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Tenebrio molitor meal in diets for European sea bass (*Dicentrarchus labrax* L.) juveniles: Growth performance, whole body composition and *in vivo* apparent digestibility

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ABSTRACT

Two trials were carried out on European sea bass (*Dicentrarchus labrax* L.) juveniles to evaluate the effects of dietary inclusion of a full-fat *Tenebrio molitor* (TM) larvae meal. A first growth trail was performed on 450 European sea bass using three isonitrogenous and isolipidic experimental diets (3 tanks/diet, 50 fish/tank) formulated to contain increasing levels of TM meal inclusion and precisely: 0 (TM0), 25 (TM25) and 50% (TM50) as fed basis. The performances, proximate body composition and fatty acid (FA) profile of whole fish fed the experimental diets were evaluated. A digestibility trial was then conducted on 180 fish to evaluate the *in vivo* apparent digestibility coefficient (ADC) of diets having 25% of TM inclusion in absence (TMD) or presence of exogenous enzymes (Carbohydrases, TM-Carb; Proteases, TM-Prot) compared to a fish meal based control diet (CD). The growth trial results showed that the highest inclusion level (TM50) led to a worsening of final body weight, weight gain, specific growth rate, and feeding rate if compared to the control diet (TM0). Regarding the whole body composition, crude protein and ether extract were not significantly influenced by the use of TM, while changes were observed in the FA profile. In particular, C18:2 n6 increased (+91% and +173% in TM25 and TM50, respectively *vs* TM0) with the inclusion of TM while sharp decreases of C20:5 n3 (−30% and −58% in TM25 and TM50, respectively *vs* TM0) and C22:6 n3 (−35% and −67% respectively *vs* TM0) were highlighted. Consequently, the $\sum$ n3/$\sum$ n6 FA ratio showed a significant decrease (−63%

Abbreviations: AIA, acid insoluble ash; ADC, apparent digestibility coefficient; ADF, acid detergent fibre; CP, crude protein; DM, dry matter; EE, ether extract; FA, fatty acids; FAME, fatty acid methyl esters; FBW, final body weight; FCR, feed conversion ratio; FM, fish meal; FR, feeding rate; HI, Hermetia illucens; HSI, hepatosomatic index; IBW, initial body weight; PER, protein efficiency ratio; TM, *Tenebrio molitor*; VSI, viscerosomatic index; WG, weight gain.

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and −84% in TM25 and TM50, respectively *vs* TM0). As far as digestibility trial is concerned, the crude protein ADC of the fish fed TMD was significantly higher than that of the fish fed CD (92.31 *vs* 89.97, respectively). The supplementation of digestive enzymes did not improve the protein and ADF digestibility.

1. Introduction

In the coming years there will be a worldwide protein shortage due to the increasing demand by the ever growing world population. In particular, European Union (EU) already suffers from important protein deficiency and imports over 70% of consumed proteins (EU report 2010/2111(INI)).

The nutritive requirements of fish, in particular of carnivorous fish, are quite high in terms of quality and quantity of protein in the diet. For this reason, fish meal (FM) with its excellent protein level and balanced amino acid profile, has been traditionally considered the best useful protein source in feed formulation. However, FM is a limited supply product (Oliva-Teles et al., 2015) and the rapid development of aquaculture has given rise to a lively debate concerning the sustainability of its production (Hardy, 2010). Moreover, according to the growing global fish consumption, the demand for aqua feed is expected to strongly increase and, as marine resources will not be sufficient to satisfy it, alternative protein sources have been widely investigated in the last 20 years. A lot of research has been conducted substituting FM with plant products (Palmegiano et al., 2005; Palmegiano et al., 2006; Oliva-Teles et al., 2015; Gai et al., 2016) and, even if a 100% substitution was achieved in some cases, plant proteins still present disadvantages such as anti-nutritional factors, high level of fibre and non-starch polysaccharides, inadequate fatty acid (FA) and amino acid profile (Gai et al., 2012), and low palatability (Gatlin et al., 2007). Moreover, some plant proteins compromise fish intestinal enterocyte integrity (Daprà et al., 2009; Merrenfield et al., 2009; Ferrara et al., 2015).

Recently, the interest of researchers turned to insect meals (Barroso et al., 2014; Henry et al., 2015; Lock et al., 2015) as they have interesting nutritional values for both fish and terrestrial animals (Makkar et al., 2014). Insects are often part of the natural animal diets, and they are claimed to be a highly sustainable source of nutrients (van Huis, 2013). Although insects grow rapidly and all stages of reproduction are controlled, the present cost price of insect meals is still not competitive if compared with other protein sources (Koeleman, 2014). Nevertheless, an increase in demand inevitably will lead to increase of production scale and thereby a reduction of insect meal prices in future (Mancuso et al., 2016).

The current European legislation does not allow the use of insect meals (as well as the use of nearly all other Processed Animal Proteins – see the Catalogue of Feed Materials – EC 68/2013) in animal feeds in response to the Bovine Spongiform Encephalopathy outbreak (EC 999/2001). Nevertheless, given the strong interest shown for insect meals by stakeholders (insects producers, feed producers and farmers), an amendment of the EC regulations to authorise their use in diets for monogastrics has been highly requested. However, research is needed as several issues on biological and chemical hazards associated with insects meals used as feed material still have to be clarified, as underlined by a recent European Food Safety Authority opinion (EFSA Scientific Committee, 2015).

Larvae from many insect species can be used for insect meal production. Among such species there is the yellow mealworm (*Tenebrio molitor* L., TM), a worldwide distributed coleopter belonging to the Tenebrionidae family (Makkar et al., 2014). Its larvae are very promising for aquaculture because they are rich in protein with an adequate amino acid profile and easy to breed and feed (De Marco et al., 2015). Currently, larvae are sold alive, dried or in powder form for fishing (bait) or pet feeding (van Huis, 2013). Recent studies reported the use of TM in poultry (Bovera et al., 2015; De Marco et al., 2015; Biasato et al., 2016; Bovera et al., 2016) and fish feed. In particular, in rainbow trout (*Oncorhynchus mykiss* Walbaum), Belforti et al. (2015) showed that a full-fat TM larvae meal could be used up to 50% of inclusion in diets (as fed basis), without negative effects on growth performances even if a reduced feed intake was observed. Similarly, Ng et al. (2001) showed good growth performances when TM meal was included up to 35% in African catfish (*Clarias gariepinus* Burchell) diets. On the contrary, Roncarati et al. (2015) showed reduced weight gain in common catfish (*Ameiurus melas* Rafinesque) fingerlings fed a diet containing TM larvae meal. So far, limited knowledge is available on TM digestibility. Some trials have been conducted *in vitro* (Marono et al., 2015; Sánchez-Muros et al., 2015; Yi et al., 2016) but to our knowledge, only one trial was performed *in vivo*, and showed a decrease of protein apparent digestibility coefficient (ADC) when TM was included at a 50% level in rainbow trout diets (Belforti et al., 2015).

The use of insect meal has not been investigated so far in the European sea bass (*Dicentrarchus labrax* L.), a major species cultured in Mediterranean region. For this, two separate trials were performed to evaluate the effects of dietary inclusion of a full-fat TM larvae meal on (i) growth performances and whole body composition (proximate constituents and FA profile) of European sea bass juveniles fed diets with increasing levels of TM, and (ii) *in vivo* apparent digestibility coefficients of diets having 25% of TM inclusion in absence or presence of exogenous enzymes.

Table 1
Growth trial: ingredients and proximate composition of TM larvae meal and experimental diets.

	TM larvae meal	TM0	TM25	TM50
Ingredients (g kg^{-1})				
Fish meal (Chile, super prime)	–	700.0	450.0	200.0
TM larvae meal[a]	–	0	250.0	500.0
Wheat gluten meal	–	50.00	75.00	150.0
Corn gluten meal	–	0	28.00	0
Wheat meal	–	92.00	90.00	80.00
Wheat bran	–	55.00	40.00	25.00
Starch gelatinized, D500	–	0	0	12.00
Fish oil	–	90.00	54.00	20.00
L-*methionine*	–	6.00	6.00	6.00
L-*lysine*	–	3.00	3.00	3.00
Choline	–	1.50	1.50	1.50
Premix[b]	–	2.50	2.50	2.50
Proximate composition[c]				
DM (g 100 g^{-1})	93.9	92.0	92.3	91.7
CP (g 100 g^{-1}, as fed)	51.9	54.8	54.5	54.6
EE (g 100 g^{-1}, as fed)	23.6	15.2	15.8	15.7
Ash (g 100 g^{-1}, as fed)	4.7	11.5	8.5	5.7
Gross energy (MJ kg^{-1}, as fed)	24.40	21.29	21.87	22.62

Abbreviations: TM, *Tenebrio molitor*; DM, dry matter; CP, crude protein; EE, ether extract.

[a] *Tenebrio molitor* larvae meal purchased from Gaobeidian Shannong Biology CO. LTD (Shannong, China).

[b] Premix (kg^{-1}): Vitamin A 4,000,000 (IU), Vitamin D3 800,000 (IU), Vitamin E 100,000 (mg), Vitamin K3 4,000 (mg), Vitamin B1 8,000 (mg), Vitamin B2 8,000 (mg), Nicotinic acid 60,000 (mg), Pantothenic acid 24,000 (mg), Vitamin B6 8,000 (mg), Vitamin B12 80 (mg), Folic acid 1,600 (mg), Biotin 320 (mg), Vitamin C (Stay C35% MONO) 80,000 (mg), Anticoagulant 71,000 (mg), Inositol 60,000 (mg), MnO 14,000 (mg), $Ca^*(IO_3)_2$ 1,600 (mg), ZnO 24,000 (mg), $FeCO_3$ 18,000 (mg), $CuSO_4 \cdot 5H_2O$ 2,800 (mg), Na_2SeO_3 60 (mg), BHA (E320) 160 (mg), Ethoxyquin (E324) 160 (mg).

[c] Values are reported as mean of duplicate analyses.

2. Materials and methods

A full-fat TM larvae meal purchased from the Gaobeidian Shannong Biology CO. LTD (Shannong, China) was used in the two trials, which were both conducted at the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) of the Hellenic Center for Marine Research (Crete, Greece). The experimental protocol was designed according to the guidelines of the current European Directive (2010/63/EU) on the protection of animals used for scientific purposes.

2.1. Growth trial

2.1.1. Fish and experimental conditions

European sea bass juveniles were obtained from the IMBBC hatchery. Four hundred and fifty fish were lightly anesthetised (2-phenoxyethanol, 150 ppm) and individually weighed (5.22 ± 0.822 g) at the beginning of the trial. The fish were randomly allotted to 9 circular tanks of 500 l supplied by open-circulation borehole aerated sea water (renewal 200% per hour). The trial was conducted from May to July under constant temperature (19.5 ± 0.5 °C), salinity (36‰), and dissolved oxygen (DO 6 ppm), under natural photoperiod.

2.1.2. Fish diets

Three experimental diets were formulated to be isonitrogenous (about 54.5 g 100 g^{-1} crude protein – CP, on as fed basis), isolipidic (about 15.5 g 100 g^{-1} ether extract – EE, on as fed basis) and isoenergetic (about 21.5 MJ kg^{-1} dry matter – DM). They were obtained including, as fed basis, graded levels of TM larvae meal [TM0 (control diet, with no TM inclusion), TM25 (25% TM inclusion) and TM50 (50% TM inclusion)]. The experimental feeds were prepared at the IMBBC laboratory. All ingredients were thoroughly mixed; water was then blended into the mixture to attain a consistency appropriate for pelleting using a 1 mm die meat grinder. After pelleting, the diets were dried at 40 °C and stored in plastic bags at −20 °C until used. The ingredients and proximate composition of the experimental diets are reported in Table 1.

Each diet was assigned to three groups of 50 fish and distributed by hand to apparent satiation, twice a day, for 7 days per week. In detail, as soon as the fish stopped eating, the pellet distribution was interrupted and any not-ingested pellet was recovered, dried and weighed. The exact quantity of feed distributed within each tank was recorded. Mortality was checked every day and the trial lasted 70 days after a 2-week period of acclimation to the tanks and diets.

2.1.3. Growth performance

At the end of the trial, fish were starved for 1 day, lightly anesthetised (2-phenoxyethanol, 150 ppm) and individually weighed. The following growth performance indexes were calculated:

- Mortality (%) = (number of dead fish/number of fish at start) × 100

Table 2
Growth trial: fatty acid profile (g 100 g^{-1} DM) of TM larvae meal and experimental diets.

	TM larvae meal	TM0	TM25	TM50
C12:0	0.04	0.01	0.02	0.03
C14:0	0.51	0.60	0.49	0.42
C14:1 *t*	–	0.01	0.01	0.01
C14:1 *c*9	0.03	0.06	0.05	0.04
C15:0	–	<0.01	<0.01	< 0.01
C16:0	3.43	1.94	2.16	2.63
C16:1 *t*	–	0.01	0.01	0.01
C16:1 *c*9	0.40	0.56	0.42	0.31
C17:0	–	0.06	0.05	0.04
C17:1 *c*9	–	0.03	0.04	0.05
C18:0	0.64	0.37	0.39	0.48
C18:1 *t*	–	0.03	0.02	0.02
C18:1 *c*9	7.58	2.87	3.56	4.62
C18:1 *c*11	–	0.38	0.27	0.18
C18:2 n6	6.97	1.20	2.21	3.57
C18:3 n3	0.27	0.36	0.28	0.22
C18:3 n6	–	0.02	0.01	0.01
C20:0	0.31	0.04	0.03	0.03
C20:1 *c*9	–	0.06	0.04	0.02
C20:1 *c*11	–	0.59	0.37	0.18
C20:2 n6	–	0.26	0.15	0.07
C20:3 n3	–	0.08	0.03	0.02
C20:3 n6	–	0.02	0.01	0.01
C20:4 n6	–	0.06	0.04	0.01
C20:5 n3	–	0.76	0.42	0.16
C22:1 n9	–	0.73	0.42	0.18
C22:5 n3	–	0.13	0.08	0.02
C22:6 n3	–	0.83	0.46	0.19
$\sum$ SFA	4.98	3.04	3.14	3.64
$\sum\sum$ MUFA	8.01	5.33	5.19	5.61
$\sum\sum$ PUFA	7.24	3.72	3.69	4.29
$\sum\sum$ n3	0.27	2.16	1.27	0.62
$\sum\sum$ n6	6.97	1.56	2.42	3.66
$\sum$ n3/$\sum$ n6	0.04	1.38	0.52	0.17
TFA	20.23	12.10	12.02	13.55

Abbreviations: TM, *Tenebrio molitor*; *t*, *trans*; *c*, *cis*; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids.
All values are reported as mean of duplicate analyses.

- Dry matter intake (DMI, g) = feed distributed (g, DM) – feed recovered (g, DM)
- Weight gain (WG, g) = [FBW (final body weight, g) – IBW (initial body weight, g)]
- Specific growth rate (SGR, % day^{-1}) = [(lnFBW – lnIBW)/number of feeding days] × 100
- Feed conversion ratio (FCR) = [total feed supplied (g, dry basis)/WG (g)]
- Protein efficiency ratio (PER) = [WG (g)/total protein fed (g, dry basis)]
- Feeding rate (FR, %) = [(total feed supplied (g, dry basis) * 100/number of feeding days)]/[$e^{(lnFBW+lnIBW)*0.5}$].

2.1.4. Chemical analyses of TM larvae meal, experimental diets and fish

Feeds were ground using a cutting mill (MLI 204–Bühler AG, Uzwil, Switzerland) and analysed for DM (#934.01), CP (#984.13) and ash (#942.05) contents according to AOAC International (2000); EE (#2003.05) was analysed according to AOAC International (2003). The gross energy content was determined using an adiabatic calorimetric bomb (IKA C7000, Staufen, Germany). The proximate composition and energy level of the TM larvae meal and of the experimental diets are shown in Table 1.

The FA composition of TM larvae meal and of the experimental diets was assessed using the method described by Schmid et al. (2009). Fatty acid methyl esters (FAME) were separated, identified and quantified on the basis of the chromatographic conditions reported by Renna et al. (2014). Tridecanoic acid (C13:0) was used as internal standard. The results were expressed in absolute values as g 100 g^{-1} DM (Table 2). All feed analyses were performed in duplicate.

At the end of the trial, 10 fish per tank were killed by over anaesthesia (2-phenoxyethanol, overdose). Fish were gutted, finely ground (Retsch ZM200, Haan, Germany), and freeze-dried (Telstar Cryodos, Terrassa, Spain). Dry matter, CP, EE, and ash contents of fish whole body (pooled per tank) were determined according to the same procedures used for feed analyses (AOAC International 2000, 2003). The freeze-dried and ground samples of fish whole body were also used to assess their FA composition, as reported by Belforti et al. (2015). Fatty acid methyl esters were separated using the same analytical instruments and temperature programme previously described for the FA analysis of feeds. Peaks were identified by injecting

Table 3
Digestibility trial: ingredients and proximate composition of the experimental diets.

	CD	TMD	TM-Carb	TM-Prot
Ingredients (g kg^{-1})				
Fish meal (Chile, super prime)	693	445.5	445.5	445.5
TM larvae meal[a]	0	247.5	247.5	247.5
Wheat gluten meal	49.50	74.25	74.25	74.25
Corn gluten meal	0	27.72	27.72	27.72
Wheat meal	91.08	89.10	89.10	89.10
Wheat bran	54.45	39.60	39.50	39.40
Fish oil	89.10	53.46	53.46	53.46
Celite	10	10	10	10
Ronozyme ProAct[b]	0	0	0	0.20
Ronozyme Multigrain[b]	0	0	0.10	0
L-*methionine*	5.94	5.94	5.94	5.94
L-*lysine*	2.97	2.97	2.97	2.97
Choline	1.485	1.485	1.485	1.485
Premix[c]	2.475	2.475	2.475	2.475
Proximate composition[d]				
DM (g 100 g^{-1})	93.7	92.9	93.2	92.9
CP (g 100 g^{-1}, as fed)	53.1	53.2	53.1	52.7
EE (g 100 g^{-1}, as fed)	16.4	14.8	14.9	14.4
ADF (g 100 g^{-1}, as fed)	1.0	2.4	2.6	2.2
Ash (g 100 g^{-1}, as fed)	9.7	9.5	10.4	9.7
Gross energy (MJ kg^{-1} as fed)[e]	21.74	21.41	21.28	21.26

Abbreviations: CD, control diet; TMD, diet with 25% of *Tenebrio molitor*; TM-Carb, TMD with Ronozyme MultiGrain – 0.01% of inclusion; TM-Prot, TMD with Ronozyme ProAct – 0.02% of inclusion; DM, dry matter; CP, crude protein; EE, ether extract; ADF, acid detergent fibre.

[a] *Tenebrio molitor* larvae meal purchased from Gaobeidian Shannong Biology CO. LTD (Shannong, China).

[b] Ronozyme ProAct (proteases) and Ronozyme Multigrain (carbohydrases: xylanase and β-glucanases) were purchased from DSM Animal Nutrition & Health, Heerlen, The Netherlands.

[c] Premix (kg^{-1}): Vitamin A 4,000,000 (IU), Vitamin D3 800,000 (IU), Vitamin E 100,000 (mg), Vitamin K3 4,000 (mg), Vitamin B1 8,000 (mg), Vitamin B2 8,000 (mg), Nicotinic acid 60,000 (mg), Pantothenic acid 24,000 (mg), Vitamin B6 8,000 (mg), Vitamin B12 80 (mg), Folic acid 1,600 (mg), Biotin 320 (mg), Vitamin C (Stay C35% MONO) 80,000 (mg), Anticoagulant 71,000 (mg), Inositol 60,000 (mg), MnO 14,000 (mg), $Ca^{*}(IO_3)_2$ 1,600 (mg), ZnO 24,000 (mg), $FeCO_3$ 18,000 (mg), $CuSO_4 \cdot 5H_2O$ 2,800 (mg), Na_2SeO_3 60 (mg), BHA (E320) 160 (mg), Ethoxyquin (E324) 160 (mg).

[d] Values are reported as mean of duplicate analyses.

[e] Determined using an adiabatic calorimetric bomb (IKA C7000, Staufen, Germany).

pure FAME standards as detailed by Renna et al. (2012). The results were expressed as g kg^{-1} whole body. All fish analyses were performed in triplicate.

2.2. Digestibility trial

2.2.1. Fish and experimental conditions

One hundred and eighty European sea bass of 65.3 ± 5.70 g initial weight obtained from the IMBBC hatchery were distributed in 12 circular fiberglass tanks (3 tanks per treatment; 15 fish per tank) of 270 l equipped with a settling column. The water and environmental conditions were the same as described for the growth trial. Four experimental isonitrogenous and isoenergetic diets were formulated based on the control diet containing about 70% FM (CD) or with about 25% of TM meal replacing 36% of FM without enzymes (TMD) or in combination with exogenous digestive enzymes, carbohydrases (xylanase and β-glucanases; Ronozyme MultiGrain – 0.01% of inclusion) (TM-Carb) or proteases (Ronozyme ProAct – 0.02% of inclusion) (TM-Prot) obtained from DSM Animal Nutrition & Health (Heerlen, The Netherlands). The ingredients and proximate composition of diets are reported in Table 3. Diets were prepared at the IMBBC laboratory following the same procedure described for the growth trial. Fish were fed the experimental diets *ad libitum* 2 times a day for 6 weeks. Faeces were collected daily for the last 3 weeks of the experiment, centrifuged and stored at −20 °C. The apparent digestibility coefficients were measured using the indirect acid-insoluble ash (AIA) method; 1% celite® (Fluka, St. Gallen, Switzerland) was added to the diets as an inert marker.

At the end of the feeding trial, 27 fish per treatment (9 fish per replicate) were randomly chosen, weighed and killed by over anaesthesia (2-phenoxyethanol, overdose) and dissected. Liver and gut were weighed to determine Hepatosomatic (HSI) and Viscerosomatic (VSI) indexes as described in Belforti et al. (2015).

2.2.2. Chemical analyses of experimental diets and faeces

Feeds and faeces were analysed for DM (#934.01), ash (#984.13), CP (#984.11), and acid detergent fibre (ADF; #973.18) according to AOAC International (2000); EE (#2003.05) was analysed according to AOAC International (2003). The AIA contents of feeds and faeces were determined according to Vogtmann et al. (1975). The gross energy content was determined using an adiabatic calorimetric bomb (IKA C7000, Staufen, Germany).

Table 4
Growth trial: mortality and growth performances of European sea bass juveniles fed the experimental diets (n = 3).

	TM0	TM25	TM50	SEM	*P*-value
Mortality (%)	4.67	8.67	6.67	1.054	0.343
IBW (g)	5.29	5.23	5.15	0.040	0.370
FBW (g)	22.08 a	20.68 ab	17.35 b	0.821	0.020
WG (g)	16.80 a	15.43 ab	12.22 b	0.792	0.018
DMI (g DM)	726.77 a	653.15 ab	560.90 b	26.773	0.026
SGR (% day^{-1})	1.99 a	1.89 ab	1.66 b	0.058	0.018
FCR	0.90	0.91	0.99	0.020	0.114
PER	2.20	2.19	2.01	0.046	0.163
FR (% day^{-1})	1.95 a	1.84 ab	1.74 b	0.033	0.004

Abbreviations: IBW, initial body weight; FBW, final body weight; WG, weight gain; DMI, dry matter intake; SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio; FR, feeding rate.
Different letters within a row indicate significant differences ($P \leq 0.05$).

The apparent digestibility coefficients of dry matter (ADC DM), crude protein (ADC CP), ether extract (ADC EE) and acid detergent fibre (ADC ADF) were calculated following Palmegiano et al. (2006).

2.3. Statistical analyses

Data were analysed using IBM SPSS Statistics 20.0. As size can significantly influence the chemical composition of fish and FBW in our trial showed significant differences among dietary treatments, data regarding proximate constituents and fatty acids of fish whole body (growth trial) were firstly analysed using an analysis of covariance (GLM Univariate procedure). The statistical model included dietary treatment as main effect and FBW as a covariate. The assumption of homogeneity of regression slopes was tested using a customized model that included dietary treatment, covariate, and their interaction. The analysis of covariance allowed us to control the effect of the covariate, which resulted in no significance for any of the considered parameters.

All data, for both the growth and digestibility trials, were therefore subjected to a one-way analysis of variance. The following models were used:

$$Y_{ij} = \mu + D_i + \varepsilon_{ij}(\text{growthtrial}) \quad \text{(i)}$$

where Y_{ij} = observation; μ = overall mean; D_i = effect of diet (TM0, TM25, TM50); ε_{ij} = residual error

$$Y_{ij} = \mu + D_i + \varepsilon_{ij}(\text{digestibilitytrial}) \quad \text{(ii)}$$

where Y_{ij} = observation; μ = overall mean; D_i = effect of diet (CD, TMD, TM-Carb, TM-Prot); ε_{ij} = residual error.

The assumption of equal variances was assessed by Levene's homogeneity of variance test. If such an assumption did not hold, the Brown-Forsythe statistic was performed to test for the equality of group means instead of the F one. Pairwise multiple comparisons were performed to test the difference between each pair of means (Tukey's test and Tamhane's T2 in cases of equal variances assumed or not assumed, respectively).

Linear regression was used to identify the relationships existing between the dietary TM meal inclusion and fish performances or some specific nutritional parameters of fish whole body composition (i.e., Σ n3 fatty acids and Σ n3 fatty acids/Σ n6 fatty acids). Significance was declared at $P \leq 0.05$.

3. Results

3.1. Growth trial

3.1.1. Growth performance

The experimental diets were well accepted by the fish and all feeds were consumed without loss. The mortality (%) and performance traits of European sea bass juveniles fed diets containing increasing levels of TM are reported in Table 4.

Fish mortality ranged from 4.67% (TM0) to 8.67% (TM25) and was not significantly different among the treatments ($P > 0.05$). IBW was comparable among the treatments. In 70 days, the fish tripled their body weight. FBW and WG were significantly lower in TM50 (17.35 and 12.22 g, respectively) if compared to TM0 (22.08 and 16.80 g; $P < 0.05$). DMI was significantly lower in fish fed TM50 if compared to TM0. SGR was higher ($P < 0.05$) in the fish fed TM0 diet (1.99% day^{-1}) compared to the highest inclusion level of insect meal (TM50: 1.66% day^{-1}). The fish fed TM50 also highlighted a significantly lower FR than those fed TM0 (1.74 *vs* 1.95% day^{-1}, respectively; $P < 0.01$); intermediate values were observed for TM25. No significant differences were observed for FCR and PER.

Table 5
Growth trial: whole body proximate (g 100 g^{-1} ww, unless otherwise stated) and fatty acid (g kg^{-1} ww) compositions of European sea bass juveniles fed the experimental diets (n = 3).

	TM0	TM25	TM50	SEM	*P*-value
Proximate composition					
DM (g 100 g^{-1})	34.67	34.20	33.37	0.340	0.324
CP	16.57	16.67	16.93	0.131	0.563
EE	13.24	12.51	12.18	0.233	0.180
Ash	3.98 b	3.98 b	4.37 a	0.076	0.020
Fatty acid composition					
C12:0	0.04 c	0.06 b	0.07 a	0.005	0.000
C14:0	3.97 a	3.29 b	2.95 b	0.157	0.001
C14:1 *t*	0.06 a	0.04 ab	0.03 b	0.005	0.011
C14:1 *c*9	0.46 a	0.38 b	0.35 b	0.017	0.002
C15:0	0.008 a	0.007 ab	0.004 b	0.001	0.036
C16:0	19.55	20.71	21.04	0.523	0.540
C16:1 *t*	0.18 a	0.13 b	0.07 c	0.002	0.000
C16:1 *c*9	5.14 a	4.32 b	3.80 b	0.213	0.005
C17:0	0.99 a	0.76 ab	0.72 b	0.053	0.036
C17:1 *c*9	0.32 b	0.38 a	0.43 a	0.017	0.004
C18:0	3.30 b	4.06 b	5.02 a	0.269	0.003
C18:1 *t*	0.19	0.16	0.15	0.012	0.403
C18:1 *c*9	30.65 b	37.55 a	41.53 a	1.758	0.006
C18:1 *c*11	3.46 a	2.83 b	2.21 c	0.189	0.001
C18:2 n6	7.94 c	15.14 b	21.66 a	1.989	0.000
C18:3 n6	0.11 c	0.23 b	0.44 a	0.049	0.000
C18:3 n3	2.23 a	1.89 ab	1.58 b	0.115	0.035
C20:0	0.96	0.94	0.89	0.017	0.160
C20:1 *c*9	0.59 a	0.46 b	0.31 c	0.041	0.000
C20:1 *c*11	4.63 a	3.18 b	2.04 c	0.381	0.000
C20:2 n6	0.51	0.60	0.60	0.030	0.414
C20:3 n6	0.08	0.08	0.06	0.007	0.609
C20:3 n3	0.44 a	0.32 b	0.17 c	0.041	0.001
C20:4 n6	0.24	0.28	0.20	0.034	0.692
C20:5 n3	3.95 a	2.75 b	1.64 c	0.350	0.001
C22:0	0.07	0.06	0.04	0.006	0.247
C22:1 n9	3.36 a	2.16 b	0.98 c	0.358	0.000
C22:5 n3	0.91 a	0.57 b	0.31 c	0.088	0.000
C22:6 n3	6.20 a	4.01 b	2.03 c	0.608	0.000
$\sum$ SFA	28.89	29.89	30.73	0.668	0.599
$\sum$ MUFA	49.03	51.59	51.90	1.074	0.554
$\sum$ PUFA	22.61 b	25.85 ab	28.69 a	0.995	0.011
$\sum$ PUFA/$\sum$ SFA	0.78 b	0.87 ab	0.94 a	0.054	0.028
$\sum$ n3	13.74 a	9.53 b	5.73 c	1.183	0.000
$\sum$ n6	8.88 c	16.33 b	22.95 a	2.046	0.000
$\sum$ n3/$\sum$ n6	1.55 a	0.58 b	0.25 c	0.500	0.000
TFA	100.54	107.34	111.31	2.456	0.205

Abbreviations: ww, wet weight; SEM, standard error of the mean; DM, dry matter; CP, crude protein; EE, ether extract; *t*, *trans*; *c*, *cis*; FA, fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA, total fatty acids.
Different letters within a row indicate significant differences ($P \leq 0.05$).

3.1.2. Fish whole body composition

The proximate whole body composition of the fish at the end of the trial is shown in Table 5. No significant differences were observed among treatments with the exception of ash that was statistically different between TM50 (4.37 g 100 g^{-1} wet weight) and the other treatments (3.98 g 100 g^{-1} ww for both TM0 and TM25).

Oleic (C18:1 *c*9), linoleic (C18:2 n6) and palmitic (C16:0) acids were the most represented FA in TM meal (7.58, 6.97 and 3.43 g 100 g^{-1} DM, respectively) (Table 2). These FA were also the most represented in the experimental diets and their concentration increased with the increase of TM meal inclusion. Long chain polyunsaturated fatty acids (PUFA) such as eicosapentaenoic (EPA, C20:5 n3), docosapentaenoic (DPA, C22:5 n3) and docosahexaenoic (DHA, C22:6 n3) acids were not detected in TM meal. As a consequence, and also due to the contemporary decrease in the fish oil content of the diets, a decrease in EPA, DPA and DHA was observed from TM0 to TM50. Similarly, the $\sum$ n3/$\sum$ n6 FA ratio decreased in the diets following the increase of TM inclusion.

The FA composition of European sea bass whole body was significantly modified by the diet (Table 5). The concentration of C18:1 *c*9 was higher in TM25 and TM50 than in TM0 (37.55, 41.53 and 30.65 g kg^{-1} ww, respectively). C18:2 n6 increased by 90% and 173% respectively for TM25 and TM50 compared to TM0. The n3 PUFA were also altered by the dietary inclusion of insect meal. In particular, if compared to TM0, a sharp decrease of EPA (−30% and −58% in TM25 and TM50, respectively), DPA (−37% and −66%, respectively) and DHA (−35% and −67%, respectively) was highlighted. In the fish fed increasing levels

Table 6
Predicting fish performance parameters and nutritional parameters of fish whole body composition from TM meal inclusion in the diet (SE and P-values associated with slope and y-intercept estimates of linear regression; n = 9).

			slope		y-intercept	
	equation	R^2	SE	*P*-value	SE	*P*-value
WG	y = 19.398 – 2.291x	0.698	0.570	0.005	1.232	0.000
SGR	y = 2.213 – 0.151x	0.721	0.035	0.004	0.076	0.000
FCR	y = 0.846 + 0.045x	0.411	0.020	0.063	0.044	0.000
PER	y = 2.319 – 0.093x	0.343	0.049	0.097	0.105	0.000
FR	y = 2.052 – 0.104x	0.834	0.017	0.001	0.038	0.000
$\sum$ n3	y = 1766.681 – 400.061x	0.952	33.806	0.000	73.029	0.000
$\sum$ n3/Σ n6	y = 2.095 – 0.650x	0.924	0.070	0.000	0.152	0.000

Abbreviations: SE, standard error; WG, weight gain; SGR, specific growth rate; FCR, feed conversion ratio; PER, protein efficiency ratio; FR, feeding rate.

Table 7
Digestibility trial: final body weight, Hepatosomatic and Viscerosomatic indexes of European sea bass fed the experimental diets (n = 3).

	CD	TMD	TM-Carb	TM-Prot	SEM	*P*-value
FBW	99.70	99.29	101.17	102.40	2.571	0.981
HSI	1.25 b	1.52 a	1.38 ab	1.42 ab	0.065	0.043
VSI	7.66	7.77	7.15	7.89	0.213	0.073

Abbreviations: CD, control diet; TMD, diet with 25% of *Tenebrio molitor*; TM-Carb, TMD with Ronozyme MultiGrain – 0.01% of inclusion; TM-Prot, TMD with Ronozyme ProAct – 0.02% of inclusion; FBW, final body weight; HSI, Hepatosomatic index; VSI, Viscerosomatic index.
Different letters within a row indicate significant differences ($P \leq 0.05$).

Table 8
Digestibility trial: digestibility of dry matter, proteins, lipids and ADF by European sea bass fed the experimental diets (n = 3).

	CD	TMD	TM-Carb	TM-Prot	SEM	*P*-value
ADC DM	0.73 b	0.80 a	0.73 b	0.76 b	0.007	0.001
ADC CP	0.90 b	0.92 a	0.90 b	0.91 b	0.003	0.003
ADC EE	0.98	0.97	0.97	0.96	0.003	0.344
ADC ADF	0.41 ab	0.45 a	0.28 bc	0.25 c	0.030	0.010

Abbreviations: CD, control diet; TMD, diet with 25% of *Tenebrio molitor*; TM-Carb, TMD with Ronozyme MultiGrain – 0.01% of inclusion; TM-Prot, TMD with Ronozyme ProAct – 0.02% of inclusion; ADC DM, dry matter apparent digestibility coefficient; ADC CP, crude protein apparent digestibility coefficient; ADC EE, ether extract apparent digestibility coefficient; ADC ADF, acid detergent fibre apparent digestibility coefficient.
Different letters within a row indicate significant differences ($P \leq 0.05$).

of TM, whole body showed a consistent increase and decrease of Σ n6 and Σ n3 FA, respectively. This led to a significant reduction of the Σ n3/Σ n6 FA ratio, which ranged from 1.55 (TM0) to 0.25 (TM50) ($P < 0.001$).

3.1.3. Regression analysis

When the dietary TM inclusion levels were plotted against performance parameters and chosen nutritional values, the equations and coefficients of determinations reported in Table 6 were obtained (SE and P-values associated with slope and y-intercept are also reported in the table). The majority of the coefficients of determination, with the exception of those found for FCR and PER, indicated that the equations fitted the data well and that the TM inclusion level in the diet made a significant contribution to predicting the considered parameters.

Particularly, a significant negative relationship was observed between the inclusion of TM meal and WG ($R^2 = 0.698$; $P < 0.01$), SGR ($R^2 = 0.721$; $P < 0.01$), and FR ($R^2 = 0.834$; $P = 0.001$).

The inclusion of insect meal also had a great influence on the nutritional value of fish whole body and both Σ n3 FA and the Σ n3/Σ n6 FA ratio showed a strong significant negative relationship with the inclusion of TM meal in the diet ($R^2 = 0.952$ and 0.924, respectively; $P < 0.001$).

3.2. Digestibility trial

The final weight of fish (100.6 ± 25.49 g) in the digestibility trial was not affected by the dietary treatment. HSI was significantly higher in the fish fed TMD if compared to the fish fed CD; intermediate values were observed for TM-Carb and TM-Prot. No significant differences were found for VSI (Table 7).

Table 8 shows the digestibility results. Dry matter and protein digestibilities showed significantly higher values for TMD if compared to the other treatments. No significant differences were found for lipid digestibility, while ADF digestibility showed the lowest values in TM-Prot and the highest in TMD.

4. Discussion

4.1. Growth trial

4.1.1. Growth performance

To our knowledge, until now no trials have been performed using TM in European sea bass diets. In the current trial, the linear regression indicated a worsening of performance parameters with the increase of TM dietary inclusion. However, ANOVA results indicated that over the period tested a dietary inclusion up to 25% of full-fat TM larvae meal is feasible without significant negative effects on performance parameters. A higher inclusion level (50%) induced decreased DMI (−23%) and FR (−11%) compared to the control diet, with a consequent 27% decrease in WG. According to Skalli and Robin (2004), the minimum dietary requirement of long-chain (LC) n3 PUFA for adequate growth of *D. labrax* juveniles is 0.7% on a DM basis; lower values worsen fish growth performance without negative effects on feed efficiency or PER. Minimal requirements of LC n3 PUFA were not fulfilled by TM50 (which provided only 0.62 g 100 g^{-1} DM of total n3 FA, including C18:3 n3) and this can at least partly explain the observed significant decrease of WG and SGR in the fish fed the highest TM inclusion level in the current trial.

Recently, Belforti et al. (2015) used the same full-fat TM larvae meal and the same inclusion levels in rainbow trout diets. These authors also reported a significant decrease of voluntary feed intake; no significant difference was reported among treatments for WG, while a significant improvement was observed for FCR, PER and SGR. Belforti et al. (2015) stated that the productive traits were not negatively affected by dietary inclusion of TM and attributed the decrease in FR to the high quantity of fat in the TM meal, as well as to its FA composition. Roncarati et al. (2015) performed a pre-fattening trial on common catfish fingerlings using a FM control diet and a TM diet where insect substituted 50% of FM; these diets were neither isonitrogenous nor isolipidic. Roncarati et al. (2015) concluded that the diet with insect meal was able to sustain growth in catfish fingerlings but the fish fed FM performed better than those fed TM. A decrease in growth performances was also reported by Ng et al. (2001) using TM in African catfish fingerlings when insect meal exceeded the level of 40% of FM substitution.

The use of meals from other insect species in substitution of FM showed contrasting results. Some studies on rainbow trout or turbot (*Psetta maxima* L.) reported that the use of insects (*Hermetia illucens* L. – HI, the black soldier fly) decreased growth performances and diet digestibility (St-Hilaire et al., 2007; Kroeckel et al., 2012), while no significant differences were observed in another trial with African catfish fingerlings fed maggots (Fasakin et al., 2003).

4.1.2. Fish whole body composition

The obtained values of whole body proximate constituents are consistent with literature for European sea bass juveniles (Messina et al., 2013; Tibaldi et al., 2015; Peixoto et al., 2016). Regarding the effect of insect meal inclusion in the diet, the obtained results contrast with Belforti et al. (2015) who reported a significant decrease of DM and EE contents, and an increase of CP content with increasing inclusion of TM larvae meal in rainbow trout diets. Also Sealey et al. (2011), in a study using a full-fat HI in rainbow trout diets showed a decrease in DM and EE in fish fillets associated with the dietary inclusion of insect meal. Kroeckel et al. (2012) used a defatted HI meal to feed turbot juveniles and found no differences for CP and ash contents, but moisture and lipid contents were significantly reduced with the increase of HI in the diets.

Independently from the dietary treatment, C18:1 *c*9, C16:0 and C18:2 n6 were the most abundant FA found in whole body of European sea bass, which is in accordance with previous published literature (Skalli and Robin, 2004; Eroldoğan et al., 2013). It is well known that the FA profile of fish mostly mirror that of administered diets, at comparable fish age and size. Data reported in this trial are in agreement with those described by Sealey et al. (2011) and by Belforti et al. (2015) who found decreasing levels of valuable n3 PUFA (such as C18:3 n3, C20:5 n3, C22:5 and C22:6 n3, well known for their beneficial effects on human health) when fish were fed diets containing insect meals. Eroldoğan et al. (2013) also reported comparable trends as those observed in our trial, as well as a noticeable reduction of the Σ n3/Σ n6 FA ratio, in European sea bass fed diets where fish oil was totally replaced by vegetable oils rich in C18:1 *c*9 and/or C18:2 n6, the two most abundant FA also found in TM larvae meal.

4.2. Digestibility trial

In our trial HSI was significantly higher in the fish fed 25% TM (TMD treatment) if compared to the fish fed the control diet; such result does not agree with the findings of Belforti et al. (2015) who reported a decreasing HSI in rainbow trout following increasing dietary inclusion level of TM (2.18 *vs* 1.79 and 1.61 for diets containing 0, 25 and 50% of TM inclusion, respectively).

A previous study on *in vitro* digestibility of TM showed that chitin varies from 4.8 to 6.7% of the meal (5.75% ± 0.012) depending on the meal sample (Marono et al., 2015). A dietary inclusion of 25% of TM, replacing 36% of dietary FM, would thus bring a maximal amount of 1.7% of chitin. Despite the common assumption that monogastric animals, including fish, cannot digest chitin (Rust, 2002), the replacement of 36% of FM by TM meal increased the digestibility of proteins and did not significantly alter lipid and carbohydrate digestibility. Chitinase activity has been shown in the digestive tract of many marine fish species (Krogdahl et al., 2005; Gutowska et al., 2004; Kawashima et al., 2016). Although the evaluation of chitinase activity was out of scope for the present research, our results suggest that chitinase activity maybe present

in European sea bass, either through the production of endogenous enzymes by the fish or through exogenous enzymes produced by intestinal bacteria. Chitinase activity due to exogenous bacteria was shown in various fish species (Ray et al., 2012) but not yet in European sea bass. Other enzyme-producing bacteria have been isolated from the gut of many fish species (Ray et al., 2012): protease- and/or lipase-producing bacteria (*Vibrio, Acinetobacter, Enterobacteriaceae, Pseudomonas*) have been isolated from sea bass larvae (Gatesoupe et al., 1997).

Many studies have shown that the addition of exogenous enzymes improves the digestibility of plant-based diets in mammals (Carneiro et al., 2008; Emiola et al., 2009) and fish (Dalsgaard et al., 2012; Castillo and Gatlin, 2015). The addition of exogenous proteases and carbohydrases (not specifically designed for chitin and insect meal) to the TM containing diet of European sea bass significantly reduced the digestibility of both proteins and carbohydrates if compared to the TMD diet. A reduction of acid and neutral detergent fibre digestibility was also obtained in pigs fed corn, soya, whey and dried distillers grains with soluble based diets enriched with similar enzymes (Kerr et al., 2013). In the current trial, the exogenous enzymes were added at a level recommended to other productive animals, the dietary enzyme concentration may have been too low to help the fish to digest proteins and carbohydrates present in the TM-enriched diets or their effect was not detectable under our experimental conditions. However, this would justify an absence of improvement of digestibility rather than the observed decreased digestibility values. As suggested in a recent review, a better explanation would be that these exogenous enzymes, added to the diet to improve its digestibility, may in fact alter the viability of the gut microflora that helps the digestive activity of the fish (Ringø et al., 2015). Indeed, β-glucanase and xylanase have been shown to influence the bacterial microbiota of broiler chickens (Jozefiak et al., 2010) and bacteria of the fish microflora, such as *Vibrio harveyi*, *Vibrio fisheri* and *Photobacterium leiognathi*, have been shown to have a chitinolytic activity (Ramesh and Venugopalan, 1989). The proteases and carbohydrases (mainly β-glucanases) present in TM-prot and TM-carb diets respectively, may have affected the proteins and carbohydrates present at the surface of Gram-negative bacteria (Beveridge, 1999) or the surface proteins present on Gram-positive bacterial membrane (Navarre and Schneewind, 1999; Desvaux et al., 2006) in turn decreasing their digestive activity towards insect chitin. Because chitin is a complex matrix of proteins, lipids and other compounds (Kramer et al., 1995), a better digestion of insect chitin will ease the access of digestive enzymes to protein and lipids, consequently increasing not only carbohydrates digestibility but also proteins and lipids digestibility (Tanaka et al., 1997). Evidently, more detailed research is required to elucidate the mode of action of exogenous enzymes in TM-supplemented feeds for sea bass.

5. Conclusions

The main results of the present study were that a full-fat *Tenebrio molitor* meal can be used up to 25% of inclusion in diets for European sea bass juveniles without affecting growth performances; such inclusion level allowed saving 36% of fish meal in the diet. At a higher TM inclusion level (50%) fish performances were negatively affected. The whole body proximate composition was not significantly influenced by the use of TM (with the exception of ashes), while a dramatic worsening was highlighted in the whole body FA profile.

Despite the chitin content of *Tenebrio molitor*, the digestibility of TM-containing diet was not reduced compared to the FM diet. Digestibility of proteins was even better in fish fed TM compared to fish fed FM. The supplementation of digestive enzymes (proteases and carbohydrases), instead of improving protein and carbohydrate digestibilities, reduced them, possibly because they affected the chitinolytic activity of some intestinal bacteria.

Further research is highly suggested to confirm these results and to investigate deeply the digestibility effects.

Conflict of interest

The authors declare that there are no conflicts of interest.

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