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When a turbot catches a fly: Evaluation of a pre-pupae meal of the Black Soldier Fly (*Hermetia illucens*) as fish meal substitute — Growth performance and chitin degradation in juvenile turbot (*Psetta maxima*)

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ABSTRACT

Larvae of black soldier flies (*Hermetia illucens*) are commercially produced on agricultural waste streams and convert these into animal body protein and fat. A feeding trial was carried out for 56 days in a recirculating aquaculture system (RAS) by replacing fish meal protein subsequently by *Hermetia* meal (HM) protein. Six diets were formulated for the replacement and contained 0%, 17%, 33%, 49%, 64%, and 76% of HM (54.1 ± 1.1% crude protein, 13.4 ± 0.7% crude lipid, dry matter basis). The diets were fed to triplicate groups of turbot 54.9 ± 0.9 g once a day by hand until apparent satiation. Feed intake was affected by dietary HM inclusion and decreased with increasing HM incorporation due to low palatability. Growth performance was high, but affected by dietary HM inclusion. SGR was lower in all treatments containing HM whereas FCR was significantly higher at HM inclusion levels > 33%. Protein retention was highest at HM inclusion ≤ 33% and decreased significantly with increasing HM supplementation. Whole-body protein content was not affected by treatment, while body lipid decreased with increasing HM inclusion levels. The apparent digestibility coefficients (ADC) of HM were low for organic matter, crude protein, crude lipid, and gross energy. Chitinase activity or chitinolytic active bacteria were not detected in the mid gut of turbot. The presence of chitin might have influenced the feed intake, availability, and digestibility of the nutrients and therefore growth performance. In general, our study shows that the incorporation of HM protein in fish diets is possible, but limited by its low nutritive value. Considering that HM is produced on local greenhouse waste streams, HM might be a feasible alternative protein source for the partial replacement of fish meal. Further research on HM meal processing to increase nutrient utilization is needed.

1. Introduction

Shortages in fish meal and oil supply resulted in rising prices of formulated diets, while they are the main expenditure in intensive aquaculture production (40–70% of production costs) (Wilson, 2002). Especially, carnivorous flatfish e.g. turbot (*Psetta maxima*), plaice (*Pleuronectes platessa*), common sole (*Solea solea*), and Atlantic halibut (*Hippoglossus hippoglossus*) have high dietary protein demands of 500 up to 650 g kg^{-1} DM, thus the aquaculture industry has to face this problem and identify alternative feed sources in order to decrease dietary costs (Lee et al., 2003). The production of flatfish in aquaculture systems in Europe, headed by turbot, is growing steadily in the last years and profound knowledge on the physiology of turbot is needed to meet the dietary requirement of this aquaculture fish species under the fluctuating feed source supply (Brown, 2002; Person-Le Ruyet, 2002). Several studies have assessed the use of alternative plant based proteins including their nutritional value in flat fish (Bonaldo et al., 2011; Burel et al., 2000a,b; Fournier et al., 2004; Nagel et al., 2012b; Regost et al., 1999; Slawski et al., 2011). Nevertheless, these plant materials are often deficient in essential amino acids, especially lysine or methione, and therefore their utilization is limited as observed in turbot (Regost et al., 1999; Slawski et al., 2011), Japanese flounder (*Paralichthys olivaceus* ; Deng et al., 2006) and halibut (Helland and Grisdale-Helland, 2006). Still, the quality of these products is high and utilization as animal feed might thus compete with its exploration for human nutrition.

Only some studies were investigated with the usage of alternative animal proteins in nutrition for turbot. The replacement of 250 g kg^{-1} FM protein by poultry by-product meal was successful in diets for Black Sea turbot (*Psetta maeotica*) (Yigit et al., 2006). However, determined ADC's

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for feather meals, poultry meat meal and hemoglobin meal were rather low in turbot when compared to gilthead sea bream (*Sparus aurata*) or European sea bass (*Dicentrarchus labrax*) (Davies et al., 2009). In Germany, commercial production of the black soldier fly was developed in order to provide an alternative protein source for feed purposes. Applied research is now needed to fill the knowledge gaps by utilizing the pre-pupae of *Hermetia illucens* in fish diets. Larvae of *Hermetia* fly feed on animal manure and plant material and are capable to convert low valued organic waste into protein rich biomass (Diener et al., 2009). Due to the amount of protein (476 g kg^{-1}, dry matter DM) and fat (118 g kg^{-1}, DM) and the well balanced essential amino acid (EAA) profile, the pre-pupae meal might be a suitable alternative for fish meal. HM meets macronutrient requirements of terrestric animals and fish; former studies showed suitability of HM as ingredient in diets for swine and broiler chickens as well as in nutrition of rainbow trout (*Oncorhynchus mykiss*), or as pre-pupae in channel catfish (*Ictalurus punctatus*) and blue tilapia (*Tilapia aurea*) (Bondari and Sheppard, 1981; Elwert et al., 2010; Newton et al., 1977; Stamer et al., 2008; St-Hilaire et al., 2007). However, the exoskeleton of the black soldier fly pre-pupae contains the polysaccharide chitin (approximately 87.0 g kg^{-1}, DM) which might affect the digestibility and the utilization of other nutrients (Diener et al., 2009; Shiau and Yu, 1999). If chitinolytic activity is observed in fish, the ingested chitin might have a substantial nutritive function of energy intake (Fines and Holt, 2010; Goodrich and Morita, 1977a,b). Chitinase activity has been detected in blood, plasma and intestinal tract of fishes, but not yet proven to actually break down the chitin fraction in 6 marine teleosts (Fänge et al., 1976, 1979) and rainbow trout (*Salmo gairdneri*, Lindsay, 1984; Lindsay et al., 1984). However, it has been documented that the marine species cobia (*Rachycentron canadum*) is capable to digest chitin from either shrimp or crab meal revealing high endogenous chitinolytic activity in the stomach (Fines and Holt, 2010). In cod (*Gadus morhua*) the enzyme chitinase was found in stomach and intestinal tract (Danulat, 1986a,b; Danulat and Kausch, 1984; Lindsay, 1987), but no chitinolytic bacteria activity was reported. No research was realized on digestibility of chitin in turbot. But due to its natural feeding profile, the ability to digest chitin might be possible. Thus, aim of this study was to elucidate the nutritional value of *Hermetia* meal for juvenile turbot by determining the growth potential, feed intake and nutrient retention efficiencies. Special emphasize was given to digestibility of the HM itself and the capability of turbot to break down chitin by the endogenous enzyme chitinase or by the activity of chitinolytic bacteria.

2. Materials and methods

2.1. Diet formulation and diet preparation

Hermetia meal (HM) was obtained from a commercial producer (Hermetia Futtermittel GbR, Baruth/Mark, Germany). For the production of HM, the pre-pupae of the black soldier fly were collected from the substrate and freezed at −24 °C. The frozen pupae were cut to enable the leakage of intracellular fat from the larvae. This material was transferred into a tincture press (Fischer Maschinenfabrik GmbH, HP-5MT-VA, Neuss, Germany) and pressed at 450 bar at 60 °C for

Table 1
Formulation (g kg^{-1} feed), proximate composition (g kg^{-1} dry matter), and amino acid composition (g kg^{-1} crude protein) of the raw materials (HM = *Hermetia* meal, FM = fish meal) and experimental diets used in the feeding trial.

	Raw material		Diets					
	FM	HM	HM%0	HM%17	HM%33	HM%49	HM%64	HM%76
Ingredients (g kg^{-1}, dm)								
Herring meal[a]			687	550	422	305	180	80
Hermetia meal[b]			0	165	332	486	640	756
Wheat protein[c]			20	20	20	20	20	20
Blood meal[d]			50	50	50	50	50	50
Wheat starch[c]			148	130	102	75	57	50
Fish oil[a]			90	80	69	59	49	39
Vit/min. mix[e]			5	5	5	5	5	5
Proximate composition (g kg^{-1}dry matter)								
Moisture	73	44	78	64	59	62	52	47
Crude lipid	98	118	128	130	142	142	132	128
Crude protein [f]	641	476	548	549	537	539	533	527
Chitin	0	96	0	16	32	47	61	73
NfE [g]	95	151	194	166	148	138	128	120
Crude ash	166	159	130	139	141	134	146	152
Calcium	21	65	31	35	39	44	48	51
Phosphorus	29	7	17	15	13	11	9	7
Gross energy (MJ kg^{-1})	22	21.1	21.6	21.5	21.8	21.9	21.5	21.3
Essential amino acids (g kg^{-1} crude protein)								
Arginine	59.3	57.1	61.2	58.2	53.4	56.1	51.2	49.2
Cystine	8.7	8.2	9.0	8.7	8.2	8.6	8.0	7.6
Histidine	20.9	33.4	26.4	27.0	29.0	28.1	29.4	30.4
Isoleucine	39.8	51.9	37.3	37.6	39.7	39.5	40.3	40.5
Leucine	71.0	86.3	76.8	76.2	77.3	77.9	77.2	77.6
Lysine	71.6	71.2	66.9	64.9	62.1	64.5	60.5	59.2
Methionine	26.4	21.8	23.3	21.9	19.9	21.3	19.1	18.1
Phenylalanine	39.9	38.9	42.1	42.6	45.2	45.7	46.3	48.3
Threonine	41.2	46.4	38.7	38.7	38.3	38.0	37.4	37.8
Valine	48.1	72.1	53.3	54.5	59.2	57.5	60.9	61.9

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[d] Euroduna-Technologies GmbH, Barmstadt, Germany.
[e] AllerAqua, Christiansfeld, Denmark.
[f] Protein content calculated by nitrogen * 6.25; protein content was corrected for chitin.
[g] NfE, nitrogen-free extract = 1000 − (crude protein + crude lipid + crude ash + chitin).

30 min. The defatted material was dried for 20 h in an oven at low temperature (60 °C) and grinded to a meal.

Proximate composition of the main ingredients FM and HM (moisture, crude protein, crude fat, ash, energy, phosphor and calcium) are presented in Table 1. All diet ingredients were mixed and experimental diets were pressed to pellets of 4 mm in diameter using a pellet-press (L 14–175; AMANDUS KAHL, Reinbeck, Germany).

For the feeding trial six experimental diets were formulated with increasing levels of HM. Therefore, fish meal protein was subsequently replaced by HM protein at 0%, 17.8%, 35.9%, 52.2%, 69.2%, and 81.7%. The diets are designated in correspondence to the total amount of HM per kg fish feed as follows: HM%0, HM%17, HM%33, HM%49, HM%64, and HM%76. All six diets were formulated to be isonitrogenous and isocaloric (Table 1). Amino acid profiles of the diets were above estimated requirements (Kaushik, 1998; Peres and Oliva-Teles, 2008) for juvenile turbot for all EAA and no supplementation of free amino acids was necessary. Fatty acid composition of the HM and all experimental diets is given in Table 2.

For the digestibility trial, two diets were prepared and supplemented with titanium dioxide as inert marker. By replacing 30% of the basal diet by the test ingredient HM, the apparent digestibility coefficients (ADC, %, Table 3) were determined according to the protocol of Glencross et al. (2010).

2.2. Experimental setup

The experiments were conducted at the Gesellschaft für Marine Aquakultur (Büsum, Germany) and juvenile turbot (*P. maxima*) were obtained from Maximus A/S (Bedsted Thy, Denmark). For the feeding trial 18 rectangular aquaria (bottom surface 0.3 m^2, total water volume 175 L) were arranged as a recirculation aquaculture system (RAS) and equipped with mechanical and biological filter and a disinfection unit (UV-filter). Water discharge was approximately 1370 L kg^{-1} feed and the photoperiod was a 12 L:12D cycle. Water parameters were determined daily (temperature 16.5 ± 0.2 °C, dissolved oxygen 8.2 ± 0.2 mg l^{-1} with a Handy Polaris; OxyGuard International A/S, Birkerod, Denmark; salinity 27 ± 1 g l^{-1} with a digital refractometer HI 96822; Hanna Instruments, Woonsocket, USA, 0.2 ± 0.1 mg l^{-1} NH_4–N, 1.4 ± 0.3 mg l^{-1} NO_2–N; Microquant test kit; Merck KGaA, Darmstadt, Germany).

Table 2
Fatty acid composition and sum of saturated fatty acids (SAFA), mono-unsaturated fatty acids (MUFA) and poly-unsaturated fatty acids (PUFA) of HM and experimental diets (% total fatty acids) used in the feeding trial.

Fatty acid	Raw material	Diets					
	HM pure	HM%0	HM17%	HM%33	HM%49	HM%64	HM%76
10:0	0.9	<0.1	0.2	0.3	0.4	0.5	0.7
12:0	47	1.2	8.8	14.1	21.6	28.4	33.9
14:0	6.5	6	6	6.1	6.2	6.3	6.3
16:0	15	16.8	16.2	15.4	15.3	15.3	15
18:0	2.2	2.8	2.7	2.5	2.4	2.4	2.3
16:1n−9	3.1	4.5	4.2	4.3	4	3.8	3.6
18:1n−9	14	15.5	15.2	14.2	14.3	14.5	14.5
18:1n−7	0.2	2.9	2.4	2.1	1.6	1.2	0.9
20:1n−9	<0.1	9.4	7.6	6.5	4.9	3.6	2.5
22:1n−11	<0.1	13.5	10.8	8.8	6.5	4.7	3.1
22:1n−9	<0.1	0.7	0.6	0.5	0.4	0.3	0.2
18:2n−6	9.4	5.5	6.4	6.8	7.7	8.5	9.1
20:4n−6	<0.1	0.5	0.4	0.4	0.3	0.2	0.2
18:3n−3	0.8	1.7	1.6	1.5	1.3	1.2	1.1
18:4n−3	<0.1	1.6	1.4	1.6	1.2	0.8	0.6
20:5n−3	<0.1	4.5	4	4.2	3.2	2.2	1.6
22:5n−3	<0.1	1	0.9	1	0.8	0.5	0.4
22:6n−3	<0.1	7	6.3	6.5	4.9	3.3	2.2
Σ SAFA	71.6	26.8	33.9	38.4	45.9	52.9	58.2
Σ MUFA	17.3	46.5	40.8	36.4	31.7	28.1	24.8
Σ PUFA	10.2	21.8	21	22	19.4	16.7	15.2

Table 3
Formulation (g kg^{-1} feed) and proximate composition (g kg^{-1} dm) of the basal (0% HM) and the test diet (30% HM) for the determination of the apparent digestibility.

	Basal diet	Test diet
Ingredients (g kg^{-1}, feed)		
Fish meal	690	483
Hermetia meal	0	300
Wheat starch	145	101.5
Fish oil	150	105
Vit/min. mix	5	3.5
Titaniumdioxid[a]	10	7
Proximate composition (g kg^{-1}, dm)		
Moisture	85	53
Crude lipid	151	177
Crude protein	590	555
NfE	119	100
Crude ash	140	168
Phosphorus	17.3	10.9
Gross energy (MJ kg^{-1})	21.82	21.91

[a] Kronos Titan GmbH & Co.oHG, Nordemham, Germany.

Fish were randomly stocked in the experimental tanks. For a two week acclimatization period to environmental conditions fish were fed with a commercial dry feed (Aller Aqua, 4 mm). After this period of acclimatization, 10 fish were sacrificed (n = 10) with an overdose of the anesthetic tricaine methanesulphonate (MS 222) and stored at −20 °C for further analysis. A total of 180 fish were weighed individually to the nearest of 0.1 g (initial weight 54.9 ± 0.9 g), length (14.8 ± 0.4 cm) was measured (0.1 cm) and animals were randomly restocked to the experimental tanks (10 individuals per tank). Prior to weighing fish were starving for 24 h. Each experimental group was reared in triplicates and fed once a day by hand (09:00) until apparent satiation over a period of 54 days. The amount of diet supplied to each tank was recorded daily and remaining feed in the tank was siphoned out, analyzed for dry matter and subtracted from feed offered (on dry matter basis) to calculate actual daily feed intake.

For the fecal sampling a total of 66 fish (96.4 ± 22 g) were housed in the RAS under same experimental conditions and fed the basal or test diet (incorporation 30% HM) once per day in duplicates at a feeding level of 1.0% BW d^{-1} over a period of 28 days. Feces of all fish were collected 10 to 12 h after the last meal by dissection of the rectum (Koven et al., 1997), and stored at −20 °C and subsequently analyzed for proximate composition (gross energy, gross protein, dry matter).

2.3. Sampling and chemical analyses

At the end of the feeding trial fish were weighed and length was measured individually. From each tank 3 fish were sacrificed and stored until further analysis at −20 °C. Additionally, 3 fish per tank were sampled for liver weight to determine hepatosomatic index (HSI). Feed, fish and feces were analyzed for their respective proximate composition. Therefore, fish and feces were freeze-dried (for 120 h and 72 h respectively), homogenized and stored at −20 °C; experimental diets were homogenized and stored at −20 °C. Dry matter was analyzed in duplicate after drying the samples at 103 °C until constant weight was obtained. Ash was determined by incineration at 550 °C for 4 h with a combustion oven (P300; Nabertherm Lilienthal, Germany). Analysis for calcium and phosphorus were carried out by the LUFA-ITL GmbH (Kiel, Germany) following the VDLUFA VII 2.2.2.6 protocol. Chitin was analyzed by the Seehof-Laboratorium (F&E GmbH, Wesselburenerkoog, Germany) according to the protocol of Lovell et al. (1968) by applying the formic acid method and nitrogen content was consequently corrected for chitin. Total nitrogen content was determined by the Kjeldahl method (InKjel 1225 M, WD 30; Behr, Düsseldorf, Germany). Protein content of the raw material, diets, fish, and feces were calculated by multiplying N with 6.25. Dietary amino acid concentrations were analyzed by Evonik industries (Hanau,

Germany) in triplicates following the protocol of a liquid based chromatography. Crude fat was analyzed gravimetrically after extraction with petroleum ether with a Soxleth system (R 106 S; Behr, Düsseldorf, Germany). Fatty acid composition was determined by LUFA-ITL GmbH (Kiel, Germany) following the protocol of DGF C VI 10a/11a. Gross energy was measured in a bomb calorimeter (C 200; IKA, Staufen, Germany). Tracer (TiO_2) concentration in feed and feces was measured photometrically (Short et al., 1996).

2.4. Chitinase assay and chitinolytic bacteria sampling

To assess the digestion of chitin, the midgut contents of 3 fish per tank were sampled for chitinase activity as well as chitinolytic bacteria after culture on chitin agar. This sampling was carried out 6 h post feeding. Here, all chitinolytic activity was purely attributed to chitinase. Chitinase activity was assayed according to Jeunniaux (1966) modified by Gutowska et al. (2004) by an end product measurement of N-acetyl-D-glucosamin (NAG) according to Reissig et al. (1955), determining NAG in duplicate from a standard dilution series. Briefly, the chitinase assay was adapted to a micro plate scale and measured with an Infinite 200® TECAN spectrometer at 585 nm absorbance (TECAN Group Ltd., Männedorf, Switzerland). As substrate, a colloidal chitin suspension was prepared at a concentration of 5 mg ml^{-1}, since synthetic chitin substrates (glycol chitosan, chitin azure) have been reported to differ in pH optima from natural homogenates (Clark et al., 1988). The intestinal contents were diluted, homogenized, and adjusted to pH 8. Chitinase activity was determined in the natural homogenate, assessing the digestion of chitin into tri- and disaccharides by chitinase and the subsequent cleavage to monomer NAG by chitobiase. In parallel, 200 µl β-glucosidase (6 U ml^{-1}, Roth, Germany) was added to compensate for low chitobiase activity, assuring the determination of total chitinase activity. The activity of chitinase was expressed as µg NAG produced per gram tissue (wet weight) per hour (µg NAG h^{-1} g^{-1}). The detection limit was determined by adding commercial chitinase from *Streptomyces griseus* (Sigma, Germany) upon addition of 200 µl β-glucosidase.

Chitinolytic active bacteria were identified on a colloid chitin agar according to Danulat and Kausch (1984), Danulat (1986a,b) and Lindsay et al. (1984). A 30% agar containing 0.02% of yeast and 5% colloidal chitin but no further nutrients was prepared with sterilized seawater and adjusted to pH 8. The intestinal contents of six fish were individually homogenized. Chitinolytic colony forming units (CFU) were determined by dilution series as colorless halo around the colony after culture at 18 °C up for 42 days. CFU were counted at day 7, day 21, and day 42.

2.5. Statistics

Data are presented as means with standard deviation. Values obtained for growth parameters, whole body composition, biometric parameters and nutrient retention were checked for normal distribution using the Kolmogoroff Smirnov Test. Same data were subjected to a one-way analysis of variance (ANOVA) using SPSS 17.0 for Windows (SPSS Inc., Chicago, US). Means of the treatments were compared by the post-hoc test Tukey HSD. Differences between treatments were considered significant at a p-level of <0.05.

3. Results

3.1. Growth performance

During the experiment no mortality of the fish was recorded. All fish accepted the experimental diets which were not strongly influenced by dietary formulation and feed intake could be determined precisely. Feed intake (%BW d^{-1}) differed between the groups and was significantly lower at higher HM inclusion (≥33%) compared to all other diets (Table 4). Fish fed the control diet had a significantly higher final weight and specific growth rate (SGR) than those found in fish fed the diets containing HM. Feed conversion ratio (FCR) was same for diet HM%0, HM%17, and HM%33. At higher inclusion, FCR significantly increased and highest value was recorded for fish fed HM%76 (Table 4).

3.2. Body composition and nutrient retention

Whole-body composition and biometric parameters are shown in Table 5. A significant effect of HM inclusion was apparent on moisture, lipid, and energy content of the whole fish. Lipid content decreased with increasing inclusion level of HM, correlated to the moisture content. CP and CA content did not differ between the treatments. Condition factor (CF) was highest in fish fed the control diet, HM%17 and HM%33. At higher inclusion levels, CF decreased significantly and was lowest for fish fed with highest HM inclusion level. Additionally, hepatosomatic index (HSI) was affected by dietary treatment and highest for fish fed the control diet, HM%17 and HM%76.

Data on nutrient retention efficiency are presented in Table 6. The protein efficiency ratio PER and the crude protein retention efficiency (GPRE) were affected by dietary treatment and decreased significantly in treatment HM%49, HM%64 and HM%76. The efficiency of crude lipid retention (GLRE) ranged from 70.4 to 17.2% and was affected by dietary treatment and decreased significantly at inclusion levels of HM 33% and higher. Gross energy retention efficiency (GERE) was affected by dietary treatment and decreased with inclusion of >17% HM significantly ($p<0.05$).

3.3. Digestibility of the diets and raw material

Feed level during the digestibility trial was maintained at 1.05 ± 0.01 and 1.08 ± 0.04% BW d^{-1} for the basal and the test diet including 30% HM. The evaluation of the growth parameters were not considered in this trial. The ADC's for the control diet and the test diet with 30% HM incorporation are given in Table 7. For all parameters, the control diet resulted in higher ADC values compared to the test diet. The calculated ADC's for *Hermetia* meal were 45.2% for organic

Table 4
Growth performance of juvenile turbot fed the control and experimental diets over 56 days (mean ± sd). Values with different superscripts are significantly different ($p<0.05$).

	HM%0	HM%17	HM%33	HM%49	HM%64	HM%76
Wi[1]	55 ± 1	54 ± 0	55 ± 1	56 ± 1	55 ± 1	54 ± 1
Wf[2]	139 ± 8^{a}	124 ± 4^{b}	120 ± 5^{b}	106 ± 3^{c}	91 ± 2^{d}	77 ± 2^{e}
FI %BW d^{-1} [3]	1.46 ± 0.1^{a}	1.30 ± 0.1^{a}	1.34 ± 0.0^{a}	1.14 ± 0.1^{b}	1.02 ± 0.0^{b}	0.85 ± 0.0^{c}
SGR[4]	1.73 ± 0.1^{a}	1.53 ± 0.1^{b}	1.43 ± 0.1^{b}	1.19 ± 0.1^{c}	0.94 ± 0.0^{d}	0.63 ± 0.0^{e}
FCR[5]	0.76 ± 0.0^{a}	0.76 ± 0.0^{a}	0.82 ± 0.0^{a,b}	0.86 ± 0.0^{b}	0.98 ± 0.0^{c}	1.21 ± 0.0^{d}

[1] Wi = initial body weight (g).
[2] W_f = final body weight (g).
[3] Daily feed intake (% body weight day^{-1}).
[4] Specific growth rate (% day) = [ln (W_f) − ln (W_i)]/feeding days × 100.
[5] FCR = feed conversion ratio = feed intake (g)/weight gain (g).

Table 5
Proximate whole body composition (% original substance) and biometric parameters of initial sample and turbot fed the control and the experimental diets over 56 days (mean ± sd). Values with different superscripts are significantly different ($p<0.05$).

	Initial fish (n = 10)	HM%0	HM%17	HM%33	HM%49	HM%64	HM%76
Proximate body composition (% original substance)							
Moisture	77.1	75.5 ± 0.3^{a}	$76.6 \pm 0.7^{a,b}$	$76.3 \pm 0.1^{a,b}$	77.2 ± 0.5^{b}	77.4 ± 0.4^{b}	77.4 ± 0.3^{b}
Crude lipid	4.3	5.8 ± 0.3^{a}	$4.8 \pm 0.6^{a,b}$	$4.8 \pm 0.3^{a,b}$	4.5 ± 0.5^{b}	4.1 ± 0.4^{b}	3.8 ± 0.4^{b}
Crude protein	14.3	15.2 ± 2.2	15.2 ± 2.8	15.5 ± 2.2	14.9 ± 1.5	15.0 ± 2.1	15.2 ± 3.2
Crude ash	4.3	3.4 ± 0.2	3.4 ± 0.1	3.5 ± 0.1	3.4 ± 0.1	3.5 ± 0.1	3.4 ± 0.2
Gross energy (MJ kg^{-1})	4.83	5.7 ± 0.2^{a}	$5.4 \pm 0.1^{a,b}$	$5.4 \pm 0.2^{a,b}$	$5.2 \pm 0.2^{b,c}$	4.0 ± 0.2^{c}	4.9 ± 0.1^{c}
Biometric parameters							
CF, g $(cm^3)^{-1}$ [1]	n.d	2.02 ± 0.03^{a}	$1.93 \pm 0.03^{a,b}$	$1.92 \pm 0.02^{a,b}$	$1.88 \pm 0.03^{b,c}$	1.81 ± 0.05^{c}	1.69 ± 0.06^{d}
HSI (%) [2]	n.d	2.79 ± 0.36^{a}	$2.24 \pm 0.34^{a,b}$	2.09 ± 0.08^{b}	1.96 ± 0.06^{b}	1.73 ± 0.24^{b}	$2.34 \pm 0.14^{a,b}$

[1] Condition factor (body weight/total length^3) * 100.
[2] Hepatosomatic index (liver weight/body weight) * 100.

matter, 63.1% for crude protein, 78.0% for crude lipid, and 54.5% for gross energy.

3.4. Chitinase activity

Standard dilution series for the NAG quantification revealed regression coefficients $R^2 > 95\%$ allowing accurate quantification. Neither chitobiase (without ß-glucosidase) nor chitinase activity was detected (addition of ß-glucosidase). The detection limit for chitinase activity was 156 µg NAG h^{-1} g^{-1} for the intestinal contents.

3.5. Chitinolytic active bacteria

On average $35.0 \pm 4.3 * 10^{-4}$ CFU per 0.1 ml of intestinal sample were observed. No halos around the CFU were detected.

4. Discussion

To our knowledge no digestibility trial with HM as alternative protein source was carried out in fish yet. In the present study the feces were collected 8 h to 10 h post feeding by killing the fish and dissection of the posterior intestine. Various techniques can be used to collect feces and should be adapted to experimental conditions e.g. fecal consistence. This dissection method was used successfully in studies by De Schrijver and Ollevier (2000), Dias et al. (2010), and Koven et al. (1997), reducing the degradation and dispersal of the feces. The use of feces collection systems was rejected because of the instable consistence of turbot feces, and stripping of turbot was hampered by the intestinal loops (De Groot, 1971).

The organic matter digestibility was 83.2% for the fish meal based diet and decreased with HM inclusion to 71.0%, resulting in a low organic matter ADC of HM itself (45.2%). The protein digestibility of the fish meal basal diet was 89.1%. The $ADC_{protein}$ for the test diet was reduced (81.1%) and are comparable to values obtained by Regost et al. (1999) by inclusion of 20 or 40% of corn gluten meal CGM in a diet of turbot. However, the $ADC_{protein}$ for the test ingredient HM, was only 63.1% and therefore substantially lower compared to values obtained for extruded peas (87.9%) , extruded lupines (96.2%), and rape seed meal (70.0–76.4%) of juvenile turbot of 110 g initial weight applying the fecal settling method (Burel et al., 2000a). In *Solea senegalensis*, CGM and soy bean meal SBM resulted in higher $ADC_{protein}$ (90.6% and 86.7% for CGM and SBM, respectively) compared to the present study at same inclusion level and same feces collection method (Dias et al., 2010). In comparison to alternative animal protein sources the $ADC_{protein}$ of HM was higher than values observed for hydrolyzed feather meal (HFM, $ADC_{protein} = 46.6\%$) or enzymatic treated feather meal ($ADC_{protein} = 36.0\%$) obtained for turbot with an initial weight of 131 g, whereas poultry meat meal (PMM) and spray-dried hemoglobin meal (SDHM) resulted in higher values for ADCprotein (78.4% and 74.8% respectively) (Davies et al., 2009). Latter authors discussed the low ADC values found for feather meals with the resistance of feather meals to proteolytic activity in the intestinal tract of fish without improving the ADC by an enzymatic treatment. Low digestibility was found for energy and lipid of HM. ADC_{lipid} was 78.0% and even lower values were obtained for energy (54.4%). Davies et al. (2009) obtained extremely low lipid and energy digestibility for PMM (ADC_{lipid}: 54.6% and ADC_{energy}: 45.2%) SDHM (ADC_{lipid}: 34.6% and ADC_{energy}: 35.6%) and HFM (ADC_{lipid}: 37.1% and ADC_{energy}: 21.7%). Those low values were reported to be a result of the short digestive tract of turbot and a low metabolic rate of turbot. Ogunji et al. (2009) determined the ADC's of housefly maggot meal and obtained higher values compared to the present study in carp (*Cyprinus carpio*, ADC_{lipid}: 96.8%, $ADC_{protein}$: 84.9%, ADC_{energy}: 74.9%) and lower values for Nile tilapia (ADC_{lipid}: 86.1%, $ADC_{protein}$: 57.7%, ADC_{energy}: 58.1%), which was explained by spawning activity and feces condition. The low lipid digestibility values observed in the present study are in line with Shiau and Yu (1999) which found that lipid digestion in diets for tilapia supplemented with chitin was significantly decreased. The influence of dietary structural polysaccharides, like chitin, is reported to inhibit nutrient absorption from intestinal tract and thereby reducing fat absorption in mice and broiler chickens (Han et al., 1999; Razdan and Pettersson, 1994). The

Table 6
Nutrient retention efficiency (% nutrient or energy intake) of turbot fed the experimental diets (mean ± sd) over 56 days. Values with different superscripts are significantly different ($p<0.05$).

	HM%0	HM%17	HM%33	HM%49	HM%64	HM%76
PER [1]	2.4 ± 0.0^{a}	2.4 ± 0.1^{a}	$2.3 \pm 0.1^{a,b}$	2.2 ± 0.1^{b}	1.9 ± 0.0^{c}	1.6 ± 0.1^{d}
GPRE [2]	37.3 ± 1.1^{a}	36.6 ± 0.5^{a}	$35.4 \pm 1.4^{a,b}$	$32.9 \pm 1.6^{b,c}$	29.9 ± 1.0^{c}	26.6 ± 0.9^{d}
GLRE [3]	70.4 ± 3.8^{a}	$53.3 \pm 8.5^{a,b}$	$44.8 \pm 5.2^{b,c}$	$38.7 \pm 10.4^{b,c}$	$29.1 \pm 7.1^{c,d}$	17.2 ± 8.9^{d}
GERE [4]	38.0 ± 2.0^{a}	$35.8 \pm 0.3^{a,b}$	$33.3 \pm 0.7^{b,c}$	29.6 ± 2.4^{c}	23.6 ± 1.4^{d}	19.7 ± 1.4^{d}

[1] Protein efficiency ratio (body weight gain, g/protein intake, g).
[2] Gross protein retention efficiency ((%final body protein content × final body weight) − (%initial body protein content × initial body weight)/crude protein intake × 100).
[3] Gross lipid retention efficiency ((%final body lipid content × final body weight) − (%initial body lipid content × initial body weight)/crude lipid intake × 100).
[4] Gross energy retention efficiency ((%final body energy × final body weight) − (%initial body energy × initial body weight)/gross energy intake × 100).

Table 7
Apparent digestibility coefficients (ADC) of the diets containing 0% (basal) and 30% (test) HM and the calculated digestibility of the test ingredient.

	Basal diet	Test diet	HM[2]
ADC organic matter [1]	83.2	71.0	45.2
ADC crude protein [1]	89.1	81.1	63.1
ADC crude lipid [1]	98.7	92.8	78.0
ADC gross energy [1]	84.9	75.0	54.5

[1] ADC_{diet} (%) = $1-[(TiO_{2diet}\times Nut_{feces})/(TiO_{2feces}\times Nut_{diet})]$ where Nut $_{feces}$ or $_{diet}$ is the amount of the nutrient (or energy) in feces or diet.

[2] ADC of nutrient of test ingredient *Hermetia* meal (%) = $(Nut_{TD}x\ ADC_{TD}-0.7\times Nut_{BD}\times ADC_{BD})/(0.3\times Nut_{Ing})$ where $Nutr_{TD:}$ is nutrient concentration in test diet; ADC_{TD}: apparent digestibility of nutrients in test diet; Nut_{BD}: nutrient concentration in the basal diet; ADC_{BD}: apparent digestibility of nutrients in the basal diet; Nut_{Ing}: nutrient concentration in test ingredient.

amount of chitin in the experimental diets including HM ranged between 1.6 and 7.3%. However, even lower incorporation of chitin into diets was reported to decrease feed intake and growth in carp, tilapia (*Oreochromis niloticus*×*O. aureus*), and Atlantic salmon at inclusion levels of 1% and higher (Gopalakannan and Arul, 2006; Olsen et al., 2006; Shiau and Yu, 1999). Inclusion levels of 10% chitin reduced growth and feed intake in rainbow trout (Lindsay et al., 1984). Low lipid utilization and the small lipid retention at higher HM inclusion levels can be attributed to the presence of chitin and its negative influence on lipid digestibility. In contrast to our study, no negative impact of chitin was observed for eel (*Anguilla japonica*), red sea bream (*Pagrus major*), and yellowtail kingfish (*Seriola quinqueradiata*) when diets were supplemented with 10% chitin (Kono et al., 1987). However, the species in the latter study were fed with a commercial dry feed for eel or experimental diets containing 10% chitin, chitosan or cellulose whereas the amount and source of dietary fiber of the commercial diet revealed unknown.

In the present study growth performance decreased with increasing dietary HM as a result of decreased diet acceptability and feed intake. Reduced palatability has been reported with regard to poor feed intake and is often discussed when substantial amounts of fish meal in a diet are replaced (Day and González, 2000; Espe et al., 2007; Fournier et al., 2004; Nagel et al., 2012a,b), particularly if the alternative protein source contains antinutritional factors (ANFs) (Francis et al., 2001; Tusche et al., 2011). Thus, the lower feed intake at inclusion level >33% reduced protein and energy intake needed for growth. Final weight and SGR were significantly affected by all inclusions of HM, whereas feed intake and feed conversion ratio were not significantly affected by HM inclusion up to 332 g kg^{-1}. However, growth rates observed in the present study were higher or at least comparable to inclusion of alternative plant proteins in diets for juvenile turbot in the past (Fournier et al., 2004; Regost et al., 1999) and recently (Bonaldo et al., 2011; Dietz et al., 2012; Nagel et al., 2012b; Slawski et al., 2011; Yigit et al., 2010). The inclusion of poultry by-product meal (PBM) in diets for turbot was successful until an inclusion level of 432 g kg^{-1} feed and similar FCR and PER were obtained to what was observed in the present study until inclusion levels up to 486 g HM kg^{-1} feed. At higher inclusion levels of PBM the significant reduction in growth was caused by a lower energy and lipid intake. With increased PBM inclusion explicit differences in the amino acid composition of the experimental diets were observed and these dietary imbalances might have reduced protein retention and growth as well. Such amino acid imbalances were not observed in the present study due to a similar amino acid profile of FM and HM.

Whole body composition analyzed in the present study did not show any influence on body crude protein content. Whole body crude ash content was comparable in all treatments. Still, dietary levels of phosphorus varied between 7.0 and 17.0 g kg^{-1} DM, but were above the requirements of fish (NRC, 2011). Thus, we conclude that phosphorus was sufficiently available and not limiting growth performance in the present study. However, dry matter and crude lipid content of whole body composition were affected by dietary treatment. With increasing amounts of HM, whole-body crude lipid and gross energy content decreased significantly mainly due to a significant decreased feed intake and therefore lipid and energy intake. Approximately 26.6–37.3% of ingested protein was retained into body protein and was greatest in fish fed diets containing maximum 33% HM. The formula applied to determine GPRE includes the body weight of the fish. Since significant differences in feed intake and growth were observed, the gross protein retention efficiency was affected by the weight of the fish and therefore decreased. In our case, the fish fed 76% HM inclusion had only half of the body weight than the control group, so a difference in GPRE must be expected. The body protein content of fish is reported to be very constant (Shearer, 1993), while moisture and lipid content can differ extremely, as it was also observed in the present study. The decreased retention efficiency promoted by the diets including HM is also linked to the low availability of protein, lipid, and energy in HM. Although not studied here, it is suggested, that the lipid availability was influenced by the defatting process and the bioavailability of the lipids might have been reduced.

In this study, the focus was set on the protein utilization of HM, but since the results of whole-body lipid and energy gain, GLRE, and GERE were affected by dietary treatment, the role of the fatty acid composition and the lipid digestion should be focused as well. Therefore, fatty acids were classified into broad categories: SAFA (saturated fatty acids), MUFA (mono unsaturated fatty acids), and PUFA (poly unsaturated fatty acids), where the ratio of SAFA mainly explains the variations in ADC (Hua and Bureau, 2009). Furthermore, it is reported, that chain length and degree of saturation might influence the digestibility of fatty acids and increases with degree of saturation of fatty acids (Colombo-Hixon et al., 2011; Olsen et al., 2006). In the present study, congruently with increasing HM supplementation, the proportion of SAFA did increase and digestibility decreased linearly (R^2 = 0.99). The amount of SAFA in the HM%17 diet was 33.6% (Table 2) of the total fatty acids and thus already higher than suggested in practical diets for salmonids (23.5% SAFA, Hua and Bureau, 2009), caused by the immense amount of SAFA in the raw material HM (71.6%) itself. High SAFA concentrations in the experimental diets may decrease lipid digestibility and energy utilizations as it was also observed in rainbow trout, Atlantic salmon and sea bream (Fountoulaki et al., 2009; Ng et al., 2004, 2010; Robaina et al., 1997), consequently explaining the observations in the present study.

Although gastrointestinal bacteria and chitinase activity was reported in some fish species, the chitin digestibility is usually very low or completely absent as in rainbow trout (Lindsay et al., 1984). Here, neither chitinase activity (below the detection limit 156 μg NAG h^{-1} g^{-1}) nor CFU with detectable chitinolytic activity observable as a typical halo was recorded in any of the HM-based diets independent of the inclusion level. Thus, despite the natural prey of turbot including crustaceans at the juvenile stage (Braber and De Groot, 1973) chitin digestion seems irrelevant in turbot. Species with higher chitinase activity are cod (500–1900 μg NAG h^{-1} g^{-1}), yellowtail kingfish (2700 μg NAG h^{-1} g^{-1}), Japanese eel (3500 μg NAG h^{-1} g^{-1}), cobia (3075 μg NAG h^{-1} g^{-1}), and red sea bream (7400 μg NAG h^{-1} g^{-1}) (Danulat, 1986a,b; Danulat and Kausch, 1984; Fines and Holt, 2010; Kono et al., 1987), largely preying on crustaceans. It has to be mentioned, that chitinase measurement in these studies was determined in stomach contents, whereas in the present study samples were taken from the mid gut. Detection of chitinase activity in the mid gut has been reported in sole (Clark et al., 1988) and considerably lower in cod (Danulat, 1986a). Most importantly, reduced chitinase activity may result from a long-term adaptation to chitin-free diet fed during on-growing of the turbot used here, i.p. during larval stages, when they fed zoo plankton. In conclusion, juvenile turbot accepted diets containing

332 g kg^{-1} HM without significant effects on feed intake and feed conversion. However, SGR was significantly lower at any inclusion of HM and at higher HM incorporation the acceptance of the diet was reduced, resulting in decreased feed intake and lower growth performance. The presence of chitin might have reduced feed intake and nutrient availability and therefore reduced growth performance and nutrient utilization. The processing of the HM, especially the defatting procedure, might have caused an integration of the lipids into the chitin structure, affecting the availability of them. *H. illucens* provide in general a high quality protein source, being especially valuable from the amino acid profile. HM can be constantly produced around the year by using local green house waste streams. HM might be a feasible alternative protein source for the partial replacement of fish meal. Further research on improvement of chitin digestibility and HM meal processing to increase nutrient utilization is needed. Additionally, to utilize chitin as a nutrient, chitin degrading enzymes or bacteria could be integrated into a diet.

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