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

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

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Abstract

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

wild-caught blackspot sea bream, a valuable finfish species potentially candidate for Mediterranean aquaculture. One hundred thirty fish were randomly divided into three groups with three replicates each. Fish were fed three diets presenting increasing levels of TM in FM substitution for 131 days: TM0, TM25 and TM50 with 0%, 25% and 50% of fishmeal replacement respectively. Daily intake ratio, feed conversion ratio and specific growth rate were not affected by different diets, like slaughter traits and carcass yield. No significant differences were detected for some fillet quality parameters, such as water holding capacity and texture characteristics (hardness, cohesiveness, resilience, gumminess and adhesiveness), whilst pH value was found lower in TM50 than in TM0 and TM25 specimens. Different diets did not affect the colour of the skin dorsal region, unlike the skin ventral region where significant variations in colour were observed, as lightness and hue were lower while redness was higher in TM50 group than in the other two groups. Regarding colour of fillet epaxial region, yellowness and chroma were higher when TM was added in the diets; instead hue resulted higher in fish fed diet containing FM as exclusive source of protein. In the fillet hypaxial region, the colour presented yellowness and chroma values lower in TM0 and TM25 groups than in TM50 group; whilst this last showed the lowest value for hue. Fillets proximate composition was not affected by the diet, unlike the fatty acids profile. Σn3FA, especially EPA, was higher in fish fed TM0 diet. On the contrary Σn6, especially linoleic acid, significantly increased with TM inclusion in the diets. The Σn3/Σn6 FA ratio was linearly (TM0>TM25>TM50) reduced by TM inclusion in the diet and TM50 specimens had the highest (*i.e.* the worst) values for Atherogenicity and Thrombogenicity Indexes. Since no detrimental effects on growth performance were found, the use of *Tenebrio molitor* meal as alternative protein source in blackspot sea bream diet seems to be encouraging, but the effects on fillet quality should be considered.

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

1.Introduction

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

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

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

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

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

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

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

2.2 Fish feeding trial

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

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

At the end of the trial, a subsample of 24 specimens (n. 8 fish per diet from tanks containing large individuals) were randomly sacrificed trough an overdose of anaesthetic (Tricaine methanesulfonate-MS222, Sigma-Aldrich, Milano, Italy; 250 mg L^{-1}). After the slaughtering, sampled fish were transported, in dry ice to the Laboratories of the Department of Agri-Food Production and Environmental Sciences (DISPAA), University of Florence (Firenze, Italy) and immediately stored at – 80 °C until analyses. Before the analyses, all whole fish were defrozen, subjected to morphometric measurements and then filleted; the fillets obtained were analysed for physical and chemical characteristics.

2.4 Morphometric and product properties

After measurements of the individual body weight, each fish (n= 24) underwent the following measurements by an orthometric meter: total length and intestinal length. Then, liver, visceral fat and viscera (digestive system, liver and visceral fat) were separated and weighed. The eviscerated fish were also weighed and subsequently filleted; the fillets (right and left) and the skin from right fillet were subsequently weighed. Afterwards, from each residual of filleting process head, fins and frame were removed and separately weighed. These data were utilized to calculate the dressed yield (DY), with skin fillet yield and without skin fillet yield (FY); head, fins, frame and total waste incidences; condition factor (CF) and relative intestinal length (RIL) as follows:

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

- without skin FY (%) = [(right fillet weight (g) + left fillet weight (g)) – (right skin weight (g) × 2) / body weight (g)] × 100;
- Head (%) = [head weight (g) / body weight (g)] × 100;
- Fins (%) = [fins weight (g) / body weight (g)] × 100;
- Frame (%) = [frame weight (g) / body weight (g)] × 100;
- Total wastes (%) = [frame + fins + head + viscera weight (g) / body weight (g)] × 100.
- CF = [body weight (g) / total length^3 (cm)] × 100;
- RIL = intestinal length (cm) / fish total length (cm);

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

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

2.5 Organoleptic characteristics

Physical analyses

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

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

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

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

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

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

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

- AI = [C12:0 + (4 × C14:0) + C16:0] / (Σn3 PUFA + Σn6 PUFA + ΣMUFA)
- TI = (C14:0 + C16:0 + C18:0) / [(0.5 × ΣMUFA) + (0.5 × Σn6 PUFA) + (3 × Σn3 PUFA) + (Σn3 PUFA / Σn6 PUFA)]
- h/H = (C18:1n9 + C18:2n6 + C20:4n6 + C18:3n3 + C20:5n3 + C22:5n3 + C22:6n3) / (C14:0 + C16:0).

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

2.6 Statistical analysis

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

$Yij = m + Pi + eij$

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

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

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

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

3. Results

3.1 Growth performance

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

3.2 *Morphometric and orthometric properties*

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

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

3.3 Physical traits of fillets

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

In the Table 7, the colour parameter values related to the skin (dorsal and ventral locations) and to the fillet (epaxial and hypaxial locations) of blackspot sea bream fed different diets are presented. Concerning skin dorsal location, no differences in colour values were found for fish fed different diets whilst, on the contrary, the diets utilized affected the colour of the ventral location. In details, TM50 group fish had the lowest L* and Hue compared to TM0 and TM25 groups, that showed similar characteristics. On the other hand, the redness (a*) was significantly higher ($p < 0.001$) in the fish fed the diet including the highest level of insect meal (TM50) if compared to TM0 and TM25 groups. For the colour parameters, a significant effect of fish body weight limitedly to lightness, redness and Hue was found. In the case of fillet muscle, yellowness (b*), colour saturation (Chroma) and Hue were affected by different diets at level of both epaxial and hypaxial locations where significantly lower values for b* and Chroma and the highest values for Hue were registered for fish of control group (TM0). The colour characteristics of fillets from fish fed diets including TM did not present differences, on the contrary to what observed for skin colour at the ventral location.

3.4 Chemical composition of fillets

The results of proximate composition of blackspot sea bream fillet muscle are presented in Table 8. The dietary treatment did not affect moisture, crude protein, ether extract, total lipids and ash contents of fillets. On the other hand, the fatty acid profile of the lipid fraction of fillet muscle was substantially affected by the dietary treatment, as shown in Table 9. Concerning FA groups, the inclusion of *T. molitor* larvae meal in the diet led to an expected increase in PUFAn6 percentage (from TM0 to TM50 groups) and to a decrease in PUFAn3 incidence, that was lower ($p < 0.01$) in fish of TM25 and TM50 groups compared to TM0 group. Linoleic acid (18:2n6) incidence progressively increased ($p < 0.001$) from TM0 to TM50 groups, whilst EPA (20:5n3) percentage slightly decreased ($p < 0.05$) in muscle when fish were fed diet where FM was partially replaced with TM at 50%. Despite no statistical differences were observed, DHA (22:6n3) content progressively decreased as the levels of TM inclusion in diet increased. The TM0 and TM25 samples showed higher ($p <0.05$) 18:3n3 incidence than TM50 group. Total SFA and MUFA percentages were not influenced by the diet but, among saturated fatty acids, 16:0 (palmitic acid) content was the highest ($p < 0.05$) in TM50 samples and no differences were found between the other two groups of fish. Among MUFA, the fatty acid 18:1n7 presented the highest ($p < 0.01$) percentage in TM0 and TM25, 20:1n9 and 22:1n11 were in the highest ($p < 0.01$) percentage in TM0 group while no differences were observed between TM25 and TM50 groups.

In Table 9, the quality indexes related to the fatty acid relationships are also reported. As expected in relation to the fatty acid profile previously described, the values of atherogenicity and thrombogenicity indexes were the highest ($p < 0.05$) in TM50 specimens while no differences were observed between the fish of TM0 and TM25 groups. Concerning Σn3/Σn6 ratio, fish fed the control diet (TM0) had higher ($p < 0.001$) value and this progressively decreased in the fish fed the diets substituting FM with the alternative protein source. No effect of the body weight was observed on fatty acids profile and nutritional indexes.

4. Discussion

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

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

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

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

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

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

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

Moreover, riboflavin (vitamin B2), yellow-coloured pigments, is present in most edible insect and ranged from 0.11 to 8.9 mg per 100 mg (8.1 mg in mealworm) as reported by Finke et al. (2002). The presence of this pigment seemed to have increased the yellowness character in flesh of fish fed 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, Sealeyet 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 Σn6 and Σn3
503 fatty acids, respectively, when *T.molitor* larvae meal was added in diets, a significant reduction of
504 the Σn3/Σn6 FA ratio was found, thus adversely affecting the nutritional/functional value of the
505 fillets. IndeedΣn3/Σn6 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 Σn3/Σn6 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 anincrease 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

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

5. Conclusion

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

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