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Protein assimilation of black soldier fly larvae *Hermetia illucens* in diets for red seabream *Pagrus major*

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Abstract: Fishmeal is a major ingredient for aquaculture feeds. Japanese fish feed manufacturers still heavily depend on imported fishmeal, and include fishmeal into fish feeds at approximately 40% in 2023. Recent price increase in the imported fishmeal makes large impact on the aquaculture practice in Japan, so that the use of alternative protein ingredients has become a crucial interest. Meal of black soldier fly (BSF) larvae is a promising candidate for a fishmeal substitute in aquaculture feeds. Recently, several private companies in Japan have started to produce BSF larvae with food residues. However, a high inclusion of BSF meal (BSFm) in fish feeds has sometimes resulted in growth retardation in several fish species. Thus, in this study, a 42-day feeding experiment was conducted in juvenile red seabream (*Pagrus major*) using diets with various inclusion levels of BSFm. Three isoenergetic diets were prepared with fishmeal to BSFm ratios of 100:0 (Group 1), 50:50 (Group 2), and 22:78 (Group 3). Then, we investigated the protein assimilation from BSFm to fish using conventional growth analysis, stable isotope analysis and metabolomic analysis. Group 1 exhibited a significantly higher specific growth rate (2.92 ± 0.22 %/day) than Group 3 (2.54 ± 0.22 %/day), while Group 2 (2.77 ± 0.21 %/day) did not show a significant difference relative to Group 1. Stable isotope analysis suggested a lower assimilation of BSF protein in Groups 2 and 3, as indicated by their δN15 ratios. *In vitro* digestion evaluation suggested that several essential amino acids (EAAs) in the diet for Group 3 were potentially deficient compared to the diet for Group 1. These results suggest that a diet comprising up to 50% BSFm does not negatively affect the growth of red seabream, but an EAA deficiency may have retarded the growth in Group 3. Therefore, further nutritional improvement is needed for diet with BSFm inclusion level above 50%.

Key words: stable isotope, amino acid balance, *in vitro* digestion, growth, digestive enzymes

Introduction

Future global food crisis that will be exacerbated by global warming and population growth, is a imminent issue. As one of the strategies that could mitigate this crisis, resource circulation in food production sectors is crucial. We should transform unutilized food resources and their residues into higher trophic forms for animal feeds and eventually, for human foods. Some insects which grow on various residues from agriculture, food processing, and livestock, are nutritious and could be used as feed ingredients for fish aquaculture. Notably, larvae of black soldier fly (BSF, *Hermetia illucens*) can grow well on residues of vegetables and livestock, and fish offal (Meneguz *et al*. 2018; Spranghers *et al*. 2017; Joly and Nikiema 2019; Yuan and Hasen 2022).

BSF is a saprophytic insect that can grow even on spoiling matter (Kortsmit *et al*. 2023). The conversion efficiency of food in BSF is higher than that of yellow mealworm (*Tenebrio molitor*) and house cricket (*Acheta domesticus*). BSF has the shortest life cycle among the candidates of insects for animal

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foods (Oonincx *et al*. 2015). BFS larvae contain up to 63% protein or 39 % fat on a dry matter basis (Barragan-Fonseca *et al*. 2017), establishing the potential of BSF larvae that convert unavailable residual nutrients into valuable ones for carnivorous fish. The production of insect proteins, which includes BSF and yellow mealworm, is expected to grow into a $1.14 billion market by 2027 (Gruber and Melton 2023).

BSFm is nutritious feed ingredient with favorable effects on fish physiology. The effectiveness of BSFm has been evaluated in various fish species (Belghit *et al*. 2019; Ido *et al.* 2021; Takakuwa *et al*. 2022). However, diets with high proportions of BSFm resulted in growth retardation in several fish species.

The aims of this study are as follows: 1) evaluation of the performance of red seabream (*Pagrus major*), a marine carnivorous fish fed diets with various substitution levels of fishmeal by BSFm, and 2) investigation of the mechanism causing growth retardation when red seabream are fed with BSFm.

Material and methods

Dried whole BSF larvae, having been raised on brewer's spent grain, were purchased from San-U Fish Farm (Osaka, Japan). They were partially defatted and then powdered using a motor-driven mill. This was designated as BSFm in this study. The formulation and nutrient composition of the diets are summarized in Table 1. Red seabream juveniles (four-month-old) were divided into groups of 15 or 16 individuals each to ensure an identical initial fish density between groups. The groups were as follows: Group 1 was served as the control and fed the 100 fishmeal/0 BSFm diet, Groups 2 and 3 were the experimental groups, and fed the 50 fishmeal/50 BSFm diet and 22 fishmeal/78 BSFm diet, respectively. The water temperature for rearing was maintained at 25.2 ± 0.4°C. The seabreams were hand-fed twice a day until apparent satiety for 42 days. Diets, protein ingredients, and muscle samples of the initial and final fish were subjected to various analyses.

An intestinal digestive enzyme complex was isolated from the intestinal content of three individuals of one-year-old red seabream. The intestinal content was extracted under the ice-cooling condition, and the solid body was removed by centrifugation. The supernatant was then dialyzed overnight, and designated as the digestive enzyme complex (EC). An *in vitro* digestion test was performed according to Andoh *et al*. (2023), with slight modifications. The amount of amino acids produced by the digestion with EC was calculated using the following equation:

$$AAa = TAAa - PAAa$$

In this formula, AAa represents the amount of free amino acids (AA) produced by the digestion by EC. TAAa stands for the total amount of free amino acids in the digesta, and PAAa is the amount of free amino acids in the diet without EC supplementation.

Muscle and diet samples were lyophilized and then defatted. The muscle samples were analyzed for measurement of δ15N. Stable isotope ratios were expressed in the conventional δ notation as parts per-mil (‰) according to the following equation:

$$\delta Xm = ((Rsample/Rstandard) - 1) \times 1000$$

Table 1 Formulation (%) and proximate composition (dry matter basis) of diets

	Group 1	Group 2	Group 3
Fishmeal:BSF powder	100:0	50:50	22:78
Fishmeal	72.6	36.3	16.1
Defatted BSF powder	0.0	36.3	56.5
Wheat gluten	8.1	8.1	8.1
Fish oil	8.9	8.9	8.9
Soybean lecitin	4.0	4.0	4.0
Choline chroride	0.8	0.8	0.8
α-Tocopherol	0.0	0.0	0.0
Others (vitamins and minerals)	5.6	5.6	5.6
Crude protein (%)	56.0	51.2	48.4
Crude fat (%)	15.0	17.4	18.7
Gross energy (kcal/g)	5.03	4.99	4.97

where Xm is δ15N and R is the corresponding δ15N ratio.

The differences in values between diet and muscle were calculated individually according to the following equation:

$$\text{Difference} = \delta Xm - \delta Xd$$

where Xd is δ15N of a diet supplied to the corresponding group.

Results

In the rearing experiment, the specific growth rate (SGR) of Group 1 (2.92 ± 0.22 %/day) was the highest and significantly higher than that of Group 3 (2.54 ± 0.22 %/day) (Table 2). The SGR of Group 2 (2.77 ± 0.21 %/day) was not significantly different from that of Group 1. There were no significant differences among the three groups in terms of condition factor, condition factor without viscera, hepatosomatic index, viscera somatic index, and intraperitoneal fat ratio. The FCR values were similar across groups (1.01 to 1.04). No differences were visually observed in the feeding behavior, external body appearance, hepatopancreas, or viscera among the three groups.

In the analysis of free amino acids in the *in vitro* digesta of diets for Groups 1, 2, and 3, 19 amino acids and taurine (Tau) were detected. Cystine was either not detected or present only in trace amounts in the diets. In the reactant of EC alone without diet, no amino acids were detected by the amino acid analyzer. Three amino acids (Leu, Phe, and Trp) showed no significant differences among three diet Groups (Fig.1). Twelve amino acids (Val, Ile, Thr, His, Ala, Ser, Tyr, Gly, Gln, Asn, Asp, Pro) were higher in Group 3 than in Group 1. The lower amino acids in Group 3 than in Group 1 were Lys, Arg, and Met, and Tau. The percentages of the amounts of amino acid of Group 3 to Group 1 were 82.6 ± 4.4% in Lys, 75.8 ± 4.1 % in Arg, 74.4 ± 3.3 % in Met, and 24.9 ± 1.1 % in Tau. The values for Group 2 were intermediate between

Table 2 Growth performance and fat content of red seabreams in the rearing experiment

	Group 1	Group 2	Group 3
Fishmeal:BSF powder	100:0	50:50	22:78
Initial number of individual	15	16	16
Final number of individual	14	16	15
Initial body weight (g)	26.4 ± 1.6^{a}	26.3 ± 1.6^{a}	26.3 ± 1.5^{a}
Final body weight (g)	90.0 ± 10.1^{a}	84.5 ± 6.1^{ab}	76.7 ± 5.6^{c}
Specific growth rate (%/day)	2.92 ± 0.22^{a}	2.77 ± 0.21^{ab}	2.54 ± 0.22^{c}
Condition factor	2.18 ± 0.18^{a}	2.18 ± 0.09^{a}	2.08 ± 0.10^{a}
Condition factor without viscera	1.96 ± 0.15^{a}	1.97 ± 0.08^{a}	1.87 ± 0.10^{a}
Feed conversion ratio	1.04	1.02	1.01

Values with a different letter indicate a significant difference ($p < 0.05$).

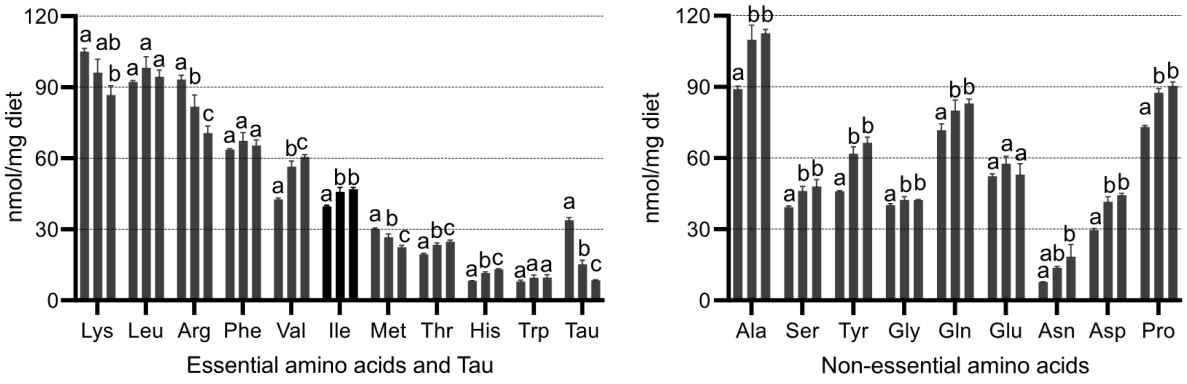

Fig.1 Amino acids produced by *in vitro* digestion with a red seabream digestive enzyme complex (EC) in three diets for Groups 1, 2, and 3

Tau is not an essential amino acid, but its essentiality has already been recognized. The bars in each amino acid indicate that the left is Group 1, the center is Group 2, and the right is Group 3. Different letters indicate a significant difference ($p < 0.05$).

Groups 1 and 3 or not significantly different from Group 3.

The amounts of total amino acid in the digestion reactants in Groups 2 and 3 were significantly higher than in Group 1 (Fig.2). The total amounts of NEAA in Groups 2 and 3 followed a trend similar to the total AA, and the total amounts of EAA were not significantly different among the three groups. Although free amino acids were also detected in the diets, they were at negligible levels except for Tau.

The δ15N ratios of BSFm and wheat gluten were lower than that of fishmeal. The δ15N ratios of the seabream muscle after 42 days of feeding changed significantly from the initial values in all groups. Compared to Group 1, the δ15N ratios of the diets for Groups 2 and 3 decreased in accordance with the dietary BSFm inclusion level (Table 3). The δ15N ratios of the muscles in these groups also decreased with the increase in the dietary BSFm level. In contrast, the difference in the δ15N ratio between the diet and its corresponding muscle in Group 1 was +2.094. For Group 2, it was +4.373, and for Group 3, it was +5.644. These increases was correlated with the increase in the BSFm inclusion level in the diet. The difference in Groups 2 and 3 is biased toward that of fishmeal.

Discussion

There were no significant differences in all parameters of growth performance between Groups 1 and 2, suggesting that substituting fishmeal with BSFm at 50 % does not affect the growth of red seabream. Conversely, a significant growth retardation compared to Group 1 was observed in final BW and SGR in Group 3 that was fed a diet with BSFm at 78 %. This suggests that growth performance becomes lower when an excess amount of BSFm as a replacement of fishmeal is included in red seabream diet. However, this growth retardation does not seem to be due to a physiological disorder, as there were no significant differences in the condition factor, condition factor without viscera, and hepatosomatic index among Groups 1, 2, and 3. Values of feed conversion ratio were also at similar levels. Visual differences in the liver color, body color, feeding behavior, and swimming activity were not observed among all groups throughout the feeding trial. There is a possibility that BSFm, as a feed ingredient, lacks some substances necessary for normal body growth of red seabream.

Three free EAAs produced by in vitro digestion, namely Lys, Arg, and Met, and Tau as well, in Group 3 were significantly lower than Group 1 (Fig.2). This difference in the free amino acid concentrations is one of the possible factors affecting the

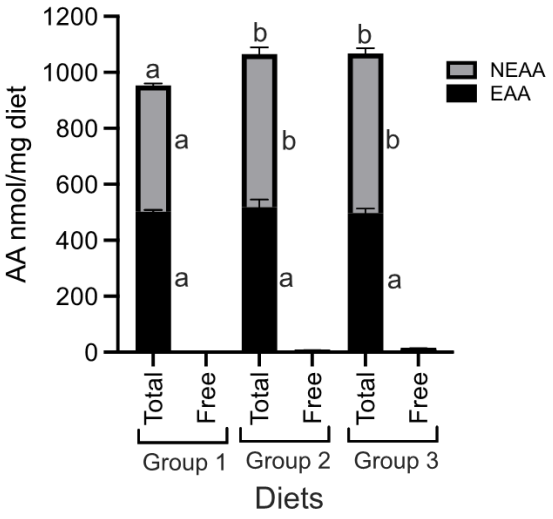

Fig.2 Total essential and non-essential amino acids produced by *in vitro* digestion with a red seabream digestive enzyme complex (EC) in three diets for Groups 1, 2, 3

"Total" indicates the sum of the amount of each amino acid produced by the EC digestion and the amount of free amino acids in each diet. "Free" indicates the amount of free amino acids in each diet. Different letters indicate a significant difference between "Total" amino acids.

Table 3 δ15N (%) of diets used, ingredients and muscle samples of red seabream

Group and ingredient	Diet and ingredient	Muscle	Difference
Initial		+14.24 ± 0.14[a]	
Group 1	+13.4	+15.49 ± 0.11[b]	2.094 ± 0.106[a]
Group 2	+8.7	+13.07 ± 0.13[c]	4.373 ± 0.128[b]
Group 3	+5.9	+11.54 ± 0.21[d]	5.644 ± 0.206[c]
Fishmeal	+15.1		
BSFm	+3.6		
Wheat gluten	+3.1		

Values are means ± SD. Values with a different letter indicate a significant difference ($p < 0.05$).

growth retardation in Group 3. In particular, Lys and Arg along with Leu are the top three EAAs that constitute the body protein in gilthead seabream (*Sparus auratus*, Kaushik 1998), a species phylogenetically close to red seabream. Deficiencies of Lys and Arg could directly affect body mass growth. Taurine deficiency is also a potential factor for the growth retardation, but no symptoms of taurine deficiency were observed in Group 3.

Compared to Group 1 fed the diet without BSFm, difference in the δ15N ratios between the diet and its corresponding muscle of seabreams increased significantly in Groups 2 and 3, which were fed diets containing BSFm,. The δ15N ratio generally increases in fish body compared to its feed, as incorporated 15N in nutrients is concentrated in fish body through metabolism (Gamboa-Delgado 2022). The difference in the δ15N ratio between the diet and muscle in Group 3 was highest (5.644), and more biased toward the δ15N ratio of fishmeal. This suggests that the assimilation of nitrogen from BSFm in the diet was inferior to that from fishmeal in Group 3. This finding is consistent with the hypothesis that the deficiency of three EAAs, namely Lys, Arg, and Met, is a causative factor in the growth retardation of seabreams fed the diet with the highest BSFm level.

In conclusion, it was demonstrated that substituting dietary fishmeal with BSFm at 50% did not affect the growth of red seabream. However, a significant growth retardation was observed when fish were fed a diet with BSFm at 78% due probably to the deficiency in certain essential amino acids. Therefore, further improvements in the nutritional quality are needed for diets with higher inclusion levels of BSFm.

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Annotated Bibliography of Key Works

(1) Andoh T, Yasuike M, Ishihara K, Fujiwara A (2023) Effects of heating duration on the digestibility of fish protein powders at 99°C *in vitro* using yellowtail *Seriola quinqueradiata* digestive enzymes. *Fish. Sci.*, **89**, 671–685.

The effects of heating duration on the digestibility of the muscle powder of three fish (pollock [WP], yellowtail [YT], and mackerel [CM]) were assessed *in vitro* using a digestive enzyme complex (EC) extracted from yellowtail pyloric caeca. This research will contribute to the establishment of optimum feed processing conditions for yellowtail. The powder samples were heated at 99°C for 0, 3, 20, 72, and 240 min, followed by hydrolysis with the EC to assess the heating effects. After 3 min of heating WP, YT, and CM, the average production of essential amino acids (EAAs) by the EC digestion decreased to 81.3%, 72.0%, and 66.9%, respectively, compared to the non-heated controls. The production further decreased with the increase of heating duration from 3 to 240 min, although most of the decrease occurred within the first 3 min in WP, YT, and CM.

(2) Takakuwa F, Tanabe R, Nomura S, Inui T, Yamada S, Biswas A, Tanaka H (2022) Availability of black soldier fly meal as an alternative protein source to fishmeal in red sea bream (*Pagrus major*, Temminck & Schlegel) fingerling diets. *Aquacult. Res.*, **53**, 36–49.

The authors replaced fishmeal (FM) in red sea bream (*Pagrus major*) diets with black soldier fly meal (BSFM) to investigate the effects of the diets on growth and feed utilization. Six isonitrogenous and isolipidic experimental diets were prepared by substituting 0%, 20%, 40%, 60%, 80%, and 100% of FM protein with BSFM (control, BSFM20, BSFM40, BSFM60, BSFM80, and BSFM100, respectively). After an eight-week feeding trial, final body weight, weight gain, specific growth rate, and feed efficiency decreased linearly with increasing BM levels ($p < 0.05$). The results suggest that BSFM can replace a maximum of 41.7% of FM in the red sea bream diet without compromising growth performance and feed efficiency for 56 days.

(3) Ido A, Ali MFZ, Takahashi T, Miura C, Miura T (2021) Growth of yellowtail (*Seriola quinqueradiata*) fed on a diet including partially or completely defatted black soldier fly (*Hermetia illucens*) larvae meal. *Insects*, **12**, 722. doi.org/10.3390/insects12080722

Yellowtail, the most popular farmed fish in Japan, is carnivorous; its diet requires a high proportion of fishmeal (FM). This study represents the first example of yellowtail fed on a diet including black solder fly (BSF) meal as an FM replacement. Partially defatted BSF meal (PDBSFM) comprised 49.0% crude protein and 23.2% crude fat, while completely defatted BSF meal (CDBSFM) that was obtained by defatting PDBSFM using hexane, achieved less than 10% crude fat, a level identical to that of FM. In their feeding trials, fish growth performances were reduced with the increase of both PDBSFM and CDBSFM. Therefore, even 10% of BSF meal inclusion, irrespective of the defatting methods, inhibited the juvenile yellowtail growth.