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Additional Information

Black soldier fly (*Hermetia illucens*) pre-pupae meal as a fish meal replacement in diets for European seabass (*Dicentrarchus labrax*)

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A B S T R A C T

A feeding trial was carried out to assess the effect of partially replacing fish meal (FM) by Black soldier fly pre-pupae meal (HM) in diets for European seabass *Dicentrarchus labrax* juveniles. A FM-based diet was used as a control and three other diets were formulated to include 6.5%, 13%, and 19.5% of HM, replacing 15%, 30% and 45% of FM respectively. Each diet was fed to triplicate groups of fish (initial weight: 50 g) for 62 days.

At the end of the trial, there were no differences among groups in growth performance or feed utilization. Plasma metabolic profiles also remained unaffected, except that plasma cholesterol was reduced with dietary HM inclusion. The apparent digestibility coefficients (ADC) of protein, lipids, dry matter, organic matter, and energy were generally high, and not affected by the dietary treatment. The ADC of arginine, histidine, and valine were higher in HM diets when compared to the control. Amylase and protease activities were not affected by dietary HM, while lipase activity was lower in HM 6.5 diets than in the control and HM 19.5 diets.

In conclusion, up to 19.5% of HM, corresponding to 22.5% of total dietary protein, may successfully replace FM in diets for juvenile European seabass, without adverse effects on growth performance, feed utilization or digestibility.

1. Introduction

Due to the expected increase in human population, the world will require an additional 23 million tons of aquatic food by 2030 to maintain current per capita fish consumption. This must come from aquaculture, as fisheries production has stabilized over the last decades (FAO, 2016). Most aquacultured fish are produced using aquafeeds, and intensive aquaculture production of carnivorous fish species employs high quality fish meal (FM) and fish oil as the main dietary ingredients (Oliva-Teles et al., 2015). However, increased demand for aquaculture feeds has led to rapid price increases for these commodities (FAO, 2016). To overcome these limitations, considerable research efforts are being made to reduce the dependency of aquafeed manufacturers on FM and fish oil (Glencross et al., 2007). Nowadays, a major challenge for aquaculture is to source sustainable supplies of terrestrial animal and plant feedstuffs for aquafeed production (Naylor et al., 2009; Oliva- Teles et al., 2015; Tacon et al., 2011).

In the last decades, attention has focused on the use of plant protein-rich feedstuffs in practical diets for carnivorous fish (Barrows et al., 2007; Gatlin et al., 2007; Oliva-Teles et al., 2015). However, plant feedstuffs have relatively low protein content, unbalanced essential amino acid profiles, low palatability, the presence of antinutrients, and competition with other food-feed industry sectors (Gatlin et al., 2007; Glencross et al., 2007; Krogdahl et al., 2010). This has pressured the search for nutritional strategies to improve utilization of plant protein- based diets (Gatlin et al., 2007; Magalhães et al., 2016; Pérez-Jimenez et al., 2012), as well as for other valuable alternatives to fish meal, such as animal feedstuffs, including slaughterhouse by-products or insect meals (IM) (Moutinho et al., 2016; Oliva-Teles et al., 2015).

Several reviews on the use of insects as ingredients for aquafeeds are available (e.g., Barroso et al., 2014; Makkar et al., 2014; Sánchez-Muros et al., 2014; van Huis, 2013; van Huis et al., 2013). Compared to conventional animal protein, insects have several advantages, including being reared on discarded organic by-products with low water input, high feed conversion efficiency, emission of low levels of greenhouse gases and ammonia, few animal welfare issues, and low risk of transmitting zoonotic infections (van Huis et al., 2013). Even though nutrient composition of IM is dependent on taxonomic group, rearing substrates and technological process, protein content is high (60–80%;

Sánchez-Muros et al., 2014) with a well-balanced essential amino acid profile (Alegbeleye et al., 2012; Barroso et al., 2014; Henry et al., 2015). Insect lipid content and fatty acid composition can be manipu- lated by rearing conditions and technological treatments (Barroso et al., 2014). Furthermore, use of defatted IM may avoid some constraints regarding fatty acid profile, mainly for marine fish species (Barroso et al., 2014; Henry et al., 2015). Insects are also good sources of minerals such as potassium, calcium, iron, magnesium, and selenium, and of several vitamins, levels of which depend on the rearing conditions (Henry et al., 2015).

Among different candidate species to produce IM, pre-pupae *H. illucens* (HM) is especially interesting, since standard

55 mass-rearing techniques for industrial production of high quality product already exist (Henry et al., 2015; van Huis et al., 56 2013). HM has an average protein content of 55% DM, a well-balanced essential amino acid profile, and circa 35% DM fat, 57 which may be reduced to 9 to 5% by defattening processes (Bußler et al., 2016). Moreover, if fish-offal or other omega-3 58 polyunsaturated fatty acid (PUFA) rich sources are included in the diet, larval omega-PUFA content will increase, making it 59 more suitable for carnivorous fish and marine fish diets (St-Hilaire et al., 2007).

60 Although HM is considered to have a nutritional value close to that of FM, replacement of FM by HM in aquafeeds 61 has not yet been as successful as hoped (Henry et al., 2015). Maximum dietary FM replacement level has ranged 62 from 6 to 25%, depending of the fish species (Henry et al., 2015), with highest levels being attained for rainbow 63 trout, *Oncorhynchus mykiss* (Sealey et al., 2011). High inclusion levels of HM reduce growth performance of channel 64 catfish, *Ictalurus punctatus*, rainbow trout, and turbot, *Psetta maxima* (Kroeckel et al., 2012; Newton et al., 2004; St- 65 Hilaire et al., 2007). In salmon, *Salmo salar*, dietary supplementation with lysine and methionine allowed dietary 66 HM level up to a maximum of 25% (Lock et al., 2016).

67 Until now, IM has been evaluated as a feed ingredient mainly for freshwater species; the only studies available for 68 marine fish are those of Kroeckel et al. (2012) for turbot and of Karapanagiotidis et al. (2014) with gilthead sea bream 69 *Sparus aurata*. As insects represent a part of most freshwater species' natural food, they may be more prone to use IM. 70 Insect utilization by marine fish may be problematic mainly related to chitin digestibility, palatability (Kroeckel et al., 71 2012), or perhaps taurine availability (El-Sayed, 2014).

72 Thus, this study aimed to evaluate the effect of dietary replacement of FM by HM on growth performance, plasma 73 metabolic profile, feed utilization, apparent digestibility, and digestive enzyme activities of European seabass juveniles.

74

75 ## 2. Materials and methods

76 ### 2.1. *Experimental diets*

77 Four experimental diets were formulated to be isoproteic and isolipidic. A FM-based diet was used as a 78 control and 3 other diets were formulated to include 6.5%, 13%, and 19.5% of IM - black soldier fly (*Hermetia illucens*) 79 pre-pupae meal (HM) - replacing 15%, 30% and 45% of FM. HM was supplied by Hermetia Deutschland GmbH & Co 80 KG, Baruth/Mark, Germany. Diets were supplemented with dicalcium phosphate to avoid phosphorus imbalance 81 and chromic oxide was incorporated as inert digestibility marker. All dietary ingredients were finely ground before mixing 82 and dry pelleted in a laboratory pellet mill (California Pellet Mill, Crawfordsville, IN, USA), through a 3 mm die. Pellets 83 were dried in an oven at 55 °C for 24 h and then stored at −20 °C until use. Proximate analysis and dietary composition 84 are presented in Table 1, and amino acid composition of the HM and of experimental diets are presented in Table 2.

85 ### 2.2. *Feeding trial*

86 The study was directed by accredited scientists (following FELASA category C recommendations) and conducted per the 87 European Union Directive (2010/63/EU) on the protection of animals for scientific purposes.

88 The feeding trial was performed at the Marine Zoological Station, University of Porto, with European seabass 89 (*Dicentrarchus labrax*) juveniles provided by *MARESA* (Mariscos de Estero S.A., Finca El Tambujal, Apdo de 90 correo 82, Ayamonte, *Huelva*).

91 The experimental system consisted of a thermo-regulated recircula- tion water system equipped with twelve fiberglass 92 tanks of 60 L water capacity, with a feces settling column connected to the outlet of each tank. Both tanks and settling columns 93 were designed according Cho et al. (1982). During the trial, water-flow per tank was about 4.5 L min^{-1}; temperature 94 averaged 25 ± 1 °C; salinity 36‰; dissolved oxygen was maintained above 8 mg L^{-1} and nitrogenous compounds were 95 kept below 0.02 mg^{-1}. Photoperiod was controlled to 12 h light as 12 h dark.

96 Fish were acclimatized to the experimental conditions for 15 days. Then, 12 homogenous groups of 10 fish with 97 an initial body weight of 50 g were established in each tank. Experimental diets were randomly allocated in triplicate 98 and fish were fed by hand, twice a day (at 9:00 and 16:00), 6 days a week, until visual apparent satiation. Utmost care 99 was taken to avoid feed waste and to assure that all feed supplied was consumed. Five days after trial start, feces were 100 collected daily for 20 days; 30 min after the afternoon meal, tanks, pipes, and settling columns were thoroughly cleaned. 101 Feces accumulated in each settling column were collected daily before the morning meal, centrifuged at 3000 × *g*, pooled 102 for each tank and stored at −20 °C until analysis. At the end of the trial, fish were bulk weighed following 1 day of 103 feed deprivation.

104 Apparent digestibility coefficients (ADC) of dry and organic matter, protein, amino acids, lipids and energy of the 105 experimental diets were calculated as follows:

106

107 ### 2.3. *Chemical analysis*

Chemical analyses of ingredients, diets, and feces were conducted as follows: dry matter, by drying the samples at 105 °C until constant weight; ash, by incineration in a muffle furnace at 450 °C for 16 h; protein (N × 6.25) by the Kjeldahl method following acid digestion, using Kjeltec digestion and distillation units (Tecator Systems, Höganäs, Sweden; models 1015 and 1026, respectively); gross energy by direct combustion of samples in an adiabatic bomb calorimeter (PARR Instruments, Moline, IL, USA; PARR model 1261); lipids in ingredients and diets, by extraction with petroleum ether using a Soxtec system (Tecator Systems, Höganäs, Sweden; extraction unit model 1043 and service unit model 1046); lipids in feces according to Folch et al. (1957); chromic oxide by acid digestion according to Furukawa and Tsukahara (1966).

Amino acid analysis was undertaken as described in Banuelos- Vargas et al. (2014). Briefly, samples were hydrolyzed for 23 h with 6 N hydrochloric acid at 110 °C under nitrogen atmosphere and derivatized with phenylisothiocyanate (PITC; Pierce) reagent before separation by high performance liquid chromatography (HPLC) in a Waters Reversed- Phase Amino Acid Analysis System (Waters auto sample model 717 plus; Waters binary pump model 1525; Waters dual absorbance detector model 2487), equipped with a PicoTag column. External standards (Pierce NC10180) were prepared along with the samples, and norleucine was used as an internal standard to detect any losses due to sample processing. Chromatographic peaks were identified, inte- grated, and quantified with the Waters Breeze software package by comparing to known amino acid standards.

2.4. Blood SAMPLING AND intestine

At the end of the trial, fish continued to be fed for 3 more days and the intestine was sampled from 3 fish per tank. To ensure that the intestines were full at sampling time, fish were continuously fed as recommended by Krogdahl and Bakke-McKellep (2005), to avoid bias due to fasting effects. Blood samples were collected from the caudal artery-vein complex using heparinized syringes. Blood was immediately centrifuged and the plasma and intestinal sections were frozen at −80 °C until analysis. Then, fish were euthanized with a sharp blow to the head and immediately eviscerated in an ice-cold tray. The digestive tract was separated from adipose and the intestine divided into anterior and posterior sections. Posterior intestine was distin- guished from the anterior intestine by the increased diameter, darker mucosa and annular rings. The anterior intestine was the portion directly after stomach and included pyloric caeca.

2.5. Digestive enzyme ACTIVITIES

For enzymatic activity measurement, each intestinal section was homogenized with ice-cold buffer (100 mM Tris–HCl, 0.1 mM EDTA, pH 7.8), centrifuged (30,000 ×*g*; 30 min; 4 °C) and the resultant supernatant collected and stored at −80 °C until analysis. All enzyme activities were determined using a PowerWavex microplate scanning spectrophotometer (Bio-Tek Instruments, USA).

Total proteolytic activity was measured by the casein hydrolysis method according to Walter (1984) and adapted by Hidalgo et al. (1999). The enzymatic determination was made using 0.1 M Tris HCl at pH 8, which is the optimum pH for physiological protease activity in seabass (Alliot et al., 1974). The reaction mixture, containing casein (1% w/v; 0.125 mL), buffer (0.125 mL) and homogenate supernatant (0.05 ml), was incubated for 1 h at 37 °C and stopped by adding 0.3 ml trichloroacetic acid (8% w/v) solution. After being kept for 1 h at 2 °C, samples were centrifuged (1800 ×*g* for 10 min) and the absorbance read at 280 nm. A tyrosine solution was used to establish a calibration curve. One unit of enzyme activity was defined as the amount of enzyme needed to catalyze the formation of 1.0 μmol of tyrosine per min.

α-Amylase (EC 3.2.1.1) activity was determined with a commercial kit (ref. 41201, Spinreact, Girona, Spain) with modification; the rate of product formation (2-chloro-4-nitrophenol) was quantified at 405 nm. Lipase (EC 3.1.1.3) activity was determined using a commercial kit (ref. 1001275, Spinreact, Girona, Spain) with modification; 1-2-O-dilauryl-rac-glycero-3-glutaric acid-60-methylresorufin-ester was used as sub- strate, and the formation rate of methylresorufin was followed at 580 nm.

Total soluble protein was determined according to Bradford (1976), using bovine serum albumin solution as standard.

Enzyme activity was expressed as specific activity; one unit (U) of activity was defined as μmol of product generated per minute.

2.6. PLASMA METABOLITES

Commercial kits from Spinreact, S.A (Girona, Spain) were used for plasma glucose (Kit, cod. 1001191), total proteins

161 (Kit, cod. 201001291), triglycerides (Kit, cod.1001312) and cholesterol (Kit, cod. 1001090), determination after validation
162 for use with marine fish species (Peres et al., 2013, 2014). All measurements were taken using a PowerWavex microplate
163 scanning spectrophotometer (Bio-Tek Instru- ments, USA).

164

165 3.Results

166 Fish promptly accepted the experimental diets and during the trial no mortality occurred. Growth performance, feed
167 intake, and feed efficiency were not affected by diet composition (Table 3). Protein efficiency ratio of fish fed diet HM19.5 was
168 lower than that of fish fed the control diet (HM0). Plasma metabolite levels were similar among diets, except for
169 cholesterol, which was lower in fish fed diet HM19

170 versus controls.appparent digestibility coefficients (ADC) of dry and organic matter, protein, lipids and energy were
171 high and unaffected by diet composition (Table 4). No differences were observed in the ADC of amino acids. However,
172 compared to the control, diet HM19.5 had a which was lower in fish fed the HM6.5 diets versus the control and HM19.5
173 diets. Independent of diet, amylase and lipase activities were higher in the posterior intestine, while no differences
174 between intestinal portions were observed for protease activity.

175

176 Discussion

177 Results of the growth trial indicate that HM can be included at least up to 19.5% in diets for European seabass
178 juveniles, replacing 45% of FM, without negative effects on growth performance. The fish promptly consumed all diets
179 and no differences on voluntary feed intake were observed, indicating that HM was palatable for seabass. Feed efficiency
180 was also unaffected by dietary composition. Similar results have been reported for other species of fish (Karapanagiotidis
181 et al., 2014; Lock et al., 2016; Sealey et al., 2011). However, reduced feed intake and feed efficiency with increasing
182 dietary HM incorporation has also observed in other studies (Gasco et al., 2016; Kroeckel et al., 2012). Protein efficiency
183 ratio decreased with increasing HM dietary incorporation. As the ADC of protein was not affected by diet composition,
184 this suggests a reduced efficiency of metabolic protein utilization.

185 Essential amino acid (EAA) patterns of the order Diptera are similar

186 to that of FM (Henry et al., 2015), which is considered as the protein with the best EAA profile for fish
187 (Oliva-Teles et al., 2015). Accord- ingly, the EAA composition of HM used in this study appear adequate, with no
188 limiting amino acid for European seabass (Kaushik, 1998; Peres and Oliva-Teles, 2006, 2007), even though lysine
189 and methionine content of the H19.5 diet was lower than the other diets. Nevertheless, the few studies that have
190 published the protein content and EAA composition of HM present some important differences (Makkar et al.,
191 2014; St-Hilaire et al., 2007; Sealey et al., 2011;). This may reflect differences in EAA composition between
192 Hermetia illucens from different sources, different larval/pupae stages employed, feeding, rearing con- ditions, and
193 other factors, such as the method of processing IM. For instance, the HM used in the present study was defatted
194 and had a crude protein content of 55.8% and a crude lipid content of 5.5%, while the average non-defatted HM
195 has a crude protein content of 42.1% and a crude lipid content of 26.0% (Makkar et al., 2014). Accordingly,
196 prior to use, the composition of each batch of HM meal should be analyzed to confirm its actual composition.

197 Almost all insect meals are considered low in lysine and tryptophan for fish and, except for HM, also limiting in
198 threonine and sulphur- amino acids (Makkar et al., 2014). The HM used in the present study had lysine and methionine
199 content very close to the estimated require- ments for European seabass. Consequently, EAA composition of all diets
200 met requirements for the species, except for a slightly low value for lysine in diet HM19.5. Thus, if higher levels of FM
201 replacement by HM are considered, dietary supplementation with lysine and methionine should be considered.

202 The taurine content of HM used in this study (0.8mg/kg) was also considerably lower than the value reported by
203 McCusker et al. (2014). In both cases, this is a very low value, indicating that HM is not a good source of taurine. Thus,
204 diet supplementation with taurine should also be considered when using HM, as taurine is required for European seabass
205 fed low-FM diets (Kanashiro et al., 2014).

206 The potential of HM as an alternative protein source for aquafeeds has already been evaluated in other species.
207 Soldier fly larvae fed alone or in combination (50:50) with high or low (45% or 30%) protein commercial diets
208 did not affect growth performance of channel catfish and tilapia (Oreochromis aureus) (Bondari and Sheppard, 1987).
209 Also, dietary replacement of 25% FM by HM did not affect yellow catfish (Pelteobagrus fulvidraco) growth
210 (Zhang et al., 2014a). In rainbow trout it was shown that growth performance was unaffected with the dietary inclusion
211 of 25% HM, but it was depressed with a dietary inclusion of 50% (St-Hilaire et al., 2007). In gilthead seabream
212 juveniles, HM may replace up to 30% FM without affecting fish performance (Karapanagiotidis et
213 al., 2014), while in turbot, growth performance was negatively affected at all dietary HM inclusion levels
214 (Kroeckel et al., 2012).

Maximum replacement level of FM by HM is, however, dependent of HM quality. Sealey et al. (2011) observed that 50% FM could be successfully replaced by fish offal-enriched HM in diets for rainbow trout, whereas replacement by normal HM negatively affected fish growth. Also, of two types of HM tested for Atlantic salmon, only one allowed total FM replacement of the diets without affecting fish performance (Lock et al., 2016).

Besides HM tested in this study, the mealworm beetle, Tenebrio molitor, was also studied as alternative insect protein source for European seabass. While for European seabass it was shown that it could replace up to 25% FM (Gasco et al., 2016) for African catfish (Clarias gariepinus) and catla-rohu hybrid (Catla × Labeo rohita) a FM replacement level of 30–40% was achieved (Nandeesha et al., 1988; Ng et al., 2001).

The profile of plasma metabolites observed herein were within reference values determined for European seabass (Peres et al., 2014) and, except for cholesterol, remained unaffected by dietary composi- tion. Cholesterolemia was, however, reduced by the inclusion of 19.5% HM in the diet. This effect may be due to the presence of chitin in HM (approximately 8.7% DM; Diener et al., 2009) as chitin contains high levels of chitosan, which was shown to have cholesterol-lowering properties in fish (Chen et al., 2014; Shiau and Yu, 1999). Chitosan has been reported to interfere with cholesterol absorption by binding with lipid (cholesterol) micelles, inhibiting their absorption, and increasing bile acid excretion (Khoushab and Yamabhai, 2010). Dietary chitin may also impair digestibility of other nutrients. Even though chitinase activity was detected in some fish species, chitinolytic action seems to be limited for most fish (Abro et al., 2014; Kroeckel et al., 2012; Krogdahl et al., 2005; Lindsay et al., 1984). Therefore, chitin mainly contributes to increased bulk, reduced feces retention time, and reduced enzyme accessibility to substrates (Zhang et al., 2014b).

Nonetheless, the ADC of the diets in the present study was high and not affected by dietary incorporation of HM. The ADC of protein, lipids, and dry matter of the diets used in the present study were very like those observed in another study with European seabass using meal- worm beetle meal incorporated at circa 25% (ADC DM: 80%; ADC CP: 92%; ADC CL: 97%; Gasco et al., 2016). This seems to indicate that insect meals are well digested by seabass. These in vivo ADC data are considerably higher than the in vitro crude protein digestibility determined for H. illucens and T. molitor determined by Marono et al. (2015), which ranged from 65.8 to 68.7%. This indicates that the in vitro method may not be adequate for estimating the ADC of protein for fish (Gomes et al., 1998; Moyano et al., 2015). Digestibility of HM was also determined for turbot, showing low/moderate digestibility for organic matter, crude protein, crude lipid, and gross energy (Kroeckel et al., 2012).

This is the first study in fish evaluating the amino acid digestibility of HM containing diets. Overall, amino acid digestibility was high and independent of dietary HM inclusion, except for arginine and histidine, which increased. The high ADC of arginine is particularly interesting, as it shows that HM, besides being a good source of arginine, has a high bioavailability for this EAA, which is usually one of the first limiting essential amino acids in plant feedstuffs.

Total protease activity was higher than amylase and lipase activ- ities, as has been observed previously with seabass (Magalhães et al., 2015). Even though amylase, lipase, and proteases are secreted into the anterior section of the intestine, and therefore their activity is expected to be higher in that portion of the intestine, this was not the case in the present or other studies (Magalhães et al., 2015; Pérez-Jiménez et al., 2009). Digestive enzyme activities and digesta transit time along the gastrointestinal tract may be affected by diet composition (Castro et al., 2013, 2015; Pérez-Jiménez et al., 2009), and our results also seem to indicate a drag of secreted enzymes to the posterior intestine rather than an increased activity of these enzymes in this intestinal portion.

The fatty acid profile of HM usually has lower LC-PUFA than FM (Barroso et al., 2014) and it is known that fish lipase has higher affinity for LC-PUFA glycerides (Bakke et al., 2011). Thus, the HM fatty acid profile may modulate lipase activity and affect the ADC of lipids. In the present study, even though there were some small differences in lipase activity among diets, no differences in ADC of lipids was observed.

In conclusion, this study indicates that up to 19.5% of black soldier fly (Hermetia illucens) pre-pupae meal (corresponding to 22.5% of dietary protein), may successfully replace 45% FM in diets for juveniles of European seabass, without any adverse effect on growth perfor- mance, feed utilization, apparent digestibility coefficients or digestive enzyme activity. Further research testing higher dietary HM inclusion levels, as well as detailed economic analysis of its incorporation in the diets, are needed to better evaluate the potential of HM inclusion rates in commercial aquafeeds.

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