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A comparative study on the effect of fish meal substitution with three different insect meals on growth, body composition and metabolism of European sea bass (*Dicentrarchus labrax* L.)

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Abstract

Insects are considered a sustainable alternative protein source in aquaculture diets. So far, most studies regarding the inclusion of inset meals in the diets of farmed fish focus on one insect species individually and they are examining the effect of different insect meal inclusion levels. This is a comparative study of the use of three different insect species in the diets of European sea bass. During the twelve-week experimental period, fish (average initial weight of 5.7 g) were fed with isonitrogenous and isoenergetic diets in which 30% of the fish meal was substituted with insect larvae meals from *Tenebrio molitor* (TM), *Hermetia illucens* (HI) or *Musca domestica* (MD) three times a day for seven days per week. Under the experimental conditions, growth performance was similar in fish fed either insect inclusion diets or the fish meal diet. The inclusion of TM resulted in a slight increase in feed conversion ratio. The inclusion of HI or MD did not significantly affect the whole-body composition and nutrient retention; however, inferior nutrient efficiencies were observed when fish meal was replaced with TM. The fatty acid content of the diets affected greatly the whole-body fatty acid composition. Fish fed on the diets in n-3 polyunsaturated fatty acids diets (FM and HI) had the highest content of n-3 fatty acids while fish in the TM and MD groups had the highest n-6 polyunsaturated fatty acid content due to the abundance of these fatty acids in the TM and MD diets. The whole-body amino acid profile was not affected by different insect diets or FM replacement. Amino acid retention varied between groups without showing any dietary effect. Plasma cholesterol levels were lowered in fish fed the TM and MD diets. Plasma triglycerides, phospholipids and lactate were not affected by the FM substitution, but differences occurred between the insect diets. Liver amino acid catabolizing enzymes activities (ALT, AST and GDH) were similar in all fish groups. Overall, this study shows the efficacy of insect meals as a fish meal substitute and that fish meal can be successfully replaced by *Tenebrio molitor*, *Hermetia illucens* and *Musca domestica* meals in 30% in the diets of European sea bass.

Keywords: fish meal replacement; insect meal; fatty acids; amino acid deposition; plasma biochemical parameters

Highlights

- 30% of fish meal was substituted with *Tenebrio molitor*, *Hermetia illucens* or *Musca domestica* in the diets of European sea bass.
- Growth performance was similar in all dietary treatments.
- A minor increase in feed conversion ratio and a decrease in nutrient retention were observed when fish meal was substituted with *Tenebrio molitor* larvae meal.
- The fatty acid but not the amino acid profile was affected by the different insect meals inclusion.

Abbreviations: Fish meal, FM; *Tenebrio molitor*, TM; *Hermetia illucens*, HI; *Musca domestica*, MD; Acid Detergent fiber, ADF; Specific Growth Rate, SGR; Feed Conversion Ratio, FCR; Saturated fatty acids, SFA; Polyunsaturated Fatty Acids, PUFA; Eicosapentaenoic acid, EPA; Docosahexaenoic acid, DHA.

1. Introduction

Due to the increasing demand and the high price of fish meal and the increasing pressure put on fish stocks for its production, its substitution in the aquafeeds has become a common practice for many years. Indeed, progress has been made in the use of alternative proteins derived from terrestrial plants. However, the use of plant ingredients as fish meal replacements conflicts with environmental sustainability as it could aggravate deforestation and water bodies' eutrophication (Ramos- Elorduy, 1997) in addition to competing with the sources of ingredients for human food (Belghit et al., 2019; Malcorps et al., 2019). Insects are claimed to be a sustainable fish meal alternative because they require small land parcels and limited water to grow and they can bio-convert large amounts of organic waste into valuable animal protein, thus preventing the organic waste to become pollutant with little to no greenhouse gas emissions (van Huis and Oonincx, 2017; Sogari et al., 2019).

Insects are becoming a favorable ingredient for the feed of livestock and aquaculture animals, mainly due to their high nutritional value. Insects contain substantial amounts of high-quality protein (up to 74.4% of dry matter) and a balanced amino acid profile, which is superior to soybean meal and, in the case of Dipterans, is similar to fish meal (Barroso et al., 2014; Gasco et al., 2019a). Additionally, some insects are rich in mono-and poly unsaturated fatty acids such as oleic and linoleic acid (C18: 1 n-9 and C18:2 n-6 respectively) (Gasco et al., 2019a). Although insects lack eicosapentaenoic and docosahexaenoic fatty acids (EPA and DHA), which limits their use in marine fish feeds, their fatty acid profile reflects their diet and insect substrate enrichment with these fatty acids can positively modify the insect fatty acid profile (Liland et al., 2017). Some of the insect species are rich in iron, phosphorus, magnesium, calcium, selenium and zinc as well as vitamins A, D and B complex (Poshadri et al., 2018; Rumpold and Schlüter, 2014). Recently, the European Commission allowed the use of insects in the feeds for aquaculture animals (EU 2017/893), but insect meals are not yet a competitive alternative to current protein sources (Koeleman, 2014) due to small scale production leading to high prices. The industrial mass production of insects can be the solution to the high price and can ensure quality and quantity consistency (Arru et al., 2019).

The utilization of insects in experimental diets of piglets (Velten et al., 2017; Biasato et al., 2019), calves (Narang and Roshan, 1985), rabbits (Gasco et al., 2019b), broilers (Dabbou et al., 2018) and laying hens (Marono et al., 2018) showed encouraging results. To date, insects are recommended as a promising alternative to fish meal in the diets of farmed fish and crustaceans. Inclusion of 25% *Tenebrio molitor* meal in the experimental diets of gilthead sea bream (*Sparus aurata*) had no adverse effects on their growth performance (Piccolo et al., 2017) and even improved the protein digestibility in the case of European sea bass (*Dicentrarchus labrax*) (Gasco et al., 2016). Furthermore, the inclusion of *Tenebrio molitor* meal did not affect growth and slaughter traits of blackspot sea bream (*Pagellus bogaraveo*) (Iaconisi et al., 2017) and rainbow trout (*Oncorhynchus mykiss*) (Iaconisi et al., 2018), but it did alter flesh quality and especially the flesh fatty acid profile.

To our knowledge, there are no comparative studies assessing the effects of fish meal substitution with different insect meals at the same trial. Thus, the objective of the present study was to investigate the effects of dietary inclusion of three insect meals derived from *Hermetia illucens, Musca domestica* and *Tenebrio molitor* larvae on European sea bass (*Dicentrarchus labrax)* growth performance, feed utilization, nutrient retention and metabolism.

2. Materials and methods

All experimental procedures were designed and conducted by the Federation of European Laboratory Animal Science Associations (FELASA) accredited scientists (functions A-D) according to the guidelines stated by the EU Directive 2010/63/EU regarding the protection of animals used for scientific purposes. The trial was conducted at the Institute of Marine Biology, Biotechnology and Aquaculture (IMBBC) of the Hellenic Centre for Marine Research (Heraklion, Greece) under the protocol 255340 issued by ethics committee of the region of Crete, Greece..

2.1 Insect meals and diets

Frozen whole *Tenebrio molitor* larvae were obtained from the Benaki Phytopathological Institute (Athens, Greece), were oven-dried at 40 °C for 24 h and homogenized in a knife mill (Grindomix GM200, Retsch GmbH, Haan, Germany). Dried whole *Musca domestica* larvae were purchased from Reptilia nostra (Athens, Greece) and then homogenized to form a fine powder. Partially defatted *Hermetia illucens* larvae meal was acquired from Hermetia Deutschland GmbH & Co. KG (Baruth/Mark, Germany) and the rest of the diet ingredients were purchased from local suppliers.

Four diets were formulated: one control diet in which fish meal (FM) was the main protein source, and three diets in which 30% of the fish meal was substituted with insect meals from the larvae of *Tenebrio molitor* (TM), *Hermetia illucens* (HI) or *Musca domestica* (MD). The insect meal inclusion level was 19.5%. A percentage of 65% fish meal was incorporated in the control/fish meal-based diet, which is comparable to the current inclusion of fish meal in the commercial feeds for sea bass fingerlings (5.7 ± 1g). Diets were designed to be isoproteic and isoenergetic (Table I) and, at this scope, the amount of some ingredients (namely fish oil, wheat and wheat gluten meal) slightly changed among diets. Moreover, according to the different insect meals amino acid profile, DL-methionine and L-lysine were included in diets to ensure an essential amino acid balance among the diets. Diets were prepared at the IMBBC laboratory. The ingredients were mixed thoroughly by hand, water was added to the mixture to obtain the preferred consistency, and the mixture was converted into pellets using a mincing machine (4 mm die). Finally, the pellets were oven-dried at 40 °C for 24 h and stored in a freezer at-20 °C until used.

2.2 Fish and experimental conditions

Juveniles *D. labrax* were obtained from the IMBBC hatchery. At the start of the experiment, 360 fish were lightly anesthetized (phenoxyethanol, 150 ppm), were individually weighted (5.7 ± 1.05g), and they were randomly distributed into 12 cylindroconical, indoor 250 L tanks equipped with a settling column. A photoperiod of 12 h light/12 h dark, a temperature of 19.3 ± 0.2 °C and a salinity of 35 psu were maintained throughout the experiment. Open-circulation borehole water with water renewal of 200% per hour provided adequate levels of dissolved oxygen, and the oxygen saturation was consistently over 80%. Fish were fed by hand, three times a day (until apparent satiation), seven days a week, for three months. Apparent satiation was achieved by monitoring fish behavior, specifically the feeding would stop when the fish feeding activity would become slower and pellets would remain uneaten. Any unconsumed feed trapped in the settling column was removed and dried daily to determine feed intake.

2.3 Growth performance and somatic indexes

At the end of the feeding trial, fish were starved for 24 h, were lightly anesthetized and their individual weight and length were recorded. In addition, five fish per tank were randomly sampled, were sacrificed by anesthesia overdose (500ppm of 2-Phenoxyethanol), and blood and liver samples were collected. Viscerosomatic index, hepatosomatic index, mesenteric fat index and relative gut length were calculated based on five fish per tank. Ten fish at the beginning of the experiment and three fish per tank at the end of the experiment were sacrificed by anesthesia overdose and stored at-20 °C for whole-body proximate composition analysis. The following growth performance and somatic indexes were calculated:

Survival (%) = 100 x final number of fish / initial number of fish

Weight gain (WG, %) = 100 x [FBW (final body weight, g) – IBW (initial body weight, g))] / IBW

Specific growth rate (SGR, % day^{-1}) = 100 x [(ln FBW - ln IBW) / number of days]

Daily feed intake (DFI, % body weight day^{-1}) = [total dry feed intake (g) x 100] / [(IBW + FBW) x 0.5] x number of days

Feed conversion ratio (FCR) = total dry feed / (FBW-IBW)

Condition factor (CF) = 100 x (body weight (g) / total $length^3$ (cm))

Hepatosomatic index (HSI) = 100 x (liver weight / body weight)

Mesenteric fat index (MSI) = 100 x (perivisceral fat weight / body weight)

Viscerosomatic index (VSI) = 100 x (viscera weight / body weight)

Relative gut length (RGL) = intestinal length (cm) / fish total length (cm)

Nutrient retention efficiency or deposition (%)= 100 x [final nutrient quantity in the body (g, wet basis) - initial nutrient quantity in the body (g, wet basis)] / nutrient consumed (g, dry basis), where nutrient can be dry matter, protein, lipid, energy, ash or amino acids

2.4 Proximate and chemical analysis of diets and fish

Fish for the whole-body analysis were frozen, homogenized (Retsch ZM200, Haan, Germany) and freeze-dried (Telstar Cryodos, Terrassa, Spain), while feeds were pulverized (Retsch ZM200). Insects, diets and fish were analyzed for dry matter (method 934.01) and ash (method 942.05) according to (AOAC, 1990), crude lipids according to (Folch et al., 1957), crude protein according to Dumas method [nitrogen content (N) x 6.25] using a nitrogen analyzer (FP-528, Leco corporation, St. Joseph, Michigan, USA), crude fiber (CF) and acid detergent fiber (ADF) corrected for ash using Fibretherm (C. Gerhardt GmbH & Co., Königswinter, Germany), nitrogen linked to acid detergent fiber (ADIN) according to (Goering, Van Soest, 1979), and energy using an adiabatic bomb calorimeter (6300, Parr Instrument Company, St. Moline, Illinois, USA). The ash-free acid detergent fiber content of the diets represent not only cellulose and lignin from the plant feedstuffs (Goering, Van Soest, 1979) but also chitin from the insect inclusion and indigestible nitrogen such as heat-damaged proteins, proteins attached to cell-walls and proteins attached to chitin (Bernard et al., 1997; Finke, 2007; Goering, Van Soest, 1979). The traditional determination of protein, where nitrogen content is multiplied by 6.25 (assuming protein is 16% nitrogen), leads to an overestimation of the crude protein content of the diets. For this reason, the nitrogen linked to acid detergent fiber (ADIN) was calculated and the protein content was adjusted by subtracting the ADIN from the diets nitrogen content and then multiplying by 6.25.

Fatty acid methyl esters (FAMEs) were prepared according to (AOCS, 1989) (Method Ce 1b-89). The FAMEs were then analyzed using an Agilent GC-7890 B gas chromatograph (Agilent Technologies, Santa Clara, CA, USA), equipped with a flame-ionization detector (GC-FID) and a DB-23 capillary column (60 m x 0.25 mm i.d. × 0.15 µm film thickness) (Agilent, Santa Clara, CA, USA). Helium was used as carrier gas at 2 mL/min constant flow; the split ratio was 1:50 and the injected volume 1.0 µL. The thermal gradient was 50 °C for 1 min, 50 °C to 175 °C at 25 °C min^{-1}, 175 °C to 230 °C at 4 °C min^{-1} and kept at 230 °C for 15 minutes. The injector and detector temperature were maintained at 250 and 280 °C, respectively. Fatty acids were identified by comparison with a known standard mixture (Supelco 37 Component FAME Mix). Fatty acid methyl ester contents were expressed as a % of total FAMEs basis.

The amino acid composition of the diets was analyzed after acid hydrolysis (6 N HCl, 11 °C, 24 h), and derivatization by AccQ-Tag™ Ultra according to the amino acid analysis application solution (Waters Corporation, Milford, MA, U.S.A.). DL-Norvaline (Sigma) 2.5 mM was used as an internal standard. UPLC was performed on an Acquity system (Waters Corporation, Milford, MA, U.S.A.) equipped with PDA detector and the detection wavelength was set at 260 nm. The column used was BEH C18 column (100 mm × 2.1 mm i.d., 1.7 µm) from Waters. The flow rate was 0.7 ml min^{-1} and the column temperature was kept at 55 °C. Peak identification and integration were performed by the software Empower v.2.0 (Waters Corporation, Milford, MA, U.S.A.) using Amino Acid Standard H (Thermo Scientific Pierce) as an external standard. All analyses were performed in duplicate. In cases

were the values between replicates didn't meet the standardized acceptance criteria based on the mean and standard deviation (<5%), new duplicate analyses were performed according to established procedures. Tryptophan was not quantified due to its susceptibility to acid hydrolysis, whereas cysteine reacts with cysteine to form cystine. Moreover, during acid hydrolysis procedure asparagine is converted to aspartate and glutamine to glutamate, so the reported values for these amino acids (Asx and Glx) represent the sum of both amino acids.

2.5 Plasma and liver enzyme activities

Blood samples were drawn by caudal venous puncture with heparinized syringes, stored on crushed ice until all samples were collected and centrifuged at 6,000 rpm for 15 min. Plasma was removed and stored at-80 °C until analysis. The quantitative determination of plasma metabolites in pooled samples (five samples of each tank were pooled) was performed by enzymatic colorimetric methods using commercial kits (glucose, cholesterol, triglycerides from BIOSIS Biotechnological Applications L.T.D, Greece and phospholipids, and lactate from Spinreact, S.A.U. Spain). Enzymatic activities of alanine aminotransferase (ALT, EC 2.6.1.2) and aspartate aminotransferase (AST, EC 2.6.1.1) were determined kinetically at 340 nm using kits according to the protocols of the manufacturer (BIOSIS Biotechnological Applications L.T.D, Greece).

After removal, livers were stored at-80 °C. Six livers per diet were homogenized individually using 10 volumes of ice-cold buffer containing 20 mM Hepes, 0.25 mM saccharose, 50 mM EDTA, 5 mM K_2PO_4 and 1 mM DTT (pH 7.2). After centrifugation (1000 rcf at 4 °C for 10 min), the supernatant was freeze-thawed three times to break the mitochondrial membranes (Antonopoulou et al., 2013) and centrifuged again (6000 rcf at 4 °C for 15 min). The supernatant from the second centrifugation was used for the determination of amino acid catabolic enzymes. Liver ALT and AST activities were measured using commercial kits as mentioned above. Liver glutamate dehydrogenase activity (GDH, EC 1.4.1.2) was assessed adding L-glutamic acid a mixture of 175 mM Tris-HCl, 100 mM semi-carbazine, 1.1 mM NAD, 1 mM ADP and 5 mM L-leucine (pH 8.5) and following the formation of NADH at 340 nm (Encarnação et al., 2004; Gómez-Requeni et al., 2004). Enzyme activities were expressed as μmol of substrate reduced/oxidized per min (U) and per mg of protein. Bovine serum albumin was used for the determination of protein in the homogenate according to (Bradford, 1976).

2.6 Statistical analysis

Data were tested for normality and equality of variances with Kolmogorov-Smirnov and Levene's tests, respectively. One-way analysis of variance (ANOVA) was performed to determine if significant differences existed between dietary treatments (results were considered statistically significant at $P<0.05$) and individual means were compared using Tukey's test. Correlational analyses

were performed using the Spearman correlation. All statistical analyses were carried out using SigmaStat 3.5 (Systat Software, Inc., San Jose, California, USA).

3. Results

3.1 Diets

Experimental diets were formulated to be isonitrogenous and isoenergetic (Table 1) and to fulfill adequately the nutritional requirements of sea bass. The inclusion of HI meal and MD meal resulted in higher acid detergent fiber content. Due to the chitin and the nitrogen linked to ADF, the inclusion of insect meals, in fact, lowered the true protein content of the diets (adjusted protein) by 4.2% for the TM inclusion, 3.0% for the HI and 4.3% for the MD. In addition, the inclusion of insect meals resulted in higher crude fiber, especially in the TM (3.78%) and HI (4.03%) diets.

The fatty acid composition of the experimental diets was greatly affected by the fatty acid profile of different insect meals (Table 2). More specifically, FM and HI diets showed a higher content of total n-3 PUFA (22.16 and 21.76% respectively), which was dominated by EPA (20:5 n-3) and DHA (22:6 n-3), because of the higher fish oil dietary inclusion. The TM diet exhibited a higher content of total n-6 PUFA (13.8%), which was dominated by linoleic acid (18:2 n-6), compared to the other three experimental diets (4.63-8.06%), due to the considerable higher content of linoleic acid in the TM meal. Finally, the HI diet had a higher content of lauric acid (12:0, 2.64%) compared to the other three diets (0.10-0.14%) reflecting the extremely higher lauric acid content in the HI meal.

The amino acid composition of the different diets was approximately similar, and it was not affected by the different insect meal inclusion, except for taurine and hydroxyproline (Table 3).Taurine and hydroxyproline were not detected in TM and MD meals and were detected in low quantity in HI meal. Consequently, the insect inclusion diets had lower content of these amino acids compared to the FM diet. Lysine and methionine contents of the MD meal were relatively close to that of fish meal, while TM and HI meals had more than two-times lower lysine and methionine contents compared to fish meal. The supplementation of crystalline lysine and methionine in the diets compensated for the dietary deficiency of these two amino acids originated from the inclusion of insect meals.

3.2 Growth, feed conversion and somatic indexes

At the start of the experimental period, the fish had an average initial weight of 5.7 g, and after 83 days, the fish had grown to over four-fold of the initial body weight (Table 4). Survival did not differ between the different dietary groups and any mortality was marked as random. Daily feed intake was not affected by the substitution of fish meal or by the different insect meals (1.47-1.56% BW day^{-1}). Weight gain and specific growth rate were not influenced by the different diets, although the final body weight of the fish fed the HI diet was significantly higher (25.23 ± 1.24 g) in comparison to the fish fed the TM diet (21.58 ± 0.71 g). The inclusion of TM meal in the

corresponding diet resulted in a significantly higher feed conversion ratio (1.09 ± 0.02) in relation to the control FM based-diet (0.99 ± 0.02), and to the diet including MD meal (1.00 ± 0.00) (Table 4). Final weight, weight gain and specific growth rate showed a positive correlation only with the adjusted protein content (r = 0.691, 0.583 and 0.583, respectively; $P < 0.05$) and not the crude protein of the diets. On the other hand, FCR had a negative correlation both with the crude protein and the adjusted protein content (r =-0.864 and -0.626, respectively; $p<0.05$). Growth performance and feed efficiency correlated positively with the n-3/n-6 fatty acid content of the diet and negatively with the ratio of oleic acid/(EPA+DHA).

The hepatosomatic index, viscerosomatic index and the relative gut length had no significant difference in fish fed the different experimental diets (Table 4). Interestingly, fish fed the FM diet presented the highest condition factor value (1.14 ± 0.01; $P < 0.05$), while the fish fed the HI diet showed 24% higher mesenteric fat index than the FM group ($P < 0.05$; Table 4). The crude fiber content of the diets was positively correlated with the FCR, VSI and MSI (r = 0.648, 0.605 and 0.648, respectively; $P < 0.05$) and the ADF fraction of the diet correlated positively only with VSI and MSI (r = 0.691 and 0.648, respectively; $P < 0.05$).

3.3 Whole-body proximate and chemical composition

The partial substitution of FM with TM meal resulted in a significantly lower carcass dry matter, crude fat and energy content (31.83 ± 0.47%, 10.04 ± 0.30% and 8.27 ± 0.14 MJ kg^{-1}, respectively) compared to all the other dietary treatments (Table 5). Diets including FM, HI and MD performed similarly ($P > 0.05$) in terms of whole-body dry matter, crude protein and energy content (Table 5). Whole-body crude fat of the FM group was significantly higher (13.65 ± 0.43%) compared to the TM and HI groups (10.04 ± 0.30% and 11.91 ± 0.10% respectively).

The fatty acid content of the diets affected the fatty acid profile of the fish carcasses (Table 6). There is a positive correlation of the individual fatty acid content of the feed and the whole-body for 18 out of 22 fatty acids, and a positive correlation between fish oil inclusion and individual fatty acid content for 15 out of 22 fatty acids. Total saturated fatty acids (SFA) did not differ among the groups. The higher concentration of n-3 PUFA in the FM and HI diets resulted in significantly higher n-3 content of the fish whole-body compared to the TM and MD groups. The myristic acid (14:0), erucic acid (22:1), EPA (20:5 n-3) and DHA (22:6 n-3) content of the fish body followed the same pattern. All the experimental fish increased their n-3 PUFA content from the beginning of the experiment, although the increase was more prominent in the FM and HI groups. On the other hand, the n-6 PUFA content decreased over time, except for the TM group. The n-6 PUFA content was significantly higher in TM group (12.18 ± 0.36%), followed by the MD group (7.57 ± 0.12%), and lastly by the FM and HI groups (5.00 ± 0.20% and 5.71 ± 0.29%, respectively), thus reflecting the fatty acid composition of the diets. Similarly, total PUFA were also significantly higher in TM group (29.35 ± 0.51%) compared to the other dietary groups (22.49-24.92%). The linoleic acid (18:2 n-6) was the dominant

fatty acid in TM. As expected, the highest content of lauric acid (12:0) in the HI diet resulted in significantly higher lauric acid in the carcasses of fish fed the HI diet (0.98 ± 0.06%) compared to the other fish groups (0.05-0.07%). Overall, the substitution of fish meal with HI meal affected slightly the fish whole-body's fatty acid content, while the substitution with TM or MD meals significantly affected the content of 14 fatty acids (of the 22 measured here) and reduced the EPA and DHA content by 23%.

The whole-body amino acid profiles (% of crude protein) of the experimental fish groups are shown in Table 7. Overall, no dramatic changes were observed between the initial and final amino acid profile of the fish bodies. The essential amino acid profile of the whole body was not affected by the different protein sources ($P > 0.05$). Among the non-essential amino acids only Asx, cysteine, Glx, taurine and tyrosine were affected by the dietary treatment. The MD group had significantly higher cysteine and tyrosine content than the FM group. Regarding cysteine, there was a positive correlation between cysteine feed and whole-body content ($r = 0.691$; $P < 0.05$), but no correlation with the feed methionine. On the contrary, whole-body tyrosine was not correlated with the tyrosine feed content but it was positively correlated to feed phenylalanine content ($r = 0.928$; $P < 0.05$).

3.4 Nutrient retention and amino acid deposition

The nutrient and energy retention of sea bass fed diets with different insect meals are shown in Table 8. The protein retention of the TM group was significantly lower (27.55 ± 0.27%) compared to the other dietary treatments (29.03-29.46%). Protein retention appeared to be positively correlated with the available protein (adjusted protein) content of the diets ($r = 0.669$; $P < 0.05$). Dry matter and energy retention followed the pattern of whole-body composition with fish fed the TM diet, showing the poorest retention compared to the other dietary treatments ($P < 0.05$). Fish fed the HI and MD diet had comparable protein, fat and energy retention as the FM fish ($P < 0.05$). Additionally, fish fed the TM diet had significantly lower protein, fat and energy retention than the FM fish ($P < 0.05$). Protein, fat, dry matter and energy retention correlated negatively with the FCR ($r = -0.678$, $r = -0.944$ and $r = -0.944$, respectively; $P < 0.05$).

The essential amino acid retention and non-essential amino acid deposition (from feeding and endogenous production) of sea bass fed diets with different insect meals are shown in Table 9. Hydroxyproline and taurine showed the highest deposition among all the amino acids in all experimental diets. However, FM diet resulted in the lowest deposition of these amino acids (72.13 and 53.59%, respectively) compared to the diets which included insect meals (84.22-93.18% and 64.45-71.58%, respectively). A negative correlation was found between the amino acid content of the diet and the amino acid deposition of hydroxyproline and taurine as well as in alanine, proline, and tyrosine. In addition, a negative correlation was found between the amino acid content of the diet and the respective amino acid retention of the essential amino acids arginine, isoleucine, methionine and valine. Among the essential amino acids, the retention of arginine was the highest, while methionine

was found to present the lowest retention in all diets (18.06-26.18%). Protein retention and amino acid retention correlated positively for the branched-chain amino acids (isoleucine, leucine and valine) and histidine. Moreover, the protein retention and amino acid deposition correlated positively for proline and the sum of glutamine and glutamate.

3.5 Plasma metabolites and liver enzyme activities

Plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activities were not significantly affected by the dietary treatments (Table 10). The highest values were observed in fish fed the FM diet followed by MD, HI and TM diets. Plasma phospholipids did not vary significantly among the dietary treatments either. Sea bass fed with TM diet showed the lowest plasma glucose levels (120.01 ± 4.97 mg dl^{-1}) compared to the other groups (165.84-200.03 mg dl^{-1}). A significantly higher plasma cholesterol level was detected in fish fed the FM and HI diets (237.75 ± 8.86 mg dl^{-1} and 229.80 ± 16.91 mg dl^{-1}, respectively), while similar plasma lactate and triglycerides levels were found between the FM group and the insect meal groups.

Liver alanine and aspartate aminotransferase activities did not vary significantly among the experimental groups (Table 11). The glutamate dehydrogenase activity of the HI group was found to be significantly higher than that of the MD group (0.09 and 0.05 μmoles mg $protein^{-1}$ min^{-1} respectively).

4. Discussion

4.1 Growth

In the present study, the results of 30% substitution of fish meal (FM) by insect meals derived from *Tenebrio molitor* (TM), *Hermetia illucens* (HI) or *Musca domestica* (MD) in the diets of European sea bass were evaluated. The daily feed intake was similar in all the fish groups indicating that all diets were palatable, well accepted and were in line with common knowledge about the nutrient requirements of sea bass (Wilson, 2003). The substitution of fish meal with insect meals had no adverse effects on growth performance, except the TM diet which resulted in a slightly higher feed conversion ratio. When 50% of FM was substituted by TM in the diets of Nile tilapia (*Oreochromis niloticus*; 31% initial FM inclusion), reduced feed efficiency and poor growth performance were observed (Sánchez- Muros et al., 2016). Gasco et al. (2016) reported no negative effects of 36% FM substitution by TM on growth performance and feed efficiency in European sea bass juveniles (70% FM inclusion in the control diet). On the contrary, 33% and 50% FM substitution with TM in the diets of gilthead sea bream (50% initial FM inclusion) (Piccolo et al., 2017) and in rainbow trout (75% initial FM inclusion) (Belforti et al., 2015), respectively, resulted in improved SGR and FCR. The presence of chitin in the insect exoskeleton has been both praised and accused of its effect on growth performance. Chitin is poorly digested by several fish species (Rust, 2002) and has been shown to reduce lipid absorption (Zacour et al., 1992) and fat digestibility (Kroeckel et al., 2012), thus resulting

in reduced growth (Adeniyi and Folorunsho, 2015). On the other hand, apart from its antioxidant (Kaya et al., 2015) and immunostimulatory effect (Henry et al., 2018), chitin has been found to increase the abundance of members of the genus *Bifidobacterium* in human gut (Stull et al., 2018), thus acting as a prebiotic to promote digestibility and growth (Munir et al., 2016).

In our study, the difference of FCR among the experimental groups is negligible (coefficient of variation 4%) and even though statistically significant difference was detected, the biological significance of this difference is not clear. The slightly higher FCR in the TM group could be possibly attributed to: (1) considerably lower adjusted protein content, (2) higher crude fiber content, (3) essential fatty acid deficiency, (4) essential amino acid deficiency, or (5) toxic compounds present in the TM larvae. Under the present experimental conditions, the crude protein and adjusted protein content of the diets did not show any correlation with the FCR; however, the higher fiber content of the TM diet, could play a role in the slightly higher FCR observed, as the positive correlation showed. In addition, the ratio oleic acid (18:1 n-9)/(EPA+DHA) is an indicator of essential fatty acid deficiency in the diets of marine fish (Montero et al., 2001) and in the TM diet; this ratio was increased due to the lower inclusion of fish oil in the diet. The content of the essential amino acids in all the experimental diets were in line with the common knowledge about the amino acid requirements of sea bass (Wilson, 2003; Tibaldi et al., 1994). Finally, TM larvae have been described to contain defensive toxic compounds (Attygalle et al., 1991) that could possibly negatively affect feed conversion.

Our results indicated that the 30% substitution of FM with HI had no negative effect on fish growth and feed conversion and is in line with the results reported by Magalhães et al., (2017) on European sea bass. These authors substituted FM up to 45% with HI larvae meal, although they used larger fish (50 g initial weight) and lower initial FM content (32.4%). Moreover, it has also been found that HI diets performed as well as fish meal diets in Atlantic salmon (*Salmo salar*) at 60% inclusion (Belghit et al., 2018), in rainbow trout with up to 50% FM substitution (Mancini et al., 2017) and in Nile tilapia with up to 50% substitution (Rana et al., 2015).

This is the first study reporting the effect of FM substitution with MD in European sea bass, indicating that no negative effects were observed on growth and feed conversion. Successful FM substitution with MD was achieved in barramundi (*Lates calcalifer*; up to 76%) (Lin and Mui, 2017), in African catfish (*Clarias gariepinus*; up to 100%) (Idowu and Afolayan, 2013; Olaniyi and Salau, 2013; Oyelese, 2007) and in Nile tilapia (up to 75%) (Ogunji et al., 2007). The literature review reveals that omnivore fish are generally more tolerant to the inclusion of insect meals in their diets, and in higher percentages, probably because insects constitute a part of their natural diet.

The condition factor of all the fish groups was higher than 1.0, indicating that all the diets were well utilized (Sogbesan and Ugwumba, 2008), although the FM group exhibited higher condition factor compared to the insect meal including diets. The type of feed and consequently the digestibility of the feed can affect the relative gut length. Fish fed digestion resistant ingredients such

as lignin or chitin, appear to have a higher relative gut length as a mechanism to increase nutrient absorption (Karachle and Stergiou, 2010; Odedeyi et al., 2014). In our study, the relative gut length was similar in all the groups and did not correlate with the insect meal inclusion, crude fiber or acid detergent fiber content of the diets. Piccolo et al., (2017) reported that 50% inclusion of TM resulted in significant higher relative gut length in gilthead sea bream (*Sparus aurata*) and in impaired nutrient digestibility. Our results indicated no differences in the hepatosomatic and viscerosomatic index. The HSI values were close to two, which is correlated with high liver fat deposition (Kaushik et al., 2004). The fish fed the HI diet had significantly higher mesenteric fat index than the FM group which could be attributed to the prominently higher fiber content of the HI diet as the positive correlation showed.

4.2 Whole-body proximate composition and retention

Whole-body proximate composition of the TM group differed significantly compared to the FM group. The TM inclusion in the diet of European sea bass affected the dry matter and fat content in proportion and therefore moisture and fat content in inverse proportion, while the protein content was similar. In contrast to our results, Gasco et al. (2016) reported no differences between the proximate composition of European sea bass fed FM and TM (36% FM substitution) using similar feed formulation (70% initial FM inclusion) and fish size (5.2 g). In mandarin fish (*Siniperca scherzeri*) and rockfish (*Sebastes schlegi*), the inclusion of TM in the diets had no effect on carcass composition but improved protein productive values (Khosravi et al., 2018; Sankian et al., 2018). Our results showed that diets including FM, HI and MD resulted in similar whole-body compositions, except for the fat content of the HI-fed fish which was lower than the FM fish (11.91 ± 0.10 and 13.65 ± 0.43 respectively). Nutrient retentions were not affected by the replacement of FM with HI and MD. Similarly, MD or defatted MD meal inclusion in the diets of African catfish (Fasakin et al., 2003; Idowu and Afolayan, 2013) and barramundi (Lin and Mui, 2017), and HI inclusion in the diets of Atlantic salmon (Belghit et al., 2018) and Jian carp (*Cyprinus carpio* var. *Jian*) (Li et al., 2016) had no effects on the whole body composition. In the present experimental conditions, the protein retention was satisfactory and ranged from 27.6 - 29.5%; but it has been shown that in European sea bass juveniles, a dietary taurine concentration of 1.13% can increase the protein retention up to 31% (Saleh et al., 2019). Martins et al. (2018), based on a dose-response model, suggested a dietary taurine requirement of 0.47 - 0.50% in European sea bass juveniles and in the present study, the taurine content in the insect inclusion diets appears limiting (0.44 – 0.46%); therefore a taurine supplementation of insect inclusion diets should be considered. Carcass fatty acid composition was greatly affected by the different insect meal inclusion, because of the insect larvae fat content which affected fish oil inclusion in the diets, and thus dietary fatty acid composition. The total SFA content was not affected by insect meal inclusion as was previously mentioned in studies using full-fat TM in European sea bass (Gasco et al., 2016) and rainbow trout (Iaconisi et al., 2018). However, when defatted HI meal was added in the diets of rainbow trout, the total SFA content of the fillet increased

with the increase of the insect inclusion, due to the extremely higher amount of lauric acid (12:0) in the HI larvae (Mancini et al., 2017). The higher concentration of n-3 PUFA in the FM and HI diets resulted in a significantly higher n-3 PUFA content in the fish bodies in the FM and HI groups. Total body n-6 PUFA content increased with the inclusion of full-fat TM and MD. A similar trend was observed when full-fat TM was added in the diets of Nile tilapia (Sánchez- Muros et al., 2016) and blackspot sea bream (*Pagellus bogaraveo*) (Iaconisi et al., 2017) with an increase in total n-6 PUFA as the insect meal inclusion increased. However, total n-6 PUFA content was not affected by the inclusion of defatted HI meal, as was previously found when defatted HI meal was added in the diet of rainbow trout (Mancini et al., 2017). Long chain PUFA are essential for the fish metabolism and cell membrane structure as, among other things, they are precursors of eicosanoids and membrane phospholipid and triglyceride components (Tocher, 2015). Despite the dietary fatty acid differences, the fish meal and fish oil contents in the diets were high enough to fulfill the EPA and DHA requirements of sea bass as described by Skalli and Robin (2004). In addition, all experimental diets resulted in a whole-body n-6/n-3 ratio (0.27-0.72) below the suggested limit for healthy human nutrition (1-2:1) (Simopoulos, 2011). Such great differences in the fatty acid composition might affect organoleptic characteristics of the fish flesh, e.g. aroma and flavor (Turchini et al., 2004; Borgogno et al., 2017; Gasco et al., 2019a), and might not be desirable from a consumer's perspective. Several practices have been suggested to tackle this problem, for example, the use of defatted insect meals and the improvement of defatting technics (Iaconisi et al., 2018), the use of n-3 rich ingredients in the diets for farmed insects (Liland et al., 2017) and the use of a n-3 rich finishing diet at the end of the farming period (Bell et al., 2003). All the experimental fish maintained or increased their n-3 PUFA content from the beginning of the experiment, especially EPA and DHA since their fatty acid requirements were met. It has been shown that EPA and DHA are "preferentially retained" in the fillet, probably due to the importance of these fatty acids to the organism (Tocher, 2015). European sea bass show very low levels of elongation and desaturation of C18 fatty acids to EPA and DHA (Mourente et al., 2005) and even though fish oil substitution induces those metabolic pathways (González-Rovira et al., 2009), the endogenous production of EPA and DHA is unlikely to contribute significantly to the whole-body levels (Bell et al., 2003).

The essential amino acid and five out of the ten non-essential amino acid contents of the fish whole-body were not affected by the different diets. In contrast with the fatty acid profile, the amino acid composition of fish whole-body shows a minor response to the diet composition (Sánchez-Muros et al., 2016), possibly to due to the lower muscle protein synthesis rates in fish (Kaushik and Seiliez, 2010). In this study, almost all essential amino acids retentions and non-essential amino acid depositions, except for threonine, the sum of asparagine and aspartate and serine, differed among the experimental groups. Regarding proline, methionine, taurine and hydroxyproline, the observed differences can be attributed to the considerable differences in the content of these amino acids in the diets, as the negative correlation showed. The differences observed in the different amino acid

retentions/depositions are attributed to their structural or metabolic role. Lysine, arginine and the branched-chain amino acids (leucine, isoleucine and valine) are major constituents of body protein, especially skeletal muscles (Li et al., 2009; NRC, 2011), hence they represent a large portion of the whole-body amino acids and they are preferentially retained. Likewise, proline and glycine represent more than 50% of the amino acids forming collagen (Li et al., 2011). Sulfur amino acids (methionine and cysteine) and histidine are less deposited in the body because of their metabolic roles with methionine being a major methyl donor and histidine involved in the imidazole and purine synthesis (Andersen et al., 2016). Among the essential amino acids, arginine retention was the highest. The experimental diets provided adequate amounts of essential amino acids compared to the known knowledge of the amino acid requirements of European sea bass for all essential amino acids except for the content of which was arginine, thus the retention of arginine was the highest. Methionine retention was the lowest, as seen also for the whole-body composition, but this can be an effect of the high metabolic utilization of methionine or of the methionine dietary inclusion far exceeding the nutrient requirements of sea bass (Tulli et., 2010).

4.3 Plasma metabolites and liver enzyme activities

Serum metabolite analyses are not widely used because of the lack of standard values but they are a very useful tool for estimating the nutritional status and welfare of farmed fish. Plasma glucose and lactate levels are associated with stress response (Roque et al., 2010). In this study, the highest levels of plasma glucose were observed in fish fed the MD diet, followed by FM and HI and lastly TM. Despite the differences, all values are inside the range measured for wild European sea bass (Coz-Rakovac et al., 2005), and the observed values for well nourished farmed European sea bass (Peres et al., 2014). Plasma lactate did not differ between the dietary treatments. High levels of energetic metabolites such as cholesterol and triglycerides can be indicators of liver pathology and high-fat diet (Coz-Rakovac et al., 2005). Cholesterol was significantly lower in the TM and MD diets compared to FM and HI and triglycerides were not affected by insect meal inclusion. Plasma cholesterol in fish can be decreased by higher dietary plant inclusion because of the phytosterols (Kaushik et al., 2004), by dietary chitin inclusion due to the inhibition of fatty acid absorption (Chen et al., 2014) or by dietary taurine supplementation due to the role of taurine in bile salt formation (Gómez-Requeni et al., 2004). In our experimental conditions, plasma cholesterol indeed showed a negative correlation with the plant and chitin inclusion, but the dietary differences were minor to cause hypocholesterolemia.

The lowering effect of EPA and DHA on cholesterol is well established in mammals (Connor, 2000; Phillipson et al., 1985). However, in fish, this effect is not clear (Castro et al., 2015) and in our study increased cholesterol was observed in fish fed high fish oil inclusion diets. It has been found that dietary cholesterol is directly related to plasma cholesterol (Kaushik et al., 1995), and the increased cholesterol level in higher fish oil-fed fish may be due to the higher intake of dietary

cholesterol as fish oil contains 766 mg 100 g $^{-1}$ (USDA, 2019) When fish meal was replaced with insect meals, it reduced the blood cholesterol levels in Jian carp, European sea bass and mandarin fish (*Siniperca scherzeri*) (Li et al., 2017; Magalhães et al., 2017; Sankian et al., 2018), and the authors suggested the presence of chitin as the main reason.

Plasma enzymes (AST and ALT) activities can be affected by the nutritional, stress and general health status of the fish (Peres et al., 2014) and an increase in plasma enzymes can be an indicator of liver, primarily, but also kidneys' and gills' function impairment (Coz-Rakovac et al., 2005). In our study, the AST and ALT levels were not affected by the different dietary treatments and were inside the range previously reported for European sea bass (Coz-Rakovac et al., 2005; Peres et al., 2014; Roncarati et al., 2006). In mammals, dietary n-3 PUFA supplementation can reduce liver inflammation and fibrosis and can act as an hepatoprotectant by reducing the levels of pro-inflammatory cytokines (e.g. the tumor necrosis factor alpha – TNFα: Yang et al., 2019). A hepatic enzyme activity lowering effect of dietary PUFA was shown also in fish (Lanari et al., 1998 from Roncarati et al., 2006). In the present study a 17% increase in total dietary PUFA when fish meal was replaced with TM, resulted in 62% lower ALT and 22% lower AST in fish fed TM, although the decrease was not significant. No difference in AST and ALT was observed with the substitution of FM with HI in Jian carp (Li et al., 2017; Zhou et al., 2018), or with TM in mandarin fish (Sankian et al., 2018).

Liver amino acid catabolizing enzymes activities (ALT, AST and GDH) were not affected by the FM replacement with insect meals. Dietary amino acids can be accumulated to protein and the excess can be catabolized to ammonia and to intermediate molecules that can be used, amongst others, for lipogenesis or for energy production by entering Krebs or gluconeogenesis cycles (Fynn-Aikins et al., 1995; Ballantyne, 2001). Consequently, the amino acid catabolism increases with higher dietary protein inclusion. In addition, dietary fat is the dominant nutrient used for energy production in fish (Wilson and Halver, 1986) and a high fat diet has a sparing effect on amino acids used for energy production or lipogenesis (Ballantyne, 2001). Under the present conditions, all the experimental diets were isonitrogenous and provided an abundance of dietay fat leading to similar catabolic processes between the different dietary groups. A significantly higher GDH activity was observed in the HI group compared to the MD group, but without any other difference in the other amino acid catabolizing enzymes or in protein retention efficiency, the biological significance of this difference is unclear.

Conclusion

Under the present experimental conditions, all three insect meal diets performed equally well as the fish meal diet in terms of growth performance, however, a minor increase in feed conversion ratio was observed when the fish meal was substituted with TM. Whole-body composition and nutrient retention were not greatly affected by the inclusion of HI or MD. However, the slightly lower

feed efficiency and lower protein content of the TM diet resulted in a reduction in nutrient efficiencies, despite the higher whole-body protein content compared to the other three diets. The fatty acid composition was greatly affected by the different insect inclusion, following the fatty acid pattern of the diets. Highest n-3 fatty acid content was observed in the fish fed FM and HI, and highest n-6 fatty acid content in the fish fed TM and MD. The amino acid content was not affected by the different diets and the retention of most of the dietary amino acids, exhibited minor variations between the dietary treatments. Overall, fish meal can be successfully substituted by *Tenebrio molitor*, *Hermetia illucens* and *Musca domestica* meals in 30% in the diets of European sea bass Though, practices for the restoration of the fish n-3 fatty acid content should be implemented.

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6. Tables

Table 1: Ingredients and proximate composition of insect meals and experimental diets

	FM	TM	HI	MD
Ingredients (%)				
Fish meal (Peru, prime)	65	45.5	45.5	45.5
Insect larvae meal [a]	0	19.5	19.5	19.5
Fish oil	10	6	9.7	6.2
Wheat	17.6	18.4	16.4	18.5
Wheat gluten meal	6.7	8.2	6.6	9.1
Vitamin & mineral mix [b]	0.25	0.25	0.25	0.25
DL-methionine	0.5	1	0.9	0.3
L-lysine	0	1.2	1.2	0.7
Proximate composition of the different insect meals (dry basis) [c]				
Crude Protein (%)		58.4	67.0	58.5
Crude Lipid (%)		24.3	5.7	23.1
Ash %		4.8	7.8	7.4
Gross energy (MJ kg^{-1})		25.3	21.4	24.8
Proximate composition of the experimental diets (dry basis) [c]				
Crude Protein (%)	61.3	59.4	59.8	60.4
Adjusted Crude Protein (%) [d]	61.0	56.9	58.0	57.8
Crude Lipid (%)	19.6	17.5	17.7	17.3
Ash (%)	10.2	8.7	10.1	9.3
Crude fiber (%)	1.63	3.78	4.03	2.72
Acid detergent fiber (%)	3.73	4.75	9.97	9.25
NFE (%) [e]	7.25	10.62	8.37	10.28
Gross energy (MJ kg^{-1})	23.1	23.17	22.8	22.9

Abbreviations: FM, Fish meal; TM, *Tenebrio molitor*; HI, *Hermetia illucens*; MD, *Musca domestica*

[a] TM larvae purchased from Benaki Phytopathological Institute (Athens, Greece), defatted HI larvae meal purchased from Hermetia Deutschland GmbH & Co. (Baruth/Mark, Germany), MD larvae purchased from Reptilia nostra (Athens, Greece)

[b] Premix (kg^{-1}): Choline 90,000 (mg), Vitamin A 0.3 (MIU), Vitamin D3 0.1 (MIU), Vitamin E 20,000 (IU), Vitamin K 1030 (mg), Vitamin B1 390 (mg), Vitamin B 960 (mg), Nicotinic acid 2600 (mg), Pantothenic acid 4400 (mg), Vitamin B6 890 (mg), Vitamin B12 15 (mg), Folic acid 290 (mg), Biotin 14 (mg),Vitamin C (Stay C 35% MONO) 20,300 (mg), Inositol 15,600 (mg),Total Mn 1200 (mg), Total Ca 72,000 (mg), Total Zn 7,000 (mg), Total Cu 450 (mg), Total Se 14 (mg), Total I 100 (mg), Betaine (mg) 71,250 (mg), BHA (E320) 3000 (mg)

[c] Mean of triplicate analyses

[d] Protein adjusted for the nitrogen linked to acid detergent fiber

[e] Nitrogen-free extract, NFE = 100 - %CP - %CL - %Ash - %CF

Table 2: Fatty acid composition (% of total fatty acids) of fish meal, insect larvae meals and the experimental diets in which 30% of the fish meal (FM) was substituted with *Tenebrio molitor* (TM), *Hermetia illucens* (HI) or *Musca domestica* (MD) larvae meal

	Raw materials (fish meal and insect larvae meals)				Experimental diets			
Name	FM	TM	HI	MD	FM	TM	HI	MD
C12:0	0.07	0.14	26.77	0.13	0.10	0.12	2.64	0.14
C14:0	4.69	1.97	6.25	2.54	6.28	4.92	6.55	5.20
C15:0	0.44	0.18	0.11	1.73	0.46	0.38	0.43	ND
C16:0	18.11	ND	ND	20.90	16.06	16.19	15.47	18.12
C16:1 n-7	5.83	1.40	4.75	14.70	5.02	3.97	4.82	7.67
C17:0	0.40	0.22	0.16	0.74	0.27	0.24	0.22	0.45
C18:0	3.35	3.28	ND	4.87	2.37	2.75	2.23	3.05
C18:1 n-7	2.55	0.41	ND	0.60	2.04	1.60	1.82	1.57
C18:1 n-9 cis	11.31	33.12	16.17	25.39	10.62	16.19	10.49	13.40
C18:2 n-6 cis	2.01	37.61	10.29	12.25	4.22	13.55	4.96	7.74
C18:3 n-3	0.94	2.13	1.13	0.55	1.21	1.53	1.29	1.11
C18:4 n-3	1.97	0.70	0.46	0.84	2.56	2.09	2.72	2.08
C20:0	0.17	0.25	ND	0.10	0.17	0.20	0.17	0.16

C20:1 n-9	2.15	ND	ND	ND	6.21	4.26	6.26	4.24
C20:2 n-6	0.36	ND	ND	ND	0.28	0.26	0.25	0.23
C20:4 n-6	ND	ND	ND	ND	0.12	ND	0.12	0.10
C20:5 n-3 (EPA)	11.15	ND	ND	ND	8.00	6.40	7.80	6.01
C21:0	0.77	ND	ND	ND	0.49	0.39	0.46	0.41
C22:1 n-11	3.06	ND	ND	ND	10.23	6.94	10.20	6.95
C22:1 n-9	0.18	ND	ND	ND	0.63	0.43	0.57	0.44
C22:6 n-3 (DHA)	15.64	ND	ND	ND	10.34	8.50	9.96	7.62
ΣSFA	28.18	6.09	33.28	31.00	26.32	25.22	28.17	27.77
ΣMUFA	27.06	34.93	21.22	40.88	36.80	34.05	35.46	35.41
ΣPUFA	32.06	40.44	11.88	14.05	26.78	32.32	27.09	24.89
ΣPUFA/ΣSFA	1.14	6.64	0.36	0.45	1.02	1.28	0.96	0.90
Σn-3	29.69	2.83	1.59	1.39	22.16	18.52	21.76	16.83
Σn-6	2.37	37.61	10.29	12.25	4.63	13.81	5.33	8.06
Σn-3/Σn-6	12.54	13.28	0.15	0.11	4.79	1.34	4.08	2.09
DHA/EPA	1.40	-	-	-	1.29	1.33	1.28	1.27
C18:1 n-9 (EPA+DHA)$^{-1}$	0.42	-	-	-	0.58	1.09	0.59	0.98

Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ND, not detected

Ingredients crude fat content (% of dry matter): FM, 14.03%; TM, 24.30%; HI, 5.71%; MD, 23.09%

Means of duplicate analyses

Table 3: Amino acid composition (% of dry matter) of fish meal, insect larvae meals and the experimental diets in which 30% of the fish meal (FM) was substituted with *Tenebrio molitor* (TM), *Hermetia illucens* (HI) or *Musca domestica* (MD) larvae meal

	Raw materials (fish meal and insect larvae meals)				Experimental diets (amino acids, % of dry matter)			
	FM	TM	HI	MD	FM	TM	HI	MD
EAA								
Arginine	3.48	2.93	2.56	2.64	2.71	2.28	2.58	2.70
Histidine	1.41	1.71	1.50	1.48	1.17	1.24	1.24	1.29
Isoleucine	2.95	2.24	2.57	2.15	2.08	2.20	2.10	2.08
Leucine	5.09	3.98	4.12	3.65	3.84	3.88	3.71	3.78
Lysine	6.17	2.96	3.25	4.65	3.59	3.15	3.18	3.40
Methionine	2.01	0.68	0.91	1.56	1.88	2.23	1.99	1.67
Phenylalanine	2.42	1.78	2.03	3.37	2.19	2.14	2.13	2.65
Threonine	2.95	2.25	2.47	2.39	2.10	1.89	2.02	2.10
Valine	3.49	3.08	3.53	2.72	2.50	2.73	2.63	2.54
NEAA								
Alanine	4.47	4.39	5.02	3.05	2.90	3.14	3.03	2.70
Asx	7.07	4.30	5.75	6.12	4.16	3.75	4.04	4.12
Cysteine	0.27	0.21			0.25	0.24	0.22	0.25
Glx	10.32	5.69	7.87	8.40	8.43	8.42	8.14	8.87
Glycine	3.47	2.94	2.98	2.08	2.76	2.70	2.69	2.60
Hydroxyproline	0.39	ND	0.06	ND	0.21	0.24	0.24	0.24
Proline	2.58	4.05	3.81	2.29	2.61	3.08	2.87	2.82
Serine	2.66	2.74	2.73	2.33	2.11	1.97	2.10	2.18
Taurine	0.84	ND	0.03	ND	0.61	0.46	0.44	0.45
Tyrosine	1.75	2.79	2.68	3.33	1.40	1.37	1.67	1.88
Met+Cys	2.29	0.89	0.91	1.56	2.13	2.47	2.21	1.92
Tyr+Phe	4.17	4.57	4.72	6.70	3.59	3.51	3.80	4.53
Sum EAA	29.97	21.59	22.94	24.81	22.06	21.74	21.58	22.21
Sum NEAA	33.82	27.10	30.93	27.59	25.54	25.37	25.44	26.11
EAA/NEAA	0.89	0.80	0.74	0.89	0.86	0.86	0.85	0.85

Abbreviations: EAA, essential amino acids; NEAA, non-essential amino acids; Asx, sum of asparagine and aspartate; Glx, sum of glutamine and glutamate ND: Not Detected
Means of duplicate analyses

Table 4: Effect of 30% substitution of fish meal with different insect meals (TM: *Tenebrio molitor*, HI: *Hermetia illucens* or MD: *Musca domestica*) on growth performance and somatic indexes of European sea bass

	FM	TM	HI	MD
Survival (%)	100±0.00	98.89±1.11	100.00±0.00	98.89±1.11
IBW (gr)	5.73±0.08	5.70±0.10	5.71±0.14	5.70±0.16
FBW (gr)	24.26±0.36^{ab}	21.58±0.71^{b}	25.23±1.24^{a}	23.49±0.26^{ab}
WG (%)	323.58±11.62	278.30±7.05	343.06±30.50	312.98±14.70
SGR (% day^{-1})	1.74±0.03	1.60±0.02	1.79±0.09	1.71±0.04
DFI (% BW day^{-1})	1.48±0.01	1.52±0.01	1.56±0.04	1.47±0.02
FCR	0.99±0.02^{b}	1.09±0.02^{a}	1.03±0.01^{ab}	1.00±0.00^{b}
Somatic indexes				
CF	1.14±0.01^{a}	1.04±0.00^{c}	1.09±0.00^{b}	1.07±0.01^{bc}
HSI (%)	1.71±0.09	1.79±0.03	1.81±0.04	1.85±0.07
VSI (%)	9.52±0.48	9.96±0.18	10.62±0.15	10.21±0.16
MFI (%)	3.94±0.29^{b}	4.21±0.13^{ab}	4.89±0.19^{a}	4.30±0.05^{ab}
RGL	0.78±0.02	0.79±0.01	0.77±0.01	0.77±0.01

Abbreviations: IBW, initial body weight; FBW, final body weight; WG, weight gain; SGR, specific growth rate; DFI, daily feed intake; FCR, feed conversion ratio; CF, condition factor; VSI, viscerosomatic index; HSI, hepatosomatic index; MFI, mesenteric fat index; RGL, relative gut length

Mean ± standard error, n = 3 tanks per diet and n = 15 fish per diet for the somatic indexes. Different letters in the same row denote statistically significant difference (P < 0.05)

Table 6: Fatty acid composition of whole-body of European sea bass fed diets in which 30% of the fish meal was substituted with different insect meals (TM: *Tenebrio molitor*, HI: *Hermetia illucens* or MD: *Musca domestica*)

Table 5: Effect of 30% substitution of fish meal with different insect meals (TM: *Tenebrio molitor*, HI: *Hermetia illucens* or MD: *Musca domestica*) on whole-body proximate composition of European sea bass

Whole-body composition (% on wet basis unless otherwise stated)					
	Initial	FM	TM	HI	MD
Dry matter	29.23	34.51±0.29^{a}	31.63±0.47^{b}	33.61±0.09^{a}	34.72±0.46^{a}
Crude protein	17.29	17.79±0.16	17.82±0.29	17.86±0.01	17.61±0.14
Crude fat	9.43	13.65±0.43^{a}	10.04±0.30^{c}	11.91±0.10^{b}	13.13±0.16^{ab}
Ash	4.03	3.75±0.08	4.01±0.24	3.69±0.03	3.46±0.13
Gross Energy (MJ kg^{-1})	7.42	9.35±0.17^{a}	8.27±0.14^{b}	9.08±0.09^{a}	9.54±0.16^{a}

Mean ± standard error, n = 9 fish per diet (n = 10 initial fish). Different letters in the same row denote statistically significant difference (P < 0.05).

	Initial	FM	TM	HI	MD
C12:0	0.07	0.07±0.01^{b}	0.05±0.01^{b}	0.98±0.06^{a}	0.06±0.01^{b}
C14:0	3.61	5.41±0.13^{a}	4.11±0.06^{b}	5.40±0.20^{a}	3.94±0.01^{b}
C15:0	0.34	0.45±0.02^{b}	0.36±0.00^{c}	0.40±0.02^{bc}	0.60±0.01^{a}
C16:0	ND	19.62±0.43	17.41±0.35	17.60±0.75	18.28±0.46
C16:1 n-7	5.02	5.14±0.12^{bc}	4.57±0.11^{c}	5.35±0.20^{b}	7.18±0.09^{a}
C17:0	0.26	0.37±0.01	0.30±0.01	0.28±0.04	0.38±0.01
C18:0	3.56	3.00±0.12	2.95±0.12	2.54±0.14	3.20±0.19
C18:1 n-9 cis	25.72	17.96±0.47^{b}	21.41±0.17^{a}	17.98±0.65^{b}	23.24±0.18^{a}
C18:1 n-7	2.75	2.41±0.03^{a}	2.09±0.01^{b}	2.29±0.08^{ab}	2.25±0.01^{ab}
C18:2 n-6 cis	10.31	4.58±0.21^{c}	11.59±0.35^{a}	5.26±0.18^{c}	7.09±0.10^{b}
C18:3 n-3	1.94	1.17±0.03^{b}	1.43±0.02^{a}	1.23±0.04^{b}	1.11±0.02^{b}
C18:4 n-3	1.02	1.76±0.03^{b}	1.50±0.02^{c}	2.05±0.01^{a}	1.55±0.03^{c}
C20:0	0.20	0.19±0.01^{a}	0.17±0.01^{ab}	0.17±0.01^{ab}	0.15±0.01^{b}
C20:1 n-9	3.28	5.17±0.05^{b}	3.94±0.01^{c}	5.47±0.05^{a}	3.87±0.06^{c}
C20:2 n-6	0.56	0.42±0.00^{b}	0.59±0.02^{a}	0.45±0.02^{b}	0.48±0.02^{b}
C20:4 n-6	0.13	ND	ND	ND	ND
C20:5 n-3 (EPA)	5.00	6.26±0.07^{a}	5.53±0.08^{b}	6.36±0.11^{a}	4.84±0.09^{c}
C21:0	0.53	0.46±0.01^{a}	0.42±0.01^{ab}	0.43±0.01^{a}	0.39±0.01^{b}
C22:1 n-11	2.87	6.26±0.10^{b}	4.40±0.05^{c}	6.71±0.03^{a}	4.24±0.07^{c}
C22:1 n-9	0.37	0.56±0.01^{a}	0.41±0.01^{b}	0.55±0.02^{a}	0.40±0.01^{b}
C22:6 n-3 (DHA)	6.64	9.13±0.08^{a}	8.46±0.09^{b}	9.10±0.15^{a}	7.00±0.13^{c}
C24:1 n-9	0.44	0.68±0.02^{a}	0.49±0.01	0.58±0.02^{b}	0.44±0.00^{c}
ΣSFA	8.58	29.44±0.55	25.47±0.32	27.81±1.20	27.04±0.69
ΣMUFA	40.06	38.27±0.71^{b}	37.05±0.08^{b}	39.01±0.89^{b}	41.72±0.21^{a}
ΣPUFA	25.93	23.31±0.34^{bc}	29.35±0.51^{a}	24.92±0.53^{b}	22.49±0.36^{c}
ΣPUFA/ΣSFA	3.02	0.79±0.02^{b}	1.14±0.04^{a}	0.90±0.06^{b}	0.83±0.03^{b}
Σn-3	14.69	18.31±0.15^{a}	16.99±0.20^{b}	18.76±0.32^{a}	14.54±0.28^{c}
Σn-6	11.00	5.00±0.26^{c}	12.18±0.36^{a}	5.79±0.21^{c}	7.66±0.12^{b}
Σn-6/Σn-3	0.74	0.27±0.01^{c}	0.72±0.02^{a}	0.30±0.01^{c}	0.52±0.01^{b}

Abbreviations: SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; ND, not detected

% of total fatty acids. Mean ± standard error, n = 9 fish per diet (n = 10 initial fish). Different letters in the same row denote statistically significant difference ($P < 0.05$).

Table 7: Amino acid profile of the whole body of European sea bass fed diets in which 30% of the fish meal was substituted with different insect meals (TM: *Tenebrio molitor*, HI: *Hermetia illucens* or MD: *Musca domestica*)

	Initial	FM	TM	HI	MD
EAA					
Arginine	5.23	5.44±0.07	5.47±0.02	5.39±0.11	5.49±0.08
Histidine	2.04	2.07±0.04	2.09±0.01	2.05±0.05	2.16±0.03
Isoleucine	3.31	3.47±0.05	3.42±0.01	3.51±0.05	3.48±0.05
Leucine	6.10	6.36±0.09	6.24±0.02	6.31±0.10	6.37±0.10
Lysine	6.72	7.18±0.12	7.00±0.02	7.19±0.07	7.14±0.11
Methionine	2.53	2.61±0.04	2.58±0.02	2.59±0.07	2.64±0.05
Phenylalanine	3.50	3.62±0.06	3.56±0.02	3.59±0.08	3.65±0.05
Threonine	3.69	3.82±0.05	3.78±0.00	3.78±0.06	3.84±0.06
Valine	3.77	3.93±0.05	3.89±0.01	3.98±0.05	3.94±0.06
NEAA					
Alanine	5.20	5.35±0.03	5.42±0.01	5.36±0.01	5.45±0.08
Asx	8.06	8.07±0.09^{ab}	8.27±0.02^{ab}	8.02±0.05^{b}	8.40±0.13^{a}
Cysteine	0.37	0.36±0.01^{b}	0.38±0.01^{ab}	0.35±0.01^{b}	0.41±0.01^{a}
Glx	12.04	12.00±0.10^{ab}	12.32±0.01^{ab}	11.91±0.04^{b}	12.42±0.19^{a}
Glycine	6.36	6.50±0.06	6.61±0.08	6.65±0.08	6.48±0.12
Hydroxyproline	1.28	1.30±0.04	1.38±0.04	1.33±0.03	1.28±0.03
Proline	3.99	4.12±0.03	4.18±0.03	4.13±0.04	4.07±0.05
Serine	3.80	3.84±0.05	3.85±0.01	3.78±0.06	3.89±0.06
Taurine	1.86	2.18±0.03^{ab}	1.92±0.05	2.06±0.08^{a}	2.19±0.04^{a}
Tyrosine	2.48	2.30±0.06^{bc}	2.56±0.[illegible]^{ab}	2.21±0.09^{c}	2.69±0.04^{a}

Abbreviations: EAA, essential amino acids; NEAA, non-essential amino acids; Asx, sum of asparagine and aspartate; Glx, sum of glutamine and glutamate

% of crude protein. Mean ± standard error, n = 9 fish per diet . Different letters in the same row denote statistically significant difference (P < 0.05).

Table 8: Effect of 30% substitution of fish meal with different insect meals (TM: *Tenebrio molitor*, HI: *Hermetia illucens* or MD: *Musca domestica*) on nutrient and energy retention of European sea bass

	FM	TM	HI	MD
Dry matter	36.37±0.89^{a}	29.51±0.20^{c}	33.96±0.29^{b}	36.07±0.30^{ab}

Protein	29.46±0.19[a]	27.55±0.27[b]	29.35±0.21[a]	29.03±0.55[a]
Fat	76.82±3.62[ab]	53.18±1.44[c]	69.63±1.00[b]	81.86±1.15[a]
Ash	36.36±0.89	41.93±3.01	34.53±0.44	34.85±1.63
Energy	43.32±1.54[a]	33.74±0.33[b]	40.89±0.63[a]	44.14±0.60[a]

Mean ± standard error, n = 9 fish per diet. Different letters in the same row denote statistically significant difference ($P < 0.05$).

Table 9: Essential amino acid retention efficiency and non-essential amino acid deposition of European sea bass fed diets in which 30% of the fish meal was substituted with different insect meals (TM: *Tenebrio molitor*, HI: *Hermetia illucens* or MD: *Musca domestica*)

	FM	TM	HI	MD
EAA				
Arginine	33.55±0.57^{b}	36.52±0.59^{a}	34.18±0.27^{b}	33.55±0.14^{b}
Histidine	30.38±0.51^{a}	26.25±0.43^{b}	27.69±0.22^{b}	27.30±0.12^{b}
Isoleucine	27.65±0.47^{a}	23.91±0.39^{b}	26.51±0.21^{a}	27.43±0.12^{a}
Leucine	27.64±0.47^{a}	25.07±0.40^{b}	27.69±0.22^{a}	27.94±0.15^{a}
Lysine	32.61±0.55^{b}	33.98±0.55^{ab}	35.55±0.28^{a}	34.24±0.12^{ab}
Methionine	23.38±0.40^{b}	18.06±0.29^{d}	21.40±0.17^{c}	26.18±0.13^{c}

Table 10: Effect of 30% substitution of fish meal with different insect meals (TM: *Tenebrio molitor*, HI: *Hermetia illucens* or MD: *Musca domestica*) on serum metabolites of European sea bass

	FM	TM	HI	MD
Glucose (mg dl^{-1})	165.8±7.1^{b}	123.8±3.5^{c}	175.8±6.9^{b}	200.0±4.7^{a}
Cholesterol (mg dl^{-1})	237.8±4.0^{a}	148.6±7.9^{b}	229.8±7.6^{a}	130.7±5.2^{b}
Triglycerides (mg dl^{-1})	698.8±28.0^{ab}	412.5±39.1^{ab}	798.1±94.5^{a}	375.5±10.0^{b}
Phospholipids (mg dl^{-1})	735.5±79.1	622.0±103.6	797.0±43.5	570.9±43.2
Lactate (mg dl^{-1})	30.6±2.8^{ab}	17.8±4.2^{b}	36.8±7.9^{ab}	56.3±11.5^{a}
ALT (u l^{-1})	15.7±3.7	6.0±1.9	10.5±2.1	8.7±2.1
AST (u l^{-1})	61.0±13.1	47.3±10.5	52.0±4.1	64.1±10.2

Mean ± standard error, n = pooled samples of 5 fish per tank. Different letters in the same row denote statistically significant difference ($P < 0.05$).

Taurine	53.59±0.91^{c}	64.45±1.05^{b}	71.58±0.57^{a}	71.31±0.31^{a}
Hydroxyproline	72.13±1.22^{d}	84.22±1.37^{c}	89.18±0.71^{b}	93.18±0.40^{a}

Abbreviations: EAA, essential amino acids; NEAA, non-essential amino acids; Asx, sum of asparagine and aspartate; Glx, sum of glutamine and glutamate

Mean ± standard error, n = 9 fish per diet . Different letters in the same row denote statistically significant difference ($P < 0.05$).

Table 11: Effect of 30% substitution of fish meal with different insect meals (TM: *Tenebrio molitor*, HI: *Hermetia illucens* or MD: *Musca domestica*) on amino acid catabolism enzymes of European sea bass

	FM	TM	HI	MD
Alanine aminotrasferase (ALT)	0.62±0.04	0.66±0.01	0.51±0.11	0.55±0.05
Aspartate aminotransferase (AST)	0.66±0.08	0.48±0.07	0.50±0.13	0.58±0.05
Glutamate dehydrogenase (GDH)	0.08±0.01^{ab}	0.08±0.00^{ab}	0.09±0.01^{a}	0.05±0.00^{b}

Expressed as µmoles min^{-1} mg $protein^{-1}$. Mean ± standard error, n = 6 fish per diet. Different letters in the same row denote statistically significant difference ($P < 0.05$).

Highlights

- 30% of fish meal was substituted with *Tenebrio molitor*, *Hermetia illucens* or *Musca domestica* in the diets of European sea bass.
- Growth performance was similar in all dietary treatments.
- A minor increase in feed conversion ratio and a decrease in nutrient retention were observed when fish meal was substituted with *Tenebrio molitor* larvae meal.
- The fatty acid but not the amino acid profile was affected by the different insect meals inclusion.

Author statement:

The authors here declare their individual contributions:

Maria Mastoraki: Investigation, Formal analysis, Writing - original draft. **Paula Molla Ferrándiz**: Investigation. **Sofia C. Vardali**: Investigation, Formal analysis. **Demetrius C. Kontodimas**: Resources, Writing - review & editing. **Yannis P. Kotzamanis**: Investigation, Formal analysis, Writing - review & editing. **Laura Gasco**: Funding Acquisition, Resources, Writing - review & editing. **Stavros Chatzifotis**: Conceptualization, Writing - review & editing. **Efthimia Antonopoulou**: Conceptualization, Funding Acquisition, Writing - review & editing.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: