / body weight (g)] × 100;
- 195 • Fins (%) = [fins weight (g) / body weight (g)] × 100;
- 196 • Frame (%) = [frame weight (g) / body weight (g)] × 100;
- 197 • Total wastes (%) = [frame + fins + head + viscera weight (g) / body weight (g)] × 100.
- 198 • CF = [body weight (g) / total length³ (cm)] × 100;
- 199 • RIL = intestinal length (cm) / fish total length (cm);

200 Finally, somatic indexes as hepato-somatic (HSI), viscero-somatic (VSI) and visceral fat (VFI) indexes
201 were also calculated as follows:

- 202 • HSI (%) = [liver weight (g)/body weight (g)] × 100;
- 203 • VSI (%) = [viscera weight (g)/body weight (g)] × 100;
- 204 • VFI (%) = [visceral fat weight (g)/body weight (g)] × 100.

205

206 *2.5 Organoleptic characteristics*

207 *Physical analyses*

208 All fish (n= 24) were subjected to individual skin and fillet muscle colour measurements, performed
209 by a Spectro-color®116 colorimeter (Bell Technology Ltd, Auckland, New Zealand) using Spectral qc
210 3.6 software, according to the CIELab system (CIE,1976). In this system, lightness (L*) is expressed
211 on a 0 – 100 scale, from black to white; redness index (a*) ranges from red (+60) to green (-60) and
212 yellowness index (b*) ranges from yellow (+60) to blue (-60). In addition, the values of Chroma =
213 $(a^{*2} + b^{*2})^{1/2}$, as a measure of colour saturation, and Hue = $\arctan(b^*/a^*)$ were calculated. Colour
214 readings were performed in triplicate on dorsal and ventral locations (cranial, medial and caudal
215 positions for both locations) of the skin of the left lateral side of the fish and on epaxial and hypaxial

216 locations (cranial, medial and caudal positions for both locations) of the right fillet muscle and the
217 three values obtained for each location were expressed as mean.

218 The measurement of muscle pH was performed in triplicate on the cranial, medial and caudal
219 positions of the right fillet epaxial region from each fish (n= 24), using a Mettler Toledo DevenGo
220 SG2™ pH-meter (Novate Milanese, Milano, Italy) equipped with an Inlab puncture electrode
221 (Mettler-Toledo, Ltd). The mean value was utilized.

222 Texture analyses were carried out using a Zwick Roell® 109 texturometer (Ulm, Germany) equipped
223 with a 1kN load cell and with the Text Expert II software version 3.0. A two-cycle compression test
224 was done utilising a 10 mm diameter cylindrical probe, moving perpendicularly to the muscle fiber
225 direction, at a constant speed of 30 mm/min to 50% of total deformation. Textural features were
226 measured on a sample of muscle (3.5 x 3.5 cm) withdrawn from the epaxial region of the right fillets
227 of each fish (n= 24), in two spots from each sample and the mean value of the two spots
228 measurements were utilized for data analysis. From texture measurements, five parameters were
229 calculated according to Veland and Torrissen (1999) and Ayala et al. (2010): *i.e.* hardness (peak force
230 of the first compression cycle), cohesiveness (ratio of positive area of the force during the second
231 compression, compared to that obtained during the first compression), resilience (ratio of upstroke
232 area to downstroke area during first compression cycle), gumminess (hardness multiplied by
233 cohesiveness), and adhesiveness (work necessary to win the attractive force between the surface
234 of the probe and that of the sample).

235 Water Holding Capacity (WHC) was determined by percentage of water loss after centrifugation,
236 according to Eide et al. (1982) with the modification proposed by Hultmann and Rustad (2002). The
237 fillet samples (n= 24) were minced and 2 g were weighed inside plastic tubes equipped with a filter
238 net, then the tubes were centrifuged at 1500 rpm (~210 g) for 5 minutes. Finally, WHC was
239 calculated as difference between the initial gross weight and the gross weight after centrifugation,

240 and the value obtained was divided for the water content of the sample, determined by the AOAC
241 (2000) 950.46 method. This analysis was performed in triplicate, and the mean value of the three
242 measurements obtained from each sample was utilized for data analysis.

243

244 *Chemical analyses of feeds and fish muscle*

245 Proximate composition of the *Tenebrio molitor* larvae meal and of the three experimental diets
246 (Table 1), as well as of the freeze-dried and ground fillets from the three groups of fish (n= 24) was
247 determined according to AOAC (2000) procedures for moisture (method 950.46), crude protein
248 (method 976.05) and ash (method 920.153). In the samples of mealworm and experimental diets,
249 crude fiber (method 985.29; AOAC, 2000), neutral detergent (NDF) and acid detergent (ADF) fibers
250 were also analyzed according to the procedure of Goering and Van Soest (1970).

251 The amino acid profile (Table 2) of TM and of the three diets was determined as described in De
252 Marco et al. (2015).

253 Regarding the total lipids, a sample of the TM meal and of the experimental diets (Table 3), and
254 samples of wet fillet muscle (~2 g) from fish (n= 24) fed the three different diets were ground and
255 extracted using chloroform-methanol (2:1 v/v) solution, according to Folch et al. (1957) modified
256 method. Then, the total lipids were quantified gravimetrically. The extracted total lipids were
257 utilised for the analysis of fatty acid (FA) profile, performed according to a modified method of
258 Morrison and Smith (1964). Lipids were saponified with 5 mL of 0.5 M KOH in methanol, and FAs
259 were hydrolyzed by adding 2.5 mL of 2 M HCl. Methyl esters were prepared by transmethylation
260 using 2 mL of boron fluoride-methanol at 14% concentration. Methylated FAs were dissolved in
261 petroleum ether (40-60), dried, and finally resuspended in 1.5 mL of hexane. FA composition was
262 determined by liquid gas chromatography (LGC). A GC Varian 430 gas chromatograph (Varian Inc.,
263 Palo Alto, CA, USA) equipped with a flame ionization detector (FID) and a SupelcoOmegawax™ 320

264 capillary column (30 m × 0.32 mm i.d., 0.25 µm film and polyethylene glycol bonded phase; Supelco,
265 Bellefonte, PA, USA) was utilized. The oven temperature was held at 100 °C for 2 minutes, increased
266 to 160 °C over 4 minutes at a rate of 12 °C/min, increased to 220 °C over 14 minutes at the rate of
267 3 °C/min, and kept at 220 °C for 25 minutes. Injector and detector temperatures were set at 220 °C
268 and 300 °C, respectively. One microlitre of sample in hexane was injected into the column with the
269 carrier gas (helium) at a constant flow of 1.5 mLmin⁻¹. The split ratio was 1:20. Chromatograms were
270 recorded with a computing integrator software (Galaxie Chromatography Data System 1.9.302.952;
271 Varian Inc., Palo Alto, CA, USA) and the FAs were identified by comparing the retention time of the
272 fatty acid methyl esters (FAME) with the standard Supelco 37 component FAME mix (Supelco,
273 Bellefonte, PA, USA). FAs were quantified through calibration curves, using tricosanoic acid (C23:0)
274 (Supelco, Bellefonte, PA, USA) as internal standard, and they were expressed as a percentage of
275 total FAME.

276 The atherogenicity index (AI) and thrombogenicity index (TI), according to Ulbricht and Southgate
277 (1991) and hypocholesterolemic/hypercholesterolemic FA ratio (h/H), according to Santos-Silva et
278 al. (2002) were also calculated as follows:

- 279 • $AI = [C12:0 + (4 \times C14:0) + C16:0] / (\Sigma n3 \text{ PUFA} + \Sigma n6 \text{ PUFA} + \Sigma MUFA)$
- 280 • $TI = (C14:0 + C16:0 + C18:0) / [(0.5 \times \Sigma MUFA) + (0.5 \times \Sigma n6 \text{ PUFA}) + (3 \times \Sigma n3 \text{ PUFA}) + (\Sigma n3$
281 $\text{ PUFA} / \Sigma n6 \text{ PUFA})]$
- 282 • $h/H = (C18:1n9 + C18:2n6 + C20:4n6 + C18:3n3 + C20:5n3 + C22:5n3 + C22:6n3) / (C14:0$
283 $+ C16:0).$

284 Furthermore, PUFAn3/PUFAn6 and PUFA/SFA ratios were also calculated.

285

286 *2.6 Statistical analysis*

287 All the data were analyzed by one way ANOVA, using the GLM procedure of SAS (2007), according
288 to the model:

$$289 Y_{ij} = m + P_i + e_{ij}$$

290 Where Y is the single observation, m the general mean, P the effect of the diet (i = TM0 or TM25 or
291 TM50 diets) and e the error.

292 Comparison between means was performed by Tukey's test (SAS,2007) at $P < 0.05$.

293 For growth performance, data were analyzed using body weight as covariate to take into account
294 the different classes of weight of the fish.

295 For morphometric and product quality properties, *i.e.* physical traits and chemical composition of
296 fish fillets, the body weight of fish was found to have a significant effect on the investigated
297 parameters, therefore all the parameters were analyzed using the body weight as covariate. In this
298 way, the possible effects of diet and body weight have been separated.

299

300 **3. Results**

301 *3.1 Growth performance*

302 Table 4 shows the relationships among diets and growth performance of fish, highlighting that the
303 diet did not affect all the parameters considered.

304

305 *3.2 Morphometric and orthometric properties*

306 Also the slaughter traits (Table 5) of blackspot sea bream were not affected by the different diets,
307 except for body weight that resulted significantly higher ($p < 0.05$) in TM0 group than TM25 and
308 TM50 groups. In addition, body weight affected the weights of all the body components, except for
309 the weight of fins. Regarding orthometric measurements, the effect of body weight was found only

310 for fish total length. Finally, the dressed and fillet yields were not affected neither by diet treatment
311 nor by body weight.

312

313 *3.3 Physical traits of fillets*

314 The results related to physical characteristics analysed on fillets are reported in Table 6. No
315 significant effect of different diets was found on WHC and texture properties (hardness,
316 cohesiveness, resilience, gumminess and adhesiveness). Regarding pH value, fish of TM50 group
317 had a significantly lower ($p < 0.01$) value whilst no differences were observed between fish of TM0
318 and TM25 groups. Moreover, body weight had no significant effect on none of these parameters.

319 In the Table 7, the colour parameter values related to the skin (dorsal and ventral locations) and to
320 the fillet (epaxial and hypaxial locations) of blackspot sea bream fed different diets are presented.

321 Concerning skin dorsal location, no differences in colour values were found for fish fed different
322 diets whilst, on the contrary, the diets utilized affected the colour of the ventral location. In details,
323 TM50 group fish had the lowest L* and Hue compared to TM0 and TM25 groups, that showed similar
324 characteristics. On the other hand, the redness (a*) was significantly higher ($p < 0.001$) in the fish
325 fed the diet including the highest level of insect meal (TM50) if compared to TM0 and TM25 groups.

326 For the colour parameters, a significant effect of fish body weight limitedly to lightness, redness and
327 Hue was found. In the case of fillet muscle, yellowness (b*), colour saturation (Chroma) and Hue
328 were affected by different diets at level of both epaxial and hypaxial locations where significantly
329 lower values for b* and Chroma and the highest values for Hue were registered for fish of control
330 group (TM0). The colour characteristics of fillets from fish fed diets including TM did not present
331 differences, on the contrary to what observed for skin colour at the ventral location.

332

333 *3.4 Chemical composition of fillets*

334 The results of proximate composition of blackspot sea bream fillet muscle are presented in Table 8.
335 The dietary treatment did not affect moisture, crude protein, ether extract, total lipids and ash
336 contents of fillets. On the other hand, the fatty acid profile of the lipid fraction of fillet muscle was
337 substantially affected by the dietary treatment, as shown in Table 9. Concerning FA groups, the
338 inclusion of *T. molitor* larvae meal in the diet led to an expected increase in PUFA_{n6} percentage
339 (from TM0 to TM50 groups) and to a decrease in PUFA_{n3} incidence, that was lower ($p < 0.01$) in fish
340 of TM25 and TM50 groups compared to TM0 group. Linoleic acid (18:2_{n6}) incidence progressively
341 increased ($p < 0.001$) from TM0 to TM50 groups, whilst EPA (20:5_{n3}) percentage slightly decreased
342 ($p < 0.05$) in muscle when fish were fed diet where FM was partially replaced with TM at 50%.
343 Despite no statistical differences were observed, DHA (22:6_{n3}) content progressively decreased as
344 the levels of TM inclusion in diet increased. The TM0 and TM25 samples showed higher ($p < 0.05$)
345 18:3_{n3} incidence than TM50 group. Total SFA and MUFA percentages were not influenced by the
346 diet but, among saturated fatty acids, 16:0 (palmitic acid) content was the highest ($p < 0.05$) in TM50
347 samples and no differences were found between the other two groups of fish. Among MUFA, the
348 fatty acid 18:1_{n7} presented the highest ($p < 0.01$) percentage in TM0 and TM25, 20:1_{n9} and 22:1_{n11}
349 were in the highest ($p < 0.01$) percentage in TM0 group while no differences were observed between
350 TM25 and TM50 groups.

351 In Table 9, the quality indexes related to the fatty acid relationships are also reported. As expected
352 in relation to the fatty acid profile previously described, the values of atherogenicity and
353 thrombogenicity indexes were the highest ($p < 0.05$) in TM50 specimens while no differences were
354 observed between the fish of TM0 and TM25 groups. Concerning $\Sigma n3/\Sigma n6$ ratio, fish fed the control
355 diet (TM0) had higher ($p < 0.001$) value and this progressively decreased in the fish fed the diets
356 substituting FM with the alternative protein source. No effect of the body weight was observed on
357 fatty acids profile and nutritional indexes.

358

359 **4. Discussion**

360 In this trial, the effect of the dietary inclusion of *Tenebrio molitor* larvae meal as novel protein source
361 in partial substitution of FM in feeding of blackspot sea bream was tested. Despite the growing
362 interest in the use of insect meal for fish, no studies are currently available in literature on the use
363 of insect meal in *Pagellus bogaraveo* production and the research on the effects determined by the
364 insect meal inclusion in the diet in final quality of the product is still scarce.

365 In our study, the inclusion of *Tenebrio molitor* larvae meal at 25% and 50% of FM substitution in the
366 diet did not lead to significant effects on the considered growth performance parameters, since the
367 diets were formulated to be isoproteic and isoenergetic and the differences found in amino acid and
368 fatty acid profiles did not produce a different response by the fish. Belforti et al. (2015) reported
369 that the inclusion of 25% or 50% of TM in rainbow trout diets did not affect the final fish weight and
370 the weight gain, but significantly improved some performances parameters as FCR, SGR and Protein
371 Efficiency Ratio (PER). On European sea bass, instead, Gasco et al.(2016) found that the 50%
372 inclusion of TM in the diet led to a worsening of final body weight, weight gain, specific growth rate,
373 and feeding rate if compared to the control diet based on FM as exclusive source of protein, while
374 no negative effects were obtained at 25% inclusion. Comparable results were obtained by Piccolo
375 et al. (2017) in a trial presenting the same inclusion rates of TM (25 and 50%) in gilthead sea bream
376 diets. The group fed TM25 showed no negative effects as regards the final weight, the specific
377 growth rate, the weight gain, the protein efficiency ratio, and the feed conversion ratio compared
378 to the control diet, while at the highest level of inclusion (50%) gilthead sea bream's nutrient
379 digestibility was penalized but this did not lead to negative effects on growth performance in
380 comparison to the control group.

381 A comparison with previous growth trial on *P. bogaraveo* is difficult to do due to the wild origin of
382 the subjects, that markedly affects genetic variability of the specimens and, consequently, their
383 behaviour. Our growth performance was in line with the results of two previous studies (Silva et al.,
384 2006; Ozorio et al., 2009) that utilized *P. bogaraveo* specimens with the same wild origin. Silva et al.
385 (2006) obtained subjects of 60 g starting from 22 g in 3 months of rearing with water temperature
386 ranging from 21 to 24°C. Ozorio et al. (2009) obtained an average individual weight gain of 85 g in
387 138 days with water temperature of 19 °C, starting from an initial body weight of 64 g. All the
388 previous studies on the same species have pointed out that *P. Bogaraveo* is a slow-growing species,
389 if compared to other species of Sparidae family (Silva et al., 2006; Ozorio et al., 2009; Figueiredo-
390 Silva et al., 2010). Our trial confirmed these findings, having obtained average SGR of 0.20, after 131
391 days of rearing in water with a temperature ranging from 14 to 21 °C. However, the values obtained
392 are very low also in comparison with the other cited studies. These results could be explained
393 considering that the temperature during the most of the duration of the trial presented constantly
394 low average monthly values (February: 13.95 ± 0.55 ; March: 14.24 ± 0.88 ; April: 14.81 ± 0.83 ; May:
395 17.41 ± 0.66 ; June: 21.15 ± 1.09) and only in the last 15 days rapidly increased. On the other hand,
396 it should be also considered that *P. bogaraveo* usually lives at high depths (Chilari et al. 2006) and
397 the specimens used in our trial were captured at depths ranging from 250 to 500 meters. The same
398 authors reported that wild *P. bogaraveo* specimens, caught in Ionian Sea, showed a slower growth
399 rate than those reported by other authors in the Atlantic, and differences in growth between regions
400 could be attributed to the differences in the environmental conditions. In our trial, temperature
401 could have contributed to the observed slow growth for sure, but it has also to be considered that
402 slow temperatures, and therefore slow growth, seem to be a “natural” condition for this species.
403 FCR values, in the range of 5, are very high compared to the ones reported in the previous trials on
404 blackspot sea bream of wild origin (Silva et al., 2006; Ozorio et al., 2009), even if these trials were

405 conducted on smaller fish kept at higher temperatures. In particular, a similar value of FCR (4.2) was
406 reported by Silva et al. (2006) for blackspot sea bream fed on a diet with a low protein content
407 (20%). As we did not observe food wastage and the fish seemed to have been adapted to the
408 experimental diets, we can only assume that in our trial low temperatures had a negative effect on
409 digestibility of nutrients and feed efficiency. In fact, nutrients digestibility, growth and feed
410 efficiency are reported to be strictly influenced by water temperature in fish species (Olsen et al.,
411 1998; Pers and Oliva Teles, 1999).

412
413 Our trial results showed that the inclusion of *Tenebrio molitor* larvae meal, as a partial replacement
414 of FM in feeds, did not lead to relevant and significant effects on the slaughter yield and somatic
415 indexes of blackspot sea bream specimens. A previous study on blackspot sea bream, in which FM
416 was partially replaced with rice protein concentrate meal, found similar results (Palmelegiano et al.,
417 2007). On the contrary, Belforti et al. (2015) obtained opposite results for HSI in rainbow trout with
418 a decrease in the value of this index at the increase of mealworm levels in the diet, confirming the
419 importance of the species-specific approach when a new ingredient for feed is studied.

420 It is widely known that the fish feeding has an important impact on several quality parameters of
421 the fish and fillet characteristics, such as colour, texture, nutritional/functional quality, shelf life and
422 lipid content (Lie, 2001; de Francesco et al., 2007; García-Romero et al., 2014; Tibaldi et al., 2015).

423 The absence of differences in some physical properties (in particular texture parameters and WHC)
424 of fillets from the three groups of fish is an important finding for the positive evaluation of this new
425 ingredient as FM partial replacer. However, the different diets utilized in this trial seemed to have a
426 significant effect on skin and fillet muscle colour parameters. Previous studies observed that the
427 inclusion of different ingredients, as aquatic or terrestrial vegetable sources, had effects on colour
428 characteristics of fish skin and fillet muscle (de Francesco et al., 2004; Rørå et al., 2005; Tibaldi et
429 al., 2015). In general insects are also a good sources of pigments and the main pigment is β -carotene

430 (Finke et al., 2002). This last belong to carotenoid group and it is considered as red-coloured
431 pigment. The main repository of fish carotenoids are the skin and the flesh (Hardy, 2002;
432 Storebakken, 2002). However, fish do not possess the power to synthesize carotenoid *de novo* so
433 their presence in fish tissues is strictly associated with alimentary carotenoids or diet
434 supplementation. In fact, the carotenoid content from the marine crab meal was an effective dietary
435 ingredient for enhancing skin colour in red porgy based on Hue, Chroma values and skin carotenoid
436 concentration (García-Romero et al., 2014)

437 Although the levels of β -carotene are less than 100 μg per 100 g in mealworm larvae (Finke et al.,
438 2002), the carotenoids content could explain the change in a^* index of skin colour when fish fed
439 insect meal at 50% of fishmeal replacement, in our study.

440 Skin lightness (L^*) was significantly affected by diets in our study and a variation in this parameter
441 seemed to be mainly related with different environmental factors (Pavlidis et al., 2006)

442 Differences are related not only to the diet composition but also to the rearing conditions. In fact,
443 farmed fish are reported to have darker skin than their wild counterparts as they are more exposed
444 to solar light and so their skin chromophores become darker (Adachi et al., 2005). In the gilthead
445 sea bream it has been found that different rearing conditions affect lightness and skin colour
446 distribution (Valente et al., 2011). Also Rincón et al. (2016) observed that significant variations were
447 determined in colour studies with a higher lightness (L^*) and redness (a^*) on the skin of the wild
448 blackspot sea bream and a higher hue than farmed one.

449 Moreover, riboflavin (vitamin B2), yellow-coloured pigments, is present in most edible insect and
450 ranged from 0.11 to 8.9 mg per 100 mg (8.1 mg in mealworm) as reported by Finke et al. (2002).
451 The presence of this pigment seemed to have increased the yellowness character in flesh of fish fed
452 diets with 25 and 50% of *T. molitor* inclusion.

453 The changes in colour should be appropriately considered since the colour is one of the most
454 important parameters and is often utilised to determine the economic value of food, since it can
455 markedly influence the consumer acceptance for meat products in general (Lie, 2001).

456 The pH is important in determining the quality of fish, and can be used as a guide of freshness
457 (Pacheco-Aguilar et al., 2000) because of its influence on bacterial growth (Obemeata et al., 2011).

458 The *post mortem* glycolysis causes the accumulation of lactic acid and therefore a reduction in pH
459 of the muscle. However, fish muscle contains a relatively low level of glycogen compared to
460 mammals, thus far less lactic acid is generated after death. Although the pH value is dependent on
461 the species, a lower limit of 6.2 has been established for fish species in a state of rest prior to
462 sacrifice (Love,1988). In our study, , the pH values were affected by dietary inclusion of insect meal,
463 resulting in a lower pH in fish fed TM50 diet while no differences were found both in fish fed TM0
464 and TM25 group Marked muscle lactic acid increase and pH decrease after death, linked to high
465 anaerobic glycolysis activity also before death, are often good early stress and muscular activity
466 indices (Poli et al., 2005). Therefore, the replacement of fishmeal could induced greater stress when
467 blackspot sea bream fed high level of *Tenebrio molitor* larvae meal (50% of TM inclusion).

468 The proximate composition of blackspot sea bream muscle seemed not to be affected by insect
469 meal inclusion. However, protein and lipid contents slightly increased when larvae meal was added
470 in the feed, although these variations have not reached the threshold of the statistical significance.

471 Similarly, a study conducted using larvae meal from *Lucilia sericata* in feed for gilthead sea bream
472 showed an increase in fat in fillets from fish fed larvae (de Haro et al., 2015). These results are
473 conflicting with those obtained by other researchers that found a significant decrease in fat and
474 increase in protein of rainbow trout fillets as effect of the dietary inclusion of full-fat *Tenebrio*
475 *molitor* larvae meal in the diet (Belforti et al., 2015).

476 Insect meal is not only a source of protein, but also contains lipids whose quantity and quality are
477 largely influenced by the substrate on which the insect larvae have fed (St-Hilaire et al., 2007;
478 Kroeckel et al., 2012). However, the main drawback to the use of insect as alternative ingredient for
479 fish feeds is that they are lacking in polyunsaturated fatty acids (PUFA) of the n3 series, such as
480 eicosapentaenoic acid (EPA, 20:5n3) and docosahexaenoic acid (DHA, 22:6n3), whilst they are rich
481 in linoleic acid (LA, 18:2n6), saturated and monounsaturated fatty acids (Sánchez-Muros et al.,
482 2014). The fish fatty acid profiles generally reflect the dietary fatty acid content and the results from
483 this study are in general agreement with this dogma, indeed the fatty acid composition of *P.*
484 *bogaraveo* muscle was significantly altered by diet and the differences in the fatty acid profile of
485 fillets reflected the differences found among the diets. Despite the *Tenebrio molitor* larvae meal had
486 significant amounts of SFA and MUFA (Table 3), this trend was not reflected in fish muscle (Table 8).
487 Nevertheless, 16:0 FA (palmitic acid) seemed to increase in fish fed at 50% of TM inclusion. On the
488 contrary, Sealey et al. (2011) found that normal and fish offal-enriched black soldier fly prepupae at
489 25 and 50% of dietary inclusion decreased the content of this saturated fatty acid in rainbow trout
490 muscle. In this trial, the high linoleic acid concentration in TM was reflected on blackspot sea bream
491 muscle, with the highest value of this PUFA-n6 fatty acid recorded in the fillets from TM50 group.
492 These results are in agreement with what found by Belforti et al. (2015) utilizing the meal from the
493 same insect although in a different fish species (rainbow trout). Regarding PUFA-n3, the most
494 important fatty acids for the growth of fish and also for the health of the fish consumers, EPA and
495 DHA, gradually diminished in fish fed insect meal even if no statistical differences were found in the
496 case of DHA percentage. Similarly, Belforti et al. (2015) noted that both these fatty acids strongly
497 decreased in rainbow trout fed feed with increasing levels of FM substitution by *Tenebrio molitor*
498 meal. However, the data obtained in the present trial differ from those found by Sealey et al. (2011)
499 who described increasing levels of EPA and DHA in muscle from trout fed fish offal-enriched black

500 soldier fly (*Hermetia illucens*) prepupae, showing a relevant plasticity in insects nutritional
501 characteristics strictly dependent on the kind of diet.

502 Since the muscle of *P. bogaraveo* fillets showed consistent increase and decrease of $\Sigma n6$ and $\Sigma n3$
503 fatty acids, respectively, when *T.molitor* larvae meal was added in diets, a significant reduction of
504 the $\Sigma n3/\Sigma n6$ FA ratio was found, thus adversely affecting the nutritional/functional value of the
505 fillets. Indeed $\Sigma n3/\Sigma n6$ ratio is considered, together with EPA and DHA contents, a useful indicator
506 of the nutritional/functional values of fish as food for humans for their extremely beneficial effects
507 for human health. Palmegiano et al. (2007) reported a similar trend for the $\Sigma n3/\Sigma n6$ ratio in muscle
508 of blackspot sea bream fed diets containing increasing levels of a rice protein concentrate meal. As
509 well as the health indices are concerned, both AI and TI had similar trends and slightly worsened
510 with increasing levels of TM inclusion in the diet. The results of AI are in disagreement with the
511 findings found by Palmegiano et al. (2007) and Belforti et al. (2015) that observed a reduction of
512 this index in fish fed alternative protein source, vegetal and animal, respectively. Regarding TI,
513 Belforti et al. (2015) found an increase in rainbow trout fed feeds with progressive increase of TM in
514 partial replacement of FM. Nevertheless, the values of both indices registered in fish flesh were
515 widely lower than those of foods derived from terrestrial animals (Palmegiano et al., 2007; Belforti
516 et al., 2015). Despite the h/H index seemed not to be affected by different diets, its value slightly
517 decreased but only when insect meal was added in feeds replacing 50% of FM, confirming the critical
518 issues on nutritional/functional characteristics of fillets due to the inclusion in aquafeed of this
519 insect meal in high percentage of replacement of fish meal.

520 Currently, the effect of fishmeal replacement on flesh quality characteristics and shelf-life results
521 scarcely investigated and the knowledge is very scarce when the insects are utilized as the source
522 of protein alternative to that from fishmeal. In a study of García-Romero et al. (2014), the

523 inclusion of crab meal, as alternative protein source, in red porgy diet affected the fillet shelf-life,
524 delaying the lipid oxidation in muscle stored at 4 °C with respect to fish fed on control diet.

525

526 **5. Conclusion**

527 Based on its high nutritive and commercial value, blackspot sea bream could represent an
528 alternative to common marine fish species produced in Mediterranean area. Nevertheless, the slow
529 growth highlighted by our study, confirmed also by previous researches, could hamper its future
530 breeding possibilities. However, while consumers demand and market price will remain high, it will
531 be worthwhile investigating further this species trying to overcome the shown limits, through the
532 development of appropriate breeding techniques and the identification of ideal rearing conditions.
533 On the basis of the results obtained in this trial, *Tenebrio molitor* larvae meal seemed to be a
534 promising candidate to be used as alternative protein source for the partial replacement of fish meal
535 in diet for blackspot sea bream. Since no detrimental effects on growth performance and on fillet
536 quality were observed, mainly when the level of FM replacement is 25%, the use of mealworm as
537 suitable ingredient in feed for *P. bogaraveo* seems to be encouraging. However, further researches
538 should be performed to improve the nutritional characteristics of this novel protein source and,
539 therefore, to limit the detrimental effects of TM inclusion in the diet on the nutritional value of the
540 lipid fraction of the blackspot sea bream fillets.

541

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548

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