Thesis for the Degree of Master of Science

Attractiveness of the various protein sources to juvenile olive flounder (*Paralichthys olivaceus*)

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February 2019

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A dissertation

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A dissertation submitted in partial fulfillment of the requirements

for the degree of

Master of Science

In the Department of Marine Bioscience & Environment,

the Graduate School of Korea Maritime & Ocean University

February 2019

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Abstract

Attractiveness and the effect of dietary supplementation of various feed protein source on feed consumption in juvenile olive flounder were investigated. Fifteen feed ingredients were distributed in to a reinforced acrylic tank composed of an acclimatization chamber and 3 equally divided rectangular attracting chambers. For the preliminary test, similar sizes of thirty fish were randomly distributed in the acclimatization chamber and tournament comparison of feed ingredients was applied to evaluate attractiveness. The best top 4 feed ingredients (jack mackerel meal, sardine meal, hydrolyzed meal and Pollack meal) were selected by tournament comparison from 1st to 9th test. Among the final top 4 feed ingredients, jack mackerel achieved greater attractiveness to olive flounder than sardine and Pollack meal in the 1st trial. The attractiveness of olive flounder for sardine meal was higher than hydrolyzed fish and Pollack meal in the 2nd trial. Six hundred juvenile fish were randomly distributed in 60 flow-through tanks for the feeding trial. Four experimental EP were prepared: the control (Con) diet, and 5% jack mackerel meal (JM), sardine meal (SM), and hydrolyzed fish meal (HFM) diets. Each diet was assigned in 15 replication and hand-fed to satiation for a week. The weight gain and specific growth rate (SGR) of olive flounder fed the JM diet were higher than those of fish fed the Con, SM and HFM diets. The higher feed consumption (g/fish) was achieved in fish fed JM diets followed by the SM, HFM and Con diets, in order. In conclusion, the top 3 strong feeding attractant response of olive flounder was observed in jack mackerel meal, followed by sardine meal and hydrolyzed fish meal in order among various (15 kinds) feed ingredients. The highest feed consumption and relative feeding activity were obtained in olive flounder fed the EPs containing jack mackerel meal, followed by sardine meal and hydrolyzed fish meal.

Keywords: Olive flounder (*Paralichthys olivaceus*); Attractiveness; Various protein sources; Fish meal; Relative feeding activity

넙치 치어내 다양한 단백질 원료의 유인도

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요 약

본 연구에서는 넙치 치어내 다양한 단백질 원료에 대한 먹이유인 정도를 평가하며, 우수한 먹이유인 정도를 보인 단백질 원료의 배합사료 내 첨가에 따른 사료섭취 향상을 위한 사료원료별 사육효과 조사를 평가하였다. 15 종류의 단백질 원료를 이용하여 3구간으로 분할 된 먹이유인 정도 평가 실험 장치를 사용하였다. 예비실험에서는 동일한 크기의 치어 30마리씩을 무작위로 수용해 먹이유인 정도 평가 실험을 평가하였다. 예비실험에서는 1회부터 9회까지 가장 높은 먹이 유인정도를 보인 4종류의 단백질 원료(전갱이, 정어리, 가수분해어분, 명태)를 나타냈다. 가장 높은 먹인 유인정도를 보인 4종류의 원료중에서, 5 반복구를 두어서 최종 먹이 유인정도를 평가한 결과, 1회차 에서는 전갱이가 정어리와 가수분해어분 보다 높게 나타났고, 2회차 에서는 정어리가 가수분해어분과 명태보다 높게 나타났다. 그리고 이들 중에서 높은 먹이유인 정도를 보인 3 종의 사료 원료와 양호한 멸치 분을 대조구로 이용하여 총 4종류의 실험 사료를 준비하여 15 반복구를 두어서 1주간의 사료섭취율 실험을 실시하였다. 1주간의 사육실험에는 600 마리의 넙치 치어를 60개의 유수식 수조에 분산 수용하였다. 대조구(Con) 사료에는 55%의 멸치분을 함유하였으며, Con 사료에 첨가한 멸치분 5%를 대신하여 우수한 유인 정도를 보인 3종류의 단백질 원료를 각 5%씩 첨가한 사료 총 4종류의 EP사료를 준비하였다. 모든 실험어는 1일 2회 만복시까지 손으로 공급하였다. EP 사료를 먹은 넙치 치어의 1주간 사료섭취율

실험에서 체중증가와 일일성장률은 JM 사료 공급구가 다른 모든 실험구보다 높게 나타났다. 사료섭취량은 JM 사료 공급구가 다른 모든 실험구보다 높게 나타났으며, 다음으로는 SM, HFM과 Con 사료 공급구 순으로 나타났다. 결론적으로, 넙치 치어내 15종류의 다양한 단백질 원료 중 전갱이. 정어리, 가수분해어분 순서로 가장 좋은 먹이 유인 정도를 나타났으며, 상대적 사료 섭취율과 사료섭취량에 있어서도 전갱이. 정어리, 가수분해어분 순서로 나타냈다.

Keywords: 넙치(*Paralichthys olivaceus*); 유인도; 다양한 단백질 원료; 어분; 상대적 사료 섭취율

1. Introduction

Olive flounder (*Paralichthys olivaceus*) is the most popular and economically important marine fish species in Eastern Asia, such as Korea, China and Japan because of its high market value and growth performance (Alam et al. 2000, Seikai 2002, Kang et al. 2008). Its annual aquaculture production sharply increased from 1037 metric tons in 1990 to 41207 metric tons in 2017 in Korea (KOSIS 2018). Recently this species has been introduced to the United States and Turkey for commercial production (Geng et al. 2017).

Most of olive flounder farmers in Korea are likely to use a frozen raw fish (mackerel or sardine) or raw fish-based moist pellet (MP) composed of frozen raw fish and binder, at a certain ratio (Lee & Jeon 1996, Cho et al. 2005). More than ten times (226817 metric tons) of MP were applied to produce olive flounder than formulated feed (19385 metric tons) in 2017 in Korea (KOSIS 2018). However, the supply of MP resulted in the increased discharge of wastes and storage costs, deteriorated water quality, increased fish production cost and reduced growth performance over extruded pellet (EP) (Lee & Lee 1994, Cho et al. 2005, Sun et al. 2006, Cha et al. 2008).

EP are commonly recommended for most of fish culture because EP can reduce the water pollution source and spread of disease, and improve the digestibility of carbohydrate, storage time and feed efficiency (Kim et al. 1992, Cho et al. 2006a & b). Satoh et al. (2003), however, reported that the preference of raw fish was higher than EP in olive flounder because of decreased feed intake and feed digestibility of fish and it eventually led to decreased growth and condition factor (CF) of fish. Because chefs prefer filleting thick olive flounder to prepare raw fish or sushi over thin fish, its CF is another factor to evaluate its commercial preference in restaurants.

Freitas et al. (2011) reported mutton snapper (*Lutjanus analis*) fed on the diet with soy protein concentrate in replacement of anchovy meal diet achieved low feed intake and growth performance due to poor feed palatability. The similar results had been observed in various fish species, such as coho salmon (*Oncorhynchus kisutch*), gilthead seabream (*Sparus aurata*) (Arndt et al. 1999, Kissil et al. 2000).

The facts that EP contains less or no feeding attractants and/or stimulants compared to MP could be reasons for the fish farmers prefer feeding olive flounder MP to EP. Therefore, development of feeding attractants in EP for olive flounder is highly needed. Furthermore, feeding attractants and/or stimulants are important means of improving palatability, increasing feed intake and reducing feed wastage by improving initial feeding and feed palatability (Lee & Meyers 1996, Zou et al. 2015). Dietary supplementations of feeding attractants, such as the extracts of mussels or tissues, various origins including chemical mixtures, natural compounds and synthetic or neutral amino acid have studied in many marine fish species (Deshimaru & Yone 1978, Dias et al. 1997, Day & Gonzalez 2000, Papatryphon et al. 2000, Xue & Cuiz 2001, Tiril et al. 2008, Kader et al. 2012). Derby et al. (2016) reported that feed attractants such as krill meal, squid meal, squid liver powder, hydrolysates, or other natural, animal-based meals, added as a low percentage of the total feed content have improved the palatability and feed intake. Harada (1991) reported that *Herba plectranthi*, *Phellodendron* bark and Sweet *Hydrangea* leaf have been studied to the common attraction activities in abalone (*Haliotis discus*), oriental weatherfish (*Misgurnus anguillicaudatus*) and yellowtail (*Seriola quinqueradiata*) among twenty-three herb crude drugs such as *Aloe*, *Coptis rhizome*, *Gentian*, *Herba plectranthi*, *Phellodendron* bark, *Sophora* root, *Amomum* seed, *Cinnamon* bark, *Citrus unshiu* peel, fennel, ginger, immature orange, *Astragalus* root, *Coix* seed, *Dioscorea* rhizome, *Fructus lycii*, jujube, *Flos rosae* *rugosae*, *Mentha* herb, sweet *Hydrangea* leaf, *Digitalis*, guarana seed, toad venom and *Nux vomica*.

Fish meal (Smith et al. 2005, Nunes et al. 2006, Singh et al. 2006), krill meal (Shimizu et al. 1990, Takaoka et al. 1990, Suresh et al. 2011), crustacean meals, such as crab meal (Carr et al. 1977), shrimp meal (Carr & Chaney 1976) and shrimp head meal (Fox et al. 1994) and mollusk meals, such as mussel meal (Nagel et al. 2014, Mongile et al. 2015), oyster meal (Carr et al. 1977), clam meal (Takaoka et al. 1995), squid liver meal (Suresh et al. 2011), squid meal (Naik et al. 2001) and squid extract (Toften et al. 1995, Xue & Cui 2001) are known to have high attractiveness to several aquatic animals. In particular, Ikeda et al. (2012) suggested that histidine resulted the highest feeding stimulant activity in olive flounder among the components of the synthetic extract. Most of studies mentioned above are the very complicated process to monitor electro(neuro)physiology of fish or analyze the synthetic chemicals in the extracts of prey in wild. However, Xue & Cui (2001) suggested that electro(neuro)physiological studies can only suggest whether the fish are able to detect a chemical compound with their chemoreceptor, but do not guaranteed that the stimulus of the chemical compound will bring in changes in feeding behavior of fish or increase feed consumption by fish.

Determining attractiveness of feed ingredients based on feeding behavior (olfactory response) of fish is a simple and sustainable aquaculture technique in formulated fish feed. An artificial supplementation of feeding attractants (stimulants) or elimination of feeding deterrent in feed ingredients is difficult in feed producing process. Furthermore, attractiveness of feed ingredient to olive flounder is still less known compared to its commercial importance.

The objectives of the present study were, therefore, to determine attractiveness of feed ingredients to olive flounder and inclusion effect of the top 3 selected feed ingredients in EP on feed consumption and growth performance of fish was compared.

2. Materials and Methods

2.1. Feeding Attractiveness Trial

2.1.1 Feed Ingredients Used to Determine Attractiveness and Chemical Composition

The sources of feed ingredients [6 kinds of fish meal (anchovy, herring, jack mackerel, hydrolyzed fish, Pollack and sardine meals), 4 kinds of crustacean meal (crab, krill, shrimp head and shrimp meals), 3 kinds of mollusk meal (mussel, squid and squid liver meals) and 2 kinds of plant-originated meal (corn gluten and defatted soybean meals)] used to determine the attractiveness of fish and their chemical composition were shown in Table 1.

The chemical composition of the feed ingredients was determined according to AOAC (1990) method. Crude protein was determined using the Kjeldahl method (Kjeltec 2100 Distillation Unit, Foss Tecator, Hoganas, Sweden); crude lipid was determined using an ether-extraction method (Soxtec TM 2043 Fat Extraction System, Foss Tecator, Sweden); moisture was determined by oven drying at 105°C for 24 h; and ash was determined using a muffle furnace at 550°C for 4 h.

2.1.2. Preparation of the Experimental Fish in the Preliminary Test

Juvenile olive flounder used in the trial were purchased from a private hatchery (Uljin City, Gyeongsangbuk-do, Korea) and acclimated to the experimental conditions for 2 weeks before attractiveness trial. During the conditioning period, fish were hand fed with a commercial extruded pellet (Woosung Feed Co. LTD, Daejeon, Korea) twice a day at a ratio of 2-3% body weight of fish. Juvenile olive flounder (average body weight of 2.1 and 5.0 g) were used to determine feeding attractiveness of fish in the preliminary test (1^{st} to 9^{th} test) and top 4 selected feed ingredients in the trials (1^{st} to 2^{nd}), respectively. Water temperature monitored daily from 12.9 to 18.5°C (mean ± SD: 16.8 ± 2.16°C) during preliminary test and from

Table 1. Sources and the chemical composition (%, DM basis) of feed ingredients

Feed ingredient		Nutrient				Supply (Nation)
		Moisture	Crude protein	Crude lipid	Ash	
Fish meal	Anchovy meal	8.7	72.3	9.7	15.0	Blumar (Santiago, Chile)
	Herring meal	6.7	66.8	8.8	15.9	FF Skagen (Skagen, Denmark)
	Jack mackerel meal	5.4	74.2	8.6	12.8	FoodCorp S.A. (Coronel, Chile)
	hydrolyzed fish meal	1.7	74.6	8.6	5.3	Sopropeche (Wimille, France)
	Pollack meal	9.9	63.2	7.4	18.9	Kodiak fish meal company (Alaska, USA)
	Sardine meal	6.6	70.2	9.5	15.7	Orizon S.A. (Santiago, Chile)
Crustacean meal	Crab meal	2.4	38.6	0.8	42.6	Bigmama Seafood Co., Ltd. (Tongyeong-si, Korea)
	Krill meal	4.7	59.5	19.8	10.3	Aker Biomarine (Lysaker, Norway)
	Shrimp head meal	8.5	50.5	8.8	20.6	Harinesa (Guayas, Ecuador)
	Shrimp meal	3.7	55.8	7.8	20.7	Fortidex S.A. (Guayas, Ecuador)
Mollusk meal	Mussel meal	1.9	61.5	11.7	8.3	Bigmama Seafood Co., Ltd. (Tongyeong-si, Korea)
	Squid meal	7.0	70.7	1.9	5.1	APM Logis Co., Ltd. (Seoul, Korea)
	Squid liver meal	5.7	46.3	18.6	6.7	Dong Woo Ind Co., Ltd. (Pohang city, Korea)
Plant-originated meal	Corn gluten meal	4.4	66.2	11.3	3.4	FairBizKorea Co., Ltd. (Seoul, Korea)
	Defatted soybean meal	5.2	58.9	2.5	8.1	Dashmesh Global LLC (Illinois, USA)

17.9 to 20.1°C (mean ± SD: 19.0 ± 1.20°C) during trial period. The photoperiod followed natural conditions.

2.1.3. Preparation of Determination to Feeding Attractiveness of Fish

Three reinforced acrylic tanks (1 m × 0.6 m × 0.5 m; water volume: 270 L) composed of three equally divided rectangular attracting chambers (0.6 m × 0.2 m × 0.5 m each) and an acclimatization chamber (0.4 m × 0.6 m × 0.5 m) were used to evaluate the attractiveness of feed ingredients to juvenile olive flounder (Figure 1). The flow rate of each attracting chamber was 3.24 L/min/chamber. A vertically movable acrylic shutter divided the attracting and acclimatization chambers. Each attracting chamber had a funnel-shaped entrance (10 and 5 cm in radius in and out, respectively) to allow fish free access to each feed ingredient placed in each attracting chamber. Funnel-shaped entrances were video-recorded to check the number of fish entering through the funnel-shaped entrance. Moderate aeration was supplied in each chamber, and the photoperiod followed natural conditions.

2.1.4. Evaluation of Feeding Attractiveness of Fish

The attractiveness of olive flounder for feed ingredients was determined by randomly selecting three kinds of feed ingredients at a time. Prior to the test, 30 randomly chosen fish were stocked in the acclimatization chamber without feeding for at least 72 h. Then, 20 g of different feed ingredient powders wrapped in 100 μm (mesh size) micromesh gauze (Samjee Tech Co., Anyang city, Gyeonggi-do, Korea) was placed into three attracting chambers, each 20 cm below the top. Then, the shutter was raised to allow free access of fish towards each feed ingredient in the attracting chambers for 30 min. The raised shutter was returned to its original place to count the number of fish in each attracting chamber. To evaluate the attractiveness of feed ingredients to olive flounder, tournament comparison was applied.

Feeding attractiveness for three feed ingredients was tested at a time. Thirty

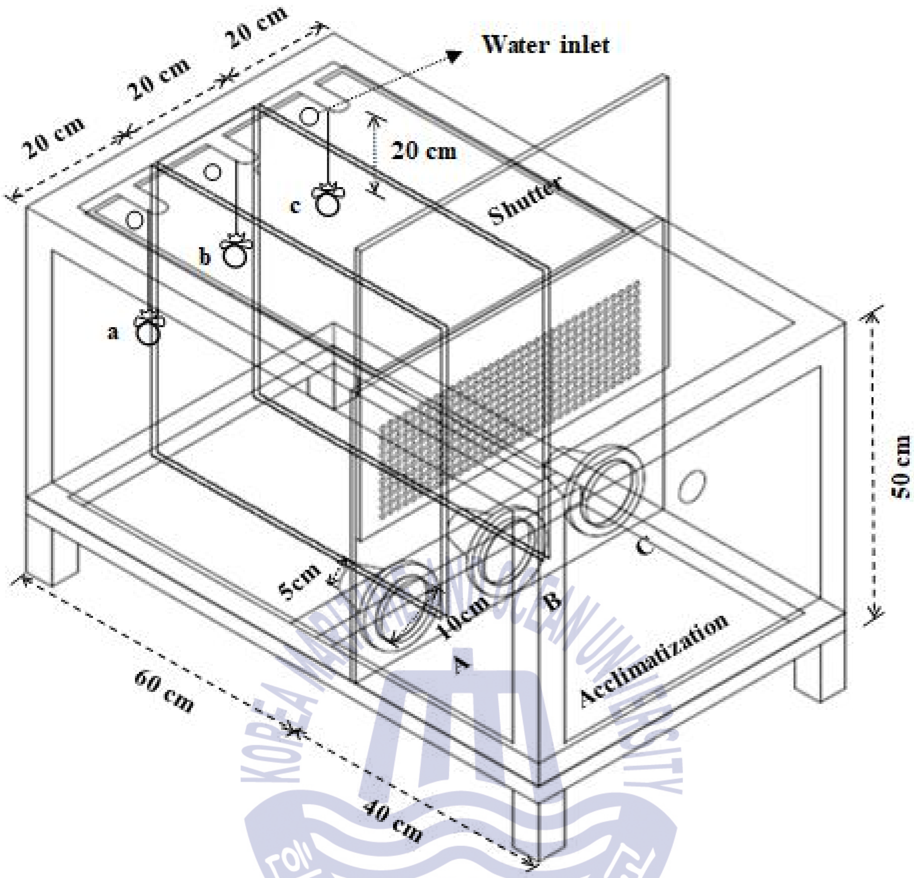

Figure 1. Drawing of the tank used to evaluate the attractiveness of olive flounder (*Paralichthys olivaceus*) responding to various feed ingredients (a, b and c are the location of different feed ingredients in each attracting chamber; A, B and C indicate the entrances of each attracting chamber)

randomly selected fish were stocked into the adaption chamber, and allowed to acclimatize for 3 days. After 3 days, 20 g of the feed ingredients to be tested was placed into three attracting chamber, respectively. And then, shutter was raised to allow access of fish to the feed ingredients. At 30 min, after the raising of the shutter, the number of fish in the attracting chamber was counted and recorded.

All assessments of feeding attractiveness to fish were tested by tournament comparison. From the 1^{st} to the 5^{th} test in the preliminary test, fifteen feed ingredients were compared to determine the highest attractiveness of fish. The relatively high attractiveness of fish for feed ingredients was compared in the 6^{th} to the 9^{th} test in the preliminary test to determine the top 4 feed ingredients. No replication was made in the preliminary test. Finally, the highest feeding attractiveness of fish for top 3 feed ingredients was determined in 5 replications for each trial.

2.2. Determination of Feed Consumption of Fish

2.2.1 The Experimental Conditions

Six hundred juvenile (an initial body weight of 5.8 g) fish were randomly chosen and distributed into 60 of 50 L flow-through tanks (water volume: 40 L) (ten fish per tank). The flow rate of water into each tank was 1.45 L/min/tank. Sand-filtered natural seawater was supplied to each tank with proper aeration. Water temperature, salinity and dissolved oxygen ranged from 16.4 to 19.3°C (mean ± SD: 17.5 ± 1.03°C), from 35.22 to 37.12‰ (mean ± SD: 36.14 ± 0.56‰) and from 8.89 to 9.40 mg/L (mean ± SD: 9.25 ± 0.13 mg/L), respectively, and the photoperiod followed natural conditions.

2.2.2 Preparation of the Experimental Diets

Four experimental EP were prepared having 15 replications for each diet (Table 2). Anchovy meal and fermented soybean meal were used as the protein source in the experimental diets. Wheat flour, and fish oil and soybean oil were used as the

Table 2. Ingredient of the experimental diets (%, DM basis).

	Experimental diets			
	Con	JM	SM	HFM
Ingredient (%)				
Anchovy meal	60	55	55	55
Jack mackerel meal		5		
Sardine meal			5	
Hydrolyzed fish meal[1]				5
Fermented soybean meal	6.5	6.5	6.5	6.5
Wheat flour	25	25	25	25
Squid liver oil	4	4	4	4
Soybean oil	2	2	2	2
Vitamin premix[2]	1	1	1	1
Mineral premix[3]	1	1	1	1
Choline	0.5	0.5	0.5	0.5
Nutrients (%)				
Dry matter	98.3	98.1	98.5	98.3
Crude protein	50.4	50.9	50.7	50.9
Crude lipid	11.9	11.8	11.8	11.8
Ash	12.4	12.3	12.0	11.5

[1]Hydrolyzed fish meal is mixture of jack mackerel, Atlantic wolffish, beaked redfish, black scabbard fish, European plaice, halibut, herring, mackerel, Atlantic cod, black bream, blue ling, European seabass, haddock, ling, pouting, roundnose grenadier, black pollack, whiting, European hake, common scallop and sardine.

[2]Vitamin premix contained the following amount which were diluted in cellulose (g/kg mix): L-ascorbic acid, 200; α-tocopheryl acetate, 20; thiamin hydrochloride, 5; riboflavin, 8; pyridoxine, 2; niacin, 40; Ca-D-pantothenate, 12; myo-inositol, 200; D-biotin, 0.4; folic acid, 1.5; p-amino benzoic acid, 20; K_3, 4; A, 1.5; D_3, 0.003; choline chloride, 200; cyanocobalamin, 0.003

[3]Mineral premix contained the following ingredients (g/kg mix): NaCl, 10, $MgSO_4{\cdot}7H_2O$, 150; $NaH_2PO_4{\cdot}2H_2O$, 250; KH_2PO_4, 320; $CaH_4(PO_4)_2{\cdot}H_2O$, 200; Ferric citrate, 25; $ZnSO_4{\cdot}7H_2O$, 4; Ca-lactate, 38.5; CuCl, 0.3; $AlCl3{\cdot}6H_2O$, 0.15; KIO_3, 0.03; $Na_2Se_2O_3$, 0.01; $MnSO_4{\cdot}H_2O$, 2; $CoCl_2{\cdot}6H_2O$, 0.1.

carbohydrate and lipid sources in the experimental diets, respectively. A 60% anchovy meal was included into the control (Con) diet. The 5% anchovy meal was substituted with equal amount of jack mackerel meal, sardine meal and hydrolyzed fish meal, referred to as the JM, SM and HFM, respectively. All experimental diets were prepared to satisfy dietary nutrient requirements for olive flounder (Kim et al. 2002, Lee et al. 2002).

The ingredients of the experimental diets were well mixed and extruder-pelletized using a twin-screw extruder (Model ATX-2, Fesco Precision Co., Daegu, Korea). Experimental diet was randomly assigned to fifteen replicate tanks and hand-fed to satiation twice daily (09:00 and 17:00 h) for 7 day. Uneaten diets were removed 30 min after feeding and deducted from feed consumption calculations.

2.2.3 Analytical Procedures of the Experimental Diets

Analysis of the chemical composition of the experimental diets was same as that of feed ingredients.

2.2.4 Evaluation of Relative Feeding Activity in the Experimental Diets

Feeding activity for the test solution was determined by daily feeding rate for the experimental diet: feed intake / 100 g of body weight / day, as reported by Ikeda et al. (1988).

$$\text{Feeding activity} = \frac{F}{\frac{W_1 + W_2}{2} \times \frac{N_1 + N_2}{2}} \times 100$$

Where F, W_1, W_2, N_1, N_2 are feed intake (dry basis) in 7 days (g), initial average body weight (g), final average body weight (g), initial number of fish, and final number of fish, respectively. The relative feeding activity for the test solution was expressed as a percentage of that for the control diet.

2.3 Statistical Analysis

The data were subjected to one-way analysis of variance (ANOVA) using SPSS version 19.0 (SPSS Inc., Chicago, IL, USA). Significant differences ($P < 0.05$) among the means were determined using Duncan's multiple range test (Duncan 1955). All percentage data were arcsine-transformed prior to statistical analysis.

3. Results

3.1 Preliminary Screening for Feeding Attractant of Olive Flounder

Feeding attractiveness of olive flounder for feed ingredients were presented in Table 3. From the 1^{st} to the 5^{th} test in the preliminary test, anchovy meal (36.7%), Pollack meal (46.7%), jack mackerel meal (40.0%), sardine meal (33.3%) and hydrolyzed fish meal (53.3%) showed the highest feeding attractiveness. Jack mackerel meal (40.0%), jack mackerel meal (40.0%), shrimp meal (40.0%) and Pollack meal (50.0%) displayed the highest feeding attractiveness from 6^{th} to the 9^{th} test. Results of the tournament comparison from 1^{st} to 9^{th} tests showed the best top 4 feeding attractants, such as jack mackerel meal, sardine meal, hydrolyzed meal and Pollack meal, in the preliminary test.

3.2 Feeding Attractiveness of Olive Flounder for the Top 4 Selected Feed Ingredients

The number of olive flounder moved towards attracting chamber and attractiveness for the top 4 feed ingredients with time were shown in Table 4. The number of fish that moved to the attracting chamber and the attractiveness of fish for jack mackerel was significantly (P < 0.05) higher than those of sardine and hydrolyzed fish meal at 10 min after observation in the 1^{st} trial. In the 2^{nd} trial, the number of fish that moved to the attracting chamber and the attractiveness of fish for sardine meal was significantly (P < 0.05) higher than those hydrolyzed fish and Pollack meal at 10 min after observation. The number of fish that moved to the attracting chamber and the attractiveness of fish for hydrolyzed fish meal was significantly (P < 0.05) higher than that of Pollack meal at 20 min after observation in the 2^{nd} trial. The strongest feeding attractant for olive flounder was jack mackerel followed by sardine meal, hydrolyzed fish and Pollack meal, in order

Table 3. Number of olive flounder moved to the attracting chambers and the attractiveness of fish responding to various feed ingredients

Test	Feed ingredient	Number of fish moved to attracting chamber	Attractiveness (%) of fish[1]
1st	Anchovy meal	11	36.7
	Herring meal	8	26.7
	Soybean meal	3	10.0
	FSA[2]	8	26.7
2nd	Pollack meal	14	46.7
	Corn gluten meal	0	0
	Shrimp head meal	12	40.0
	FSA[2]	4	13.3
3rd	Krill meal	9	30.0
	Squid meal	4	13.3
	Jack mackerel meal	12	40.0
	FSA[2]	5	16.7
4th	Shrimp meal	10	33.3
	Squid liver meal	2	6.7
	Sardine meal	13	43.3
	FSA[2]	5	16.7
5th	hydrolyzed fish meal	16	53.3
	Crab meal	2	6.7
	Mussel meal	7	23.3
	FSA[2]	5	16.7
6th	hydrolyzed fish meal	6	20.0
	Jack mackerel meal	12	40.0
	Sardine meal	8	26.7
	FSA[2]	4	13.3
7th	Jack mackerel meal	12	40.0
	Sardine meal	8	26.7
	Shrimp meal	3	10.0
	FSA[2]	7	23.3
8th	Shrimp meal	12	40.0
	Krill meal	7	23.3
	Shrimp head meal	2	6.7
	FSA[2]	9	30.3

9th	Herring meal	3	10.0
	Pollack meal	15	50.0
	Anchovy meal	9	23.3
	FSA[2]	3	10.0

[1]Attractiveness (%) of fish = number of fish moved into each attracting chamber×100/total number of fish in acclimatization chamber

[2]FSA, Number of fish stayed in the acclimatization chamber after 30 min exposing to each ingredient.

Table 4. The number of olive flounder moved to attracting chambers and the attractiveness of fish responding to the top 4 selected feed ingredients with time

Trial	Feed ingredient	Elapsed time (min)					
		10		20		30	
		Number of fish moved to attracting chamber	Attractiveness (%) of fish[1]	Number of fish moved to attracting chamber	Attractiveness (%) of fish[1]	Number of fish moved to attracting chamber	Attractiveness (%) of fish[1]
1st	Jack mackerel meal	10.4 ± 1.12[a]	33.3 ± 3.74[a]	12.0 ± 0.32[a]	40.0 ± 1.05[a]	12.2 ± 0.37[a]	40.7 ± 1.25[a]
	Sardine meal	6.2 ± 0.73[bc]	20.7 ± 2.45[a]	7.6 ± 0.24[b]	25.3 ± 0.82[b]	8.0 ± 0.32[b]	26.7 ± 1.05[b]
	Hydrolyzed fish meal	5.0 ± 0.32[c]	16.7 ± 1.05[a]	6.0 ± 0.32[c]	20.0 ± 1.05[c]	6.0 ± 0.32[c]	20.0 ± 1.05[c]
	FSA[2]	8.4 ± 1.03[ab]	28.0 ± 3.43[a]	4.4 ± 0.24[d]	14.7 ± 0.82[d]	3.8 ± 0.37[d]	12.7 ± 1.25[d]
2nd	Pollack meal	4.0 ± 0.84 [c]	13.3 ± 2.79[c]	5.0 ± 0.55[c]	16.7 ± 1.83[v]	5.0 ± 0.55[b]	16.7 ± 1.83[c]
	Sardine meal	10.8 ± 1.02[a]	36.0 ± 3.40[a]	11.4 ± 0.68[a]	38.0 ± 2.26[a]	9.8 ± 0.37[a]	38.7 ± 1.70[a]
	Hydrolyzed fish meal	8.4 ± 0.40[b]	28.0 ± 1.33[b]	9.6 ± 0.40[b]	32.0 ± 1.33[b]	9.8 ± 0.37[a]	32.7 ± 1.25[b]
	FSA[2]	6.8 ± 0.66[b]	22.7 ± 2.21[b]	4.0 ± 0.63[c]	13.3 ± 2.11[v]	3.6 ± 0.40[c]	12.0 ± 1.33[c]

Values (means of quintuplicate ± SE) in the same column sharing the same superscript letter are not significantly different (P > 0.05).
[1]Attractiveness (%) of fish = number of fish moved into each attracting chamber×100/total number of fish in acclimatization chamber.
[2]FSA, Number of fish stayed in the acclimatization chamber after 30 min exposing to each ingredient

Once fish moved to the attracting chamber from the acclimatization chamber through the funnel-shaped entrance, no fish returned back to the acclimatization chamber during the 30 min observation.

3.3 Feed Consumption of Fish

Survival (%), weight gain (g/fish) and specific growth rate (SGR) of olive flounder fed the experimental diets for a week were given in Table 5. No significant difference in survival of olive flounder was obtained. However, weight gain and SGR of fish fed the JM diet were significantly (P < 0.05) higher than those of fish fed the Con, SM and HFM diets.

Feed consumption (g/fish), relative feeding activity (%), feed efficiency (FE) and protein efficiency ratio (PER) of olive flounder fed the experimental diets for a week are given in Table 6. Feed consumption of fish fed the JM diet was significantly (P < 0.05) higher than that of fish fed the Con, SM and HFM diets. Feed consumption of fish fed the SM diet was significantly (P < 0.05) higher than that of fish fed the Con diet, but not significantly (P > 0.05) different from that of fish fed the HFM diet. FE of fish fed the JM diet was significantly (P < 0.05) higher than that of fish fed all other diets. However, no significant (P > 0.05) difference in PER was observed among the diets. Jack mackerel meal has the highest relative feeding activity, followed by sardine meal and hydrolyzed fish meal in order.

Table 5 Survival (%), weight gain (g/fish) and specific growth rate (SGR, %/day) of olive flounder fed the experimental diets containing feed ingredients with high feeding attractants for a week

Experimental diets	Initial weight (g/fish)	Final weight (g/fish)	Survival (%)	Weight gain (g/fish)	SGR[1] (%/day)
Con	5.8 ± 0.00^{a}	6.3 ± 0.01	100.0 ± 0.00^{a}	0.51 ± 0.01^{c}	1.20 ± 0.020^{c}
JM	5.8 ± 0.01	6.5 ± 0.02	100.0 ± 0.00^{a}	0.68 ± 0.01^{a}	1.58 ± 0.018^{a}
SM	5.8 ± 0.01	6.4 ± 0.01	100.0 ± 0.00^{a}	0.56 ± 0.01^{b}	1.30 ± 0.023^{b}
HFM	5.8 ± 0.01	6.3 ± 0.02	100.0 ± 0.00^{a}	0.53 ± 0.01^{c}	1.24 ± 0.022^{c}

Values (means of 15 replication ± SE) in the same column sharing the same superscript letter were not significantly different ($P > 0.05$).

[1]Specific growth rate (SGR, %/day) = (Ln final weight of fish − Ln initial weight of fish) × 100/days of feeding trial.

Table 6. Feed consumption (g/fish), relative feeding activity (%), feed efficiency (FE) and protein efficiency ratio (PER) of olive flounder fed the experimental diets containing the various feed attractants for a week

Experimental diets	Feed consumption (g/fish)	Relative feeding activity[1] (%)	FE[2]	PER[3]
Con	0.63 ± 0.010[c]	100.0	0.81 ± 0.005[b]	1.99 ± 0.011[a]
JM	0.80 ± 0.010[a]	125.1	0.85 ± 0.004[a]	1.99 ± 0.006[a]
SM	0.68 ± 0.011[b]	107.3	0.81 ± 0.006[b]	2.00 ± 0.006[a]
HFM	0.66 ± 0.010[bc]	104.0	0.80 ± 0.008[b]	2.00 ± 0.006[a]

Values (means of 15 replication ± SE) in the same column sharing the same superscript letter are not significantly different ($P > 0.05$).

[1]Relative feeding activity (%) = Feed consumed×100/100 g of body weight.

[2]Feed efficiency (FE) = Weight gain of fish/feed consumed.

[3]Protein efficiency ratio (PER) = Weight gain of fish/protein consumed.

4. Discussion

Feeding stimulants and/or attractants have been studied as an important part in influencing nutrient metabolism and the animal growth (Gaber 2005) and they strongly related to feeding behaviors and aquatic ecology (Carr et al. 1996, Kasumyan & Nikolaeva 2002, Smith et al. 2005). Feed ingredients of marine animal origins, such as fish meal, fish hydrolysates, krill meal, shrimp meal, fish solubles, fish oil and various protein hydrolysates are noted for their positive palatability to many fish species (Barrows 2000). Relatively constant and high crude protein and lipid content in various sources of fish meal compared to other protein sources (crustacean, mollusk and plant-originated meals) in this study, seemed to play an important roles as not only effective feeding attractants to aquatic animals, but also good protein sources in aquafeed. To determine the attractiveness of olive flounder for feed ingredients, fish were cued by mainly olfactory stimulus and approached to them in the three equally divided attracting chambers over 50 cm apart away from the acclimatization chamber. In the preliminary test, a strong feeding attractant response of olive flounder was observed in four kinds of fish meal (jack mackerel meal, sardine meal, hydrolyzed fish meal and Pollack meal), however, weak or moderate responses were observed in the other plant-originated meals, fish meals, crustacean meals and mollusk meals.

Squid meal a are great sources of protein ingredient which normally used as feeding stimulant in the diets of gibel carp and red snapper (Xue & Cui 2001, Kader et al. 2010). However, In our study, the attractiveness of fish towards squid meal and squid liver meal was relatively lower than other protein sources tested. Previously, Toften & Jobling (1997) reported that squid extract had no significant effect on feed intake, growth or feed digestibility as feeding attractants and/or stimulants in Atlantic salmon (*Salmo salar*). Hua et al. (2015) also reported that three feeding stimulants, such as betaine, squid extract and squid meal did not showed any significant effect on growth and feed intake in crayfish (*Procambarus clarkia*).

Most of the olive flounder of all trials were moved towards the specific feed ingredients in the acclimatization chamber within 10 min, cued by olfactory stimulus originated from the aqueous extracts of feed ingredients placed in the attracting chambers. A few fish were also moved towards the attracting chamber in either 20 or 30 min after observation. After a few minutes of feed ingredients placement in the attracting chambers, all fish were started to move actively upward and downward. However, the rest fish that did not move towards any attracting chamber from the acclimatization chamber were observed for 30 min. This probably occurred that fish, which did not make a choice of preference among the feed ingredients within 12.5 min (total water input of 121.5 L from the attracting chambers in 12.5 min) and move towards any attracting chamber, rather acclimated to the mixed aqueous attractants of feed ingredients in the acclimatization chamber (water volume: 120 L) and stayed there after 30 min observation.

The strongest feeding attractiveness to olive flounder was obtained in jack mackerel, followed by sardine meal and hydrolyzed fish meal, in order, based on the behavioral response (movement towards the attracting chamber from the acclimatization chamber) of fish in this study. The feed ingredients of marine origin specially fish meals and fish solubles are previously stated as stimulant and palatability enhancers because they are rich in nucleotides, fish protein hydrolysates and also contain free amino acids (NRC 2011). Kohbara et al. (2000) reported that gustatory receptors of yellowtail are involved in discriminating food items during feeding and that the olfactory receptors may detect food as some distance. Ikeda et al. (2012) also reported that aqueous and synthetic extracts of jack mackerel had higher feeding stimulant activity in olive flounder than the diet without the extract of jack mackerel and concluded that histidine in the synthetic extracts of jack mackerel played as a feeding stimulant. Barroso et al. (2013) reported that dietary inclusion of 10% fish meal hydrolysate showed the greatest attractant potential among four marine originated meals (fish meal hydrolysate, polychaete meal, mussel meal and squid meal) as feeding attractants in juvenile Senegalese sole (*Solea senegalensis*) and achieved the highest feed intake of fish. Galtin & Li (2008) supported that the amino acid, glycine has been shown feeding stimulant in several carnivorous fish species at levels up to 2% in diet. The higher attractiveness of olive flounder towards fish meal may be liked with the various amino acids present in it. Further work is necessary to determine the exact reason behind it.

Relatively high feeding activity of olive flounder fed the JM, SM and HFM diets compared to the Con diet in this study indicated feed consumption of fish was relatively well reflected from attractiveness of fish. Similarly, Ikeda et al. (2012) demonstrated that complete synthetic extracts including amino acids, nucleotides and other bases of jack mackerel influenced the feeding stimulant activity of Japanese flounder, among which, amino acids (Lysine, arginine, histidine and ornithine) specially histidine showed the highest feeding stimulant activity compared to diet without the extract of jack mackerel.

However, no study has been conducted on dietary inclusion effect of feed ingredients having high feeding attractiveness to a targeting fish species based on feeding behavior on growth performance and feed utilization of fish so far. Improvement in the growth performance and feed consumption of olive flounder fed the JM and SM diets compared to the Con diet in this study indicated that 5% inclusion of jack mackerel and sardine meals in olive flounder diet acted as the feeding attractants and/or stimulants. Adding the selected feed ingredients that possess high feeding attractiveness of olive flounder to a diet at small amount (5%) could improve the growth and feed consumption of fish efficiently without omission test of the synthetic chemicals of the prey or monitoring electro(neuro)physiological study of fish. Similarly, small amount (<10%) of fish solubles, krill meal, polychaete meal or blood meal in alternative diets effectively improved feed intake of fishes (Day et al. 1997, Kolkovski et al. 2000, Tusche et al. 2011, Kader et al. 2012). Determination of feeding attractiveness for feed ingredients of a targeting fish based on feeding behavior is a practical technique to develop fish feed to enhance the performance and feed consumption in fish. Similarly, Lie et al. (1989) concluded that addition of minced squid or shrimp at 10% in fish feeds increased feed intake, growth and nutrient retention in Atlantic cod (*Gadus morhua*). Feed attractants can play an important role in acceptance of diets in fish as well as enhancing growth resulted from higher consumption (Gaber 2005). Adding attractants or stimulants will be very useful technique to improve feed consumption of fish in low-fish meal diet or starter feed for larval fish.

5. Conclusion

In conclusion, the top 3 strong feeding attractant response of olive flounder was observed in jack mackerel meal, followed by sardine meal and hydrolyzed fish meal in order among various (15 kinds) feed ingredients. The highest feed consumption and relative feeding activity were obtained in olive flounder fed the EPs containing jack mackerel meal, followed by sardine meal and hydrolyzed fish meal. Feed consumption of juvenile olive flounder was well reflected from attractiveness of feed ingredients.

6. Acknowledgements

I would like to express my genuine gratitude to professor Sung Hwoan Cho, Department of Marine Bioscience & Environment, Korea Maritime and Ocean University, Korea for his precious advice and constant encouragement throughout this study. I wish to thanks to my committee, Jin-Hyung Yoo and Kyoung-Duck Kim their critical advices for my thesis, and to professors Cheol young Choi, In-Seok Park, Young Wan Seo, Ki Hwan Moon and Sun Young Lim for their considerate advices and interests in this thesis. I also wish to thank all staffs in Gangneung-Wonju National University for their suppling experimental facilities.

For my Lab. seniors and juniors, Sang Mok Lee, Bum Hee Park, Sung Choon Ji, Chung Il Kim, Young Jin Cho, In-Cheol Choi, Kyoung Tae Kim, Sung Hyo Myung, Won Gwan Jung, Ye Eun Kim, Ki Wook Lee, Hyeon Jong Kim, Ka Hee Kim, Ah Reum Kim, Bok Il Jang, Sang Hyun Lee, A Young Yoon, June Kim, Hae Seung Jung, Seong Il Baek, Ji Hyung Im, Most Waheda Rahman Ansary, Lei Li Yin and Ye Lin Ban at the Feed Nutrition and Engineering Lab. in Korea Maritime and Ocean University. I warmly thank for their help at practical things as well as sharing the good and bad moments of research and life.

Finally, I would like to express my cordial thanks to my family who have continuously loved, encouraged and supported me.

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