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Protein requirement in granulated microdiets for olive flounder (*Paralichthys olivaceus*) larvae

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넙치 자치어의 과립형 미립자 초기사료내 적정 단백질 요구량

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요 약

본 연구에서는 넙치 자치어의 과립형 미립자 초기사료내 적정 단백질 요구량에 대하여 조사하였다. 실험사료는 조단백질 함량이 42%에서부터 58%로서 4%씩 증가시키는 5종류의 과립형 미립자 초기사료(CP42, CP46, CP50, CP54, CP58사료)를 준비하였으며, 이때 에너지 함량은 4.42 kcal g^{-1} diet로 일정하게 유지시켰다. 실험사료를 공급하는 실험구는 3반복구를 두었다. 부화 후(DAH) 14일령의 넙치 자치어 7,500미를 무작위로 추출하여 15개의 70 L 사각형플라스틱 탱크에 탱크당 500마리씩 각각 수용하였다. 과립형 미립자 초기사료의 단백질 함량이 증가함에 따라 모든 필수아미노산(AA) 및 비필수아미노산의 함량은 증가하였다. 사육실험 종료시 넙치 자치어의 생존율은 실험사료에 따른 유의적인 차이를 보이지 않았다. 그러나 넙치 자치어의 마리당 증체량(weight gain)과 성장률(growth rate)은 CP54와 CP58사료 공급구에서 CP42, CP46와 CP50사료 공급구보다 유의적으로 높게 나타났다. 또한 자치어의 전장(total length)은 CP54와 CP58사료 공급구에서 CP42와 CP46사료 공급구보다 유의적으로 길게 나타났다. 넙치 자치어의 조지방 함량은 CP58사료 공급구에서 CP42, CP46와 CP50사료 공급구보다 유의적으로 높았다. 사육 실험 종료시 생존한 어체의 체구성 아미

노산 분석 결과, 실험사료의 종류에 따른 유의적인 차이는 보이지 않았다.

이상의 결과를 고려할 때, 넙치 자치어의 과립형 미립자 초기사료내 단백질 요구량은 성장률(growth rate)에 근거하면 55.2%인 것으로 평가된다.

Keywords: 넙치(*Paralichthys olivaceus*); 자치어; 과립형 미립자 초기사료; 단백질 요구량, 필수 아미노산, 조단백질

I. Experiment

Protein requirements in granulated microdiets for olive flounder (*Paralichthys olivaceus*) larvae

Abstract

The optimal protein requirements in granulated microdiets were determined for larval olive flounder. Five granulated microdiets (CP42, CP46, CP50, CP54 and CP58), containing different levels of protein ranging from 42 to 58% and at a constant estimated energy level (4.42 kcal g^{-1} diet),were prepared in triplicate. 14 days after hatching (DAH), 7500 larvae were placed in 15 indoor 70 L square plastic tanks. As the protein levels increased in the granulated microdiets, all essential and nonessential amino acid (AA) contents increased. The weight gain and growth rate of the flounderlarvae fed the CP54 and CP58 diets were greater than of larvae fed the other (CP42, CP46 and CP50) diets. The total length of larval flounder fed the CP54 and CP58 diets was greater than of the flounder fed the CP42 and CP46 diets. The crude lipid content of the larval flounder fed the CP58 diet was higher than of the flounder fed the CP42, CP46 and CP50 diets. None of the whole body AA profiles of the founder larvae was affected by the protein levels in the granulated microdiets. In conclusion, dietary protein requirement was estimated to be 55.2% based on the growth rate of the larval olive flounder.

KEY WORDS: olive flounder (*Paralichthys olivaceus*),larvae, granulated microdiet, protein requirement, essential amino acid, crude protein

1. Introduction

For the past three decades, the olive flounder (*Paralichthys olivaceus*) has been the most commercially important marine finfish for aquaculture in Korea due to its fast growth, high tolerance to fluctuation of water temperature and resistance against disease. The annual aquaculture production of this fish reached 45,737 tons in Korea in 2015 (MFAFF 2016).

To increase the aquaculture production of marine fish, healthy larval and juvenile fish production should be secured. Therefore, the successful production of larval and juvenile marine fish has been reported by several researcher groups. The larvae of olive flounder are commonly supplied with live foods, such as rotifer (*Brachionus* sp.) and *Artemia* nauplii, microdiets and/or their combination after the newly hatched larval fish have consumed their yolk-sac. The development of a weaning diet to replace live food is critical for lowering production costs and for sustaining a constantly high quantity and quality of larval marine fish production. Kanazawa (2003) emphasized that the development of microparticulate diets as a substitute for live foods is necessary to further increase the productivity of larval marine fish.

The development of microdiets for larval marine fish, such as the Pacific bluefin tuna (*Thunnus orientalis*) (Haga *et al.* 2010, 2011; Takeuchi & Haga 2015), red sea bream(*Pagrus major*) (López-Alvarado & Kanazawa 1994a; Teshima *et al.* 2004), olive flounder (Bai *et al.* 2001; Takeuchi *et al.* 2003; Wang *et al.* 2004; Ji *et al.* 2013), gilthead seabream (*Sparus aurata*) (Saleh *et al.* 2013), European sea bass (*Dicentrachus labrax*) (Person-Le Ruyet *et al.* 1993) and Atlantic cod (*Gadus morhua*) (Baskerville-Bridges & Kling 2000; Johnson *et al.* 2009) have been

successfully made. Especially, Takeuchi *et al*. (2003) reported that a mixture of microparticle diets containing two different molecular weight peptides (1000-2000 and 30,000 Da) was a good source of protein and this type of diet can be given to olive flounder from the larval to juvenile stage. The apparent digestibility coefficients of the protein in the microparticulate diet in 8-week old Atlantic cod larvae were higher (ranging from 76 to 86%) than of those coefficients in *Artemia* (ranging from 47 to 58%) (Johnson *et al*. 2009). Cahu & Infante (2001) reviewed the substitution of live food with formulated diets in marine fish larvae emphasizing the physical aspects of the diet, which must be considered, and the digestibility of the diets in the larval fish and demonstrated that the nutritional requirements are not similar between larvae and juvenile fish. Kolkovski (2013) demonstrated the techniques and methods for manufacturing the microdiet particles and described the chemical and physical properties of the microdiet particles. Still, more studies on the development of microdiets are needed for the stable production of larval marine fish.

The possibility of the complete replacement live food with microdiet alone for some marine fish larvae seems to be limited (Baskerville-Bridges & Kling 2000; Takeuchi *et al*. 2003; Wang *et al*. 2004; Faulk & Holt 2009; Tang *et al.* 2010; Alam *et al.* 2013; Li *et al.* 2013), but the combination of microdiet with live food effectively improved the survival and growth of the larval stages of marine fish (Kolkovski *et al.* 1997; Yúfera *et al.* 2000; Teshima *et al*. 2004; Ji *et al*. 2013). Teshima *et al*. (2004), in particular, showed that live food could be completely replaced with a microdiet containing a molecular weight of 1000 Da soybean peptides or with a microdiet that incorporated live food for 30 days after hatching (DAH) in red sea bream larvae however, for olive flounder larvae, the microdiet incorporated live food only for 15 DAH. However, live food could not

be replaced with the microdiet containing soybean peptides with a molecular weight of 3000 Da for the larvae of both fish species in their study.

Protein is one of the most important and expensive components in feed formulations for fish. The optimal dietary protein level for the maximum growth and survival of olive flounder larvae were suggested to be 60% or higher when microparticulate diets containing 40, 50 and 60% crude protein levels were supplied for 75 days (Bai *et al*. 2001). However, crude proteinlevels with 10% increments in the diets are too wide to determine accurate optimal protein levels for larval olive flounder, and the microparticulate diets used in their study was lab-pelletized. Therefore, the outcome of their study has little applicability to the formulation of practical microdiets for olive flounder larvae.

Therefore, the optimal protein requirement in the granulated microdiet was determined for larval olive flounder in this study.

2. Materials and Methods

2.1. Spawning and larval rearing conditions

The 1-day old fertilized eggs were purchased from Borame hatchery (Jeju Special Self-Governing Province, Korea) and transported to Garolim flounder hatchery (Seosan-si, Chungcheongnam-do, Korea). The 1-day old fertilized eggs were incubated in 5 ton round tanks at 18°C (4 million eggs per tank). Continuous aeration was provided throughout the incubation. The newly hatched larvae were started to be fed on rotifers at 3 DAH, with this food ending on 15 DAH, and the larvae were then fed *Artemia* nauplii (Great Salt Lake, Utah, USA) beginning on 14 DAH and ending on 25 DAH, with the microdiet feeding experiment beginning on 19 DAH. Rotifers and *Artemia* nauplii were enriched with AlgaMac-3050 Plus according to the manufacturer's recommendation to improve highly unsaturated fatty acids (HUFA) before fed to larvae. Rotifers and *Artemia* nauplii were daily supplied based on the feeding schedule and ration (Table 1). The designated amount of the granulated microdiet was handfed to larval fish 6-12 times a day between 06:00 and 18:00 hours.

2.2. Preparation of the experimental diets

The ingredients and feed formulation of the experimental diets are given in Table 2. Pollack and krill meals, wheat gluten and taurine are the protein sources in the experimental diets. Alpha-starch and dextrin were used as the carbohydrate sources, and fish oil was used as the lipid source in the experimental diets. Five granulated microdiets (CP42, CP46, CP50, CP54 and CP58), containing different levels of crude protein ranging from 42 to 58% with 4% increments at the expense of

Table 1 Feeding schedule and ration for olive flounder larvae by days after hatching (DAH) in this study

DAH	Rotifer (NumbermL^{-1})	*Artemia* (Number mL^{-1})	Amount of microdiet (#3) (g^{-1} time)	Amount of mixture of #3 and #4 microdiets at 1:1 (g^{-1} time)	Amount of microdiet (#4) (g^{-1} time)	Daily feeding frequency
0						
3-9	4-5					
10-13	9-10					
14-15	8-10	4-5				
16-18		10-12				
19-21		5-6	0.05			6
22-25		2-3	0.06			8
26-29			0.06			12
30-34			0.07			12
35-40				0.08		12
41-44					0.09	12

Size of #3 and #4 microdiets were 0.31-0.48 and 0.48-0.63 μm, respectively.

Table 2 Feed ingredients of the experimental microdiets (%, dry matter basis)

	Experimental diets				
	CP42	CP46	CP50	CP54	CP58
Ingredients (%)					
Pollack meal[1]	28	32.5	37	41.5	46
Krill meal	30.5	32	33.5	35	36.5
Wheat gluten	4	4	4	4	4
Taurine	2.5	2.5	2.5	2.5	1.7
α-starch	2	2	2	2	0
Dextrin	20	14.3	8.6	2.9	0
Fish oil	6.5	6.2	5.9	5.6	5.3
Soybean lecithin	0.65	0.65	0.65	0.65	0.65
Vitamin premix[2]	4	4	4	4	4
Choline chloride (50%)	0.85	0.85	0.85	0.85	0.85
Mineral premix[3]	1	1	1	1	1
Nutrients (%)					
Dry matter	94.0	94.0	94.2	94.1	94.2
Crude protein	42.6	46.2	50.4	54.3	58.0
Crude lipid	16.0	16.2	16.5	17.1	17.3
Ash	9.0	9.3	10.0	10.4	11.1
Estimated energy (kcal g^{-1})[4]	4.42	4.42	4.42	4.42	4.42

[1] Pollack meal imported from Russia.

[2] Vitamin premix contained the following amount which were diluted in brewer's yeast (mg kg^{-1} diet): L-ascorbic acid, 51.24: DL-α

-tocopheryl acetate, 150.0: thiamin hydrochloride, 20.0: riboflavin, 40.0: pyridoxine hydrochloride, 20.0: nicotinic acid, 150.0: D-calcium-pantothenate, 70.0: inositol, 300.0: D-biotin, 0.2: folic acid, 10.0: p-aminobenzoic acid, 18.2; menadione sodium hydrogen sulfite, 10.0; retinyl acetate, 6.0; cyano cobalamin, 0.001.

[3] Mineral premix contained the following amount which were diluted in brewer's yeast (mg kg^{-1} diet): $MgSO_4 \cdot 7H_2O$, 496.92; $C_4H_2FeO_4$, 65.8; $FeSO_4$, 103.04; $CuSO_4$, 5.97; $CoSO_4.7H_2O$, 3.42; CaI_2, 3.91; $ZnSO_4$, 68.85; $Al(OH)_3$, 3.81; $MnSO_4 \cdot H_2O$, 65.8.

[4] Estimated energy calculated based on 4 kcal g^{-1} for protein and carbohydrate, and on 9 kcal g^{-1} for lipid (Garling & Wilson 1976).

dextrin and at a constant estimated energy level (4.42 kcal g^{-1} diet), were prepared in triplicate.

All ingredients, except for the fish oil, were ground by an air Z-mill (SK Z-mill 0405, Seishin Co. Ltd., Japan) and mixed well. The mixed ingredients were granulated with a granulator (Flow-Z granulator, Okawara Co. Ltd., Japan). The granulated microdiets were dried at 60C by a dryer (Horizontal Fluid Bed Dryer, Okawara Co. Ltd., Japan). The granulated microdiets were sieved and grouped into the two sizes (0.31-0.48 and 0.48-0.63 μm). The debris of the granulated microdiets was sent back to the granulator, but the oversized granular diets were sent to the roll mill, reground, and sieved again. The granulated microdiets were fish-oil coated and packed. The experimental microdiets were manufactured by Daehan Feed Ltd. (Incheon, Korea).

2.3. Experimental conditions

For the feeding trial, 7500 larvae were placed in 15 indoor 70 L square plastic tanks (500 larvae per tank) at 14 DAH. Two size groups of granulated microdiets (0.31-0.48 and 0.48-0.63 μm) were supplied for 26 days as the fish grew (Table 1): the former for larval fish at 19 to 40 DAH and the latter for larval fish at 35 to 44 DAH. At 45 DAH, most of the fish larvae were bottom-settled after metamorphosis for all diet treatments.

Effective mircroorganisms (EM) (Boreong Agricultural Technology Center, Boryeong city, Chungcheongnam-do, Korea) were applied daily to each tank to purify the water at a concentration of 9.6 mL $tank^{-1}$ throughout the feeding trial. The bottom of each tank was siphon-cleaned twice a week. The water temperature ranged from 16.0 to 23.5°C (mean ± SE: 20.6 ± 0.39°C) throughout the feeding trial. The water exchanged rate was 0.17 L $tank^{-1}$ min^{-1}. The photoperiod followed

natural conditions. At the end of the 26-day feeding trial, all surviving fish from each tank were collectively weighed and sampled for growth and nutritional analysis.

2.4. Analytical procedures for the microdiets and larval fish

All surviving larval fish from each tank had been frozen and later were thawed for chemical analysis. The fifty larval fish that had been randomly chosen from each tank were measured for total weight with an electronic analytical balance (ATX224, Shimadzu Corporation, Kyoto, Japan) and for total length by an eyepiece micrometer OM-500N (NaRiKa, Tokyo, Japan) while being viewed under a microscope (Eclipse E200, Nikon, Tokyo, Japan).

Prior to further examination, all samples were homogenized and used for proximate analysis. The crude protein content was determined by the Kjeldahl method (Auto Kjeldahl System, Buchi B-324/435/412, Switzerland), crude lipid was determined using an ether-extraction method, 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. All methods were in accordance with AOAC (1990) practices. The amino acid (AA) composition of the experimental microdiets and larval fish were determined by using a high speed AA analyzer (Hitachi L-8800, Tokyo, Japan) after which the samples were hydrolyzed in 6 N HCl for 24 h at 110°C.

2.5. Statistical analysis

A one-way ANOVA and Duncan`s multiple range test (Duncan 1955) were used to determine the significance of the differences among the means of the treatments by using SAS version 9.3 program (SAS Institute, Cary, NC, USA). Broken-line analysis (Robbins, Norton & Baker 1979) was used to determine dietary protein

requirement of flounder larvae. Percentage data were arcsine-transformed prior to statistical analysis.

3. Results

As the protein levels increased in the granulated microdiets, all essential and nonessential AA contents increased (Table 3).

The survival (%), weight gain (g $fish^{-1}$), growth rate (%) and total length (mm) of the larval flounder fed the granulated microdiets containing the various levels of crude protein are presented in Table 4. The survival, which ranged from 52.1 to 55.0%, was not significantly ($P > 0.05$) affected by the protein levels in the granulated microdiets. However, the weight gain and growth rate of flounder larvae fed the CP54 and CP58 diets were significantly ($P < 0.003$ and $P < 0.006$, respectively) higher than those of the larvae fed the other (CP42, CP46 and CP50) diets. The total length of the larval flounder fed the CP54 and CP58 diets was also significantly ($P < 0.02$) longer than that of the flounder fed the CP42 and CP46 diets, but not significantly ($P> 0.05$) different from that of the flounder fed the CP50 diet.

The moisture, crude protein and ash content of the whole body of larval flounder were not significantly ($P > 0.05$) affected by the protein levels in the granulated microdiets (Table 5). However, the crude lipid content of the whole body of larval flounder fed the CP58 diet was significantly ($P < 0.02$) higher than that of the flounder fed the CP42, which was lowest, CP46 and CP50 diets, but was not significantly ($P > 0.05$) different from that of flounder fed the CP54 diet.

None of whole-body AA profiles of the founder larvae was significantly affected by the protein levels in the granulated microdiets (Table 6).

Dietary protein requirement was estimated to be approximately 55.2% based on growth rate of the larval olive flounder at the various protein levels (Fig. 1).

Table 3 Amino acid (AA) profiles of the main protein sources and experimental microdiets (% of the diet)

			Experimental diets				
	Pollack meal	Krill meal	CP42	CP46	CP50	CP54	CP58
Alanine	4.16	2.96	2.49	2.68	2.76	3.06	3.44
Arginine	4.27	3.34	2.61	2.78	2.90	3.20	3.53
Aspartic acid	7.17	5.77	4.25	4.50	4.71	5.13	5.70
Cystine	0.82	0.39	0.38	0.39	0.46	0.51	0.53
Glutamic acid	9.23	7.22	6.53	6.82	7.10	7.60	8.55
Glycine	3.49	2.46	2.48	2.68	2.82	3.09	3.53
Histidine	1.52	1.13	0.95	1.01	1.04	1.15	1.27
Isoleucine	3.29	2.92	2.08	2.21	2.36	2.58	2.79
Leucine	5.52	4.47	3.39	3.57	3.83	4.18	4.53
Lysine	5.89	4.06	3.38	3.62	3.74	4.13	4.63
Methionine	2.37	1.58	1.10	1.14	1.27	1.42	1.55
Phenylalanine	3.35	2.53	1.88	1.99	2.03	2.21	2.48
Proline	2.41	2.06	2.00	2.14	2.23	2.45	2.73
Serine	3.04	2.19	1.79	1.89	2.07	2.24	2.40
Threonine	3.11	2.41	1.83	1.95	2.11	2.31	2.46
Tyrosine	2.30	1.21	1.39	1.46	1.58	1.78	1.83
Valine	3.84	2.83	2.20	2.34	2.50	2.73	2.96

Table 4 Survival (%), weight gain (mg fish^{-1}), growth rate (%) and total length (mm) of olive flounder larvae at the end of feeding trial

Experimental diets	Initial weight (mg fish^{-1})	Final weight (mg fish^{-1})	Survival (%)	Weight gain (mg fish^{-1})	Growth rate[1] (%)	Total length (mm)
CP42	12.5 ± 0.03	58.6 ± 0.84^{c}	52.1 ± 1.07^{a}	46.0 ± 0.69^{b}	467.2 ± 6.04^{b}	17.3 ± 0.34^{c}
CP46	12.5 ± 0.01	59.1 ± 0.27^{c}	53.0 ± 1.31^{a}	46.5 ±0.30^{b}	471.7 ± 2.59^{b}	17.9 ± 0.22^{bc}
CP50	12.5 ± 0.01	59.6 ± 0.31^{bc}	53.2 ± 1.33^{a}	47.1 ± 0.33^{b}	475.7 ± 2.64^{b}	18.1 ± 0.04^{ab}
CP54	12.5 ± 0.01	61.2 ± 0.35^{a}	54.6 ± 1.51^{a}	48.6 ± 0.35^{a}	487.0 ± 2.78^{a}	18.8 ± 0.32^{a}
CP58	12.6 ± 0.01	61.1 ± 0.58^{ab}	55.0 ± 1.10^{a}	48.6 ± 0.15^{a}	487.2 ± 1.05^{a}	18.8 ± 0.20^{a}

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

[1]Growth rate = Final weight of fish/initial weight of fish ×100

Table 5 Proximate composition (% of wet weight) of the whole body of olive flounder larvae at the end of feeding trial

Experimental diets	Moisture	Crude protein	Crude lipid	Ash
CP42	81.0 ± 0.38[a]	11.4 ± 0.24[a]	2.4 ± 0.06[c]	1.8 ± 0.01[a]
CP46	81.4 ± 0.42[a]	11.5 ± 0.29[a]	2.5 ± 0.05[bc]	1.7 ± 0.03[a]
CP50	82.1 ± 0.31[a]	11.3 ± 0.18[a]	2.5 ± 0.03[bc]	1.7 ± 0.05[a]
CP54	82.1 ± 0.22[a]	11.5 ± 0.13[a]	2.6 ± 0.02[ab]	1.7 ± 0.04[a]
CP58	81.9 ± 0.43[a]	11.9 ± 0.27[a]	2.7 ± 0.04[a]	1.7 ± 0.04[a]

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

Table 6 Whole-body amino acid (AA) profiles of olive flounder larvae at the end of the feeding trial (% of wet weight)

	Experimental diets				
	CP42	CP46	CP50	CP54	CP58
Alanine	0.74 ± 0.015	0.73 ± 0.013	0.74 ± 0.049	0.77 ± 0.024	0.76 ± 0.020
Arginine	0.66 ± 0.018	0.71 ± 0.010	0.65 ± 0.049	0.70 ± 0.052	0.67 ± 0.038
Aspartic acid	1.10 ± 0.032	1.16 ± 0.026	1.09 ± 0.087	1.16 ± 0.080	1.10 ± 0.068
Cystine	0.13 ± 0.003	0.13 ± 0.003	0.13 ± 0.003	0.14 ± 0.009	0.12 ± 0.003
Glutamic acid	1.60 ± 0.047	1.68 ± 0.027	1.61 ± 0.098	1.72 ± 0.075	1.65 ± 0.064
Glycine	0.80 ± 0.015	0.80 ± 0.010	0.79 ± 0.038	0.81 ± 0.035	0.80 ± 0.025
Histidine	0.27 ± 0.003	0.28 ± 0.003	0.27 ± 0.006	0.27 ± 0.018	0.29 ± 0.033
Isoleucine	0.50 ± 0.003	0.52 ± 0.007	0.50 ± 0.023	0.53 ± 0.024	0.51 ± 0.024
Leucine	0.93 ± 0.003	0.95 ± 0.012	0.92 ± 0.031	0.95 ± 0.048	0.94 ± 0.055
Lysine	0.96 ± 0.013	0.95 ± 0.026	0.95 ± 0.039	1.01 ± 0.046	0.97 ± 0.034
Methionine	0.35 ± 0.009	0.35 ± 0.018	0.35 ± 0.015	0.37 ± 0.015	0.32 ± 0.012
Phenylalanine	0.49 ± 0.003	0.48 ± 0.009	0.47 ± 0.026	0.49 ± 0.023	0.45 ± 0.020
Proline	0.45 ± 0.015	0.46 ± 0.012	0.42 ± 0.046	0.44 ± 0.028	0.45 ± 0.017
Serine	0.52 ± 0.029	0.54 ± 0.017	0.51 ± 0.044	0.53 ± 0.038	0.53 ± 0.035
Threonine	0.58 ± 0.009	0.60 ± 0.003	0.57 ± 0.023	0.58 ± 0.034	0.56 ± 0.027
Tyrosine	0.34 ± 0.031	0.32 ± 0.020	0.33 ± 0.012	0.38 ± 0.012	0.37 ± 0.015
Valine	0.60 ± 0.003	0.62 ± 0.006	0.61 ± 0.023	0.63 ± 0.023	0.63 ± 0.038

None of AA profiles of the whole body of fish (means of triplicate ± SE) was significantly affected by the crude protein levels in granulated microdiets ($P > 0.05$).

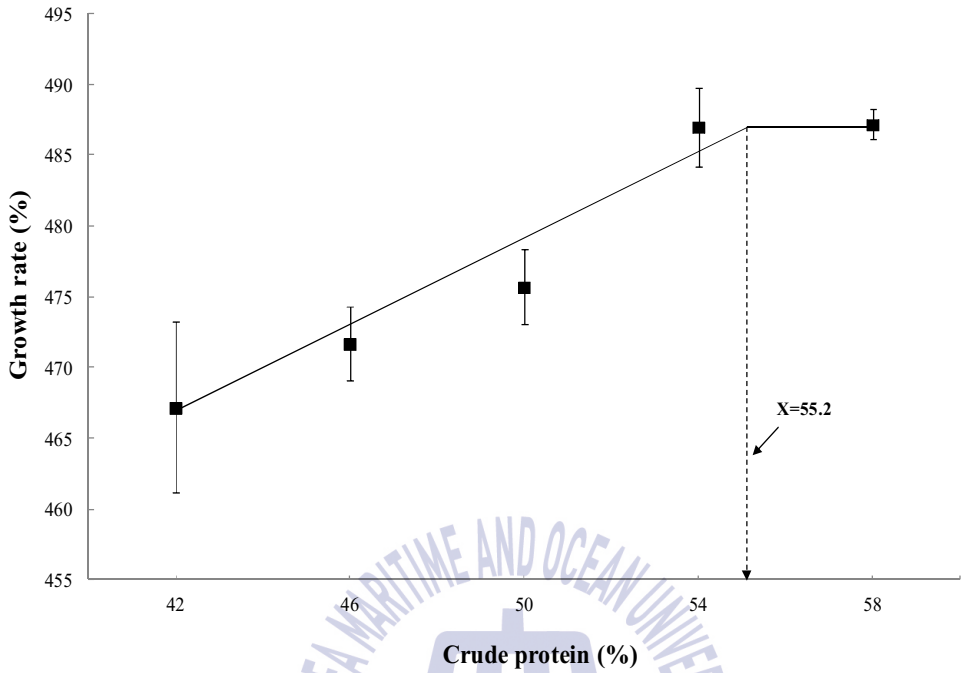

Figure 1 Effect of protein levels in granulated microdiets on growth rate of olive flounder larvae (means of triplicate ± SE).

4. Discussion

Bai *et al*. (2001) suggested that the dietary optimal protein level for maximum growth and survival of olive flounder larvae should be 60% or higher when the microparticulate diets containing fish muscle as main protein source at crude protein levels of 40, 50 and 60% were supplied to larval fish for 75 days from beginning on 8 DAH. However, the suggested values could have been overestimated because growth performance of larval flounder fed the experimental diets was poorer compared to fish larvae fed the commercial diet containing 55.8 to 61.2% protein levels. The poorer performance of the founder larvae fed on even the highest (60%) protein diet compared to the commercial diet probably resulted from the fact that larval fish might consume less protein content than the designated amount since nutrients in the all crumbled experimental diets could have more easily leached out than the commercial diet.

Our broken-line model (Fig. 1) indicated that 55.2% is the optimal protein level based on the growth rate of flounder larvae. Since the supply of protein content in excess in a diet may result in high feed costs and a deterioration of water quality, the optimum amount should be included in the microdiet. Similarly, the best growth and survival were observed in 15 DAH sea bass larvae fed the 50% protein diet when the 30, 40, 50, and 60% protein microencapsulated diets were fed for 21 days (Péres *et al*. 1996).

Cahu & Infante (2001) demonstrated that the nutritional requirements are not similar between larval and juvenile fish. Juvenile olive flounder (an initial weight of 6 g) required 50% protein for best growth when eight experimental diets with four protein (41, 44, 47 and 50%) and two energy levels (20 and 19 kJ g^{-1}) were fed for 45 days (Yigit, Koshio, Teshima & Ishikawa 2004). Kim *et al.* (2003) reported that the dietary protein requirements for the maximum growth of juvenile

olive flounder (an initial weight of 13.3 g) were estimated to be between 40 and 44% when fish meal and casein-based diets containing protein levels from 30 to 60%, with 6% increments and at a constant energy level of 17 kJ g^{-1}, were fed for 8 weeks. These results indicated that the smaller or younger fish require higher protein content in their diets than is required for the larger or older fish. This is in agreement with other studies (Einen & Roem 1997; Sweilum *et al.* 2005) showing that small fish required a high-protein and low-energy diet, whereas large fish required a low-protein and high-energy diet to achieve the bestproduction in Nile tilapia (*Oreochromis niloticus*) and Atlantic salmon (*Salmo salar*). Mangalik (1986) also reported that 3 g channel catfish (*Ictarulus punctatus*) required almost 4 times more protein per day than 250 g fish for maximum growth.

Unlike the study by Bai *et al*. (2001) in which larval flounder were fed with live food (*Artemia* nauplii) to 45 DAH, *Artemia* was supplied to larval fish until 18 DAH in this study. The survival and body weight (total length) of the larval fish at 45 DAH, ranging from 52.1 and 58.6 (17.3) to 55.0% and 61.2 mg (18.8 mm), respectively, in this study, were higher and heavier (longer) than those of larval fish at 41 DAH, which ranged from 43.0 and 54.8 (17.2) to 46.0% and 55.3 mg (17.5 mm), respectively, in the study by Bai *et al*. (2001). This indicated that the initiation of weaning to feeding with granulated microdiets at 18 DAH in this study was appropriate. Survival (66.1%) and total length (28.1 mm) of larval flounder at 40 DAH after being fed the live food (enriched rotifer and *Artemia* nauplii) were superior to those (7.4-24.5% and 19.0-20.7 mm) of larval fish fed a the combination of microparticle diet and one-third the amount of live food or one-third the amount of live food alone (Wang *et al*. 2004). Takeuchi *et al*. (2003) reported that olive flounder larvae at 11 DAH fed a combination of peptides of two molecular weights and live food at one-third the amount of live food for 10 days showed improved survival and growth rate, which were as good observed in

the larval fish fed the live food alone.

The growth of red sea bream larvae at 20 DAH was enhanced by increasing the arginine level up to 2.4% of the diet when larvae were fed on the zein microbound diets containing various levels of arginine from 2.3 to 3.1% for 28 days (López-Alvarado & Kanazawa 1994a). Similarly, the arginine requirement for flounder larvae seemed to be slightly more than the 3.2% of the diet in this study. The nutritional requirements of marine fish larvae have focused primarily on the fatty acid requirements since a few decades ago (Izquierdo *et al.* 2000; Kanazawa 2003), but AA requirements have changed in recent years (Hamre *et al.* 2013; Li *et al.* 2013 Saavedra *et al.* 2015; Canada *et al.* 2016). In particular, histidine appeared to be the limiting AA in live food (enriched rotifers and *Artemia* nauplii) and in dry feed tested for meagre (*Agryrosomus regius*) larvae when essential AA profiles of fish carcass and diet were compared (Saavedra *et al.* 2015) or in enriched rotifers when they were fed to *Diplodus puntazzo* larvae at 4 DAH (Saavedra *et al.* 2007).

Neither chemical composition nor AA profiles of the whole body of larval founder differed among the granulated microdiets containing different levels of crude protein, except for the crude lipid content, in this study. Crude lipid content of the whole larval body was relatively well reflected from that of the granulated microdiets. Similarly, Bai *et al.* (2001) demonstrated that protein content in microparticulate diets did not change either the chemical composition or the whole-body fatty acid composition of larval flounder. The different dietary levels of crystalline AA or dietary arginine content did not affect any whole-body AA contents of olive flounder (López-Alvarado & Kanazawa 1994b) and red sea bream larvae (López-Alvarado & Kanazawa 1994a). However, unlike these studies, the chemical composition of the larval olive flounder was affected by either the different feed or the feeding ration (Wang *et al.* 2004), or the whole-body crude

protein and lipid contents of large yellow croaker (*Larimichthys crocea*) larvae were affected by the dietary AA patterns (Li *et al*. 2013).

In conclusion, granulated microdiet protein requirements were estimated to be approximately 55.2% for larval olive flounders based on the growth rate.

II. Conclusion

The optimal protein requirements in granulated microdiets were determined for larval olive flounder. In conclusion, granulated microdiet protein requirements were estimated to be approximately 55.2% for larval olive flounders based on the growth rate.

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IV. References

Alam, M.S., Watanabe, W.O., Rezek, T.C., Myers, A.R., Carroll, P.M. & Daniels, H.V. (2015)

Growth performance, survival and body composition of southern flounder *Paralichthys lethostigma* larvae fed different formulated microdiets. *Aquacult.Res.,* **46**, 1924-1936.

AOAC (1990) Official methods of analysis (15th edition). Association of Official Analytical Chemists, Arlington, VA, USA.

Bai, S.C., Cha, Y. & Wang, X. (2001) A preliminary study on the dietary protein requirement of larval Japanese flounder *Paralichthys olivaceus*. *North Ame. J.Aquacult*., **63,** 92-98.

Baskerville-Bridges, B. & Kling, L.J. (2000) Development and evaluation of microparticulate diets for early weaning of Atlantic cod *Gadus morhua* larvae. *Aquacult. Nutr*., **6,** 171-182.

Cahu, C. & Infante, J.Z. (2001) Substitution of live food by formulated diets in marine fish larvae. *Aquaculture*, **200,** 1-180.

Canada, P., Engrola, S., Mira, S., Teodósio, R., Fernandes, J.M.O., Sousa, V., Barriga-Negra, L., Conceição, L.E.C. & Valente, L.M.P. (2016) The supplementation of a microdiet with crystalline indispensable amino-acids affects muscle growth and the expression pattern of related genes in Senegalese sole (*Solea senegalensis*) larvae. *Aquaculture*, **458,** 158-169.

Duncan D.B. (1955) Multiple range and multiple F tests. *Biometrics,* **11**, 1-42.

Einen, O. & Roem, A.J. (1997) Dietary protein/energy ratios for Atlantic salmon in relation to fish size: growth, feed utilization and slaughter quality. *Aquacult. Nutr*., **3,** 115-126.

Faulk, C.K. & Holt, G.J. (2009) Early weaning of southern flounder, *Paralichthys lethostigma*, larvae and ontogeny of selected digestive enzymes. *Aquaculture* **296,** 213-218.

Garling, D.L. & Wilson, R.P. (1976) Optimum dietary protein to energy ratios for channel catfish fingerlings, *Ictalurus punctatus*. *J. Nut*., **106,** 1368-1375.

Haga, Y., Naiki, T., Tazaki, Y., Takebe, T., Kumon, K., Tanaka, Y., Nakamura, T., Ishida, Y., Ide, K. & Takeuchi, T. (2010) Effect of feeding microdiet and yolk-sac larvae of spangled emperor *Lethrinus nebulosus* at different ages on survival and growth of Pacific bluefin tuna *Thunnus orientalis* larvae. *Aquaculture Sci*., **58,** 491-500.

Haga, Y., Naiki, T., Tazaki, Y., Shirai, T., Takaki, Y., Tanaka, Y., Kumon, K., Shiozawa,S., Masuma, S., Nakamura, T., Ishida, Y. & Takeuchi, T. (2011) Improvement in the feeding activity, early growth and survival of Pacific bluefin tuna *Thunnus orientalis* larvae fed a casein peptide-based microdiet supplemented with inosine monophosphate. *Fish. Sci*., **77**, 245-253.

Hamre, K., Yúfera, M., Rønnestad, I., Boglione, C., Conceição, E.C. & Izquierdo, M. (2013) Fish larval nutritionand feed formulation: knowledge gaps and bottlenecks for advances in larval rearing. *Rev. Aquacult*., **5,** 526-558.

Izquierdo, M.S., Socorro, J., Arantzamendi, L. & Hernadez-Cruz, C.M. (2000) Recent advances in lipid nutrition in fish larvae. *Fish Physiol. Biochem*., **22**, 97-107.

Ji, L., Haga, Y., Masuda,R., Takahashi, K., Ohta, H., Ishida, S. & Satoh,S. (2013) Growth, survival, digestive enzyme activities, and RNA/DNA ratio in Japanese flounder *Paralichthys olivaceus* larvae fed live food and casein peptide- and fish meal-based microdiets. *Aquaculture Sci*., **61,** 81-93.

Johnson, R.B., Cook, M.A., Nicklason, P.M. & Rust,M.B. (2009) Determination of

apparent protein digestibility of live *Artemia* and microparticulate diet in 8-week-old Atlantic cod *Gadus morhua* larvae. *Aquaculture*, **288,** 290-298.

Kanazawa, A. (2003) Nutrition of marine fish larvae. *J. App. Aquacult.*, **13,** 103-143.

Kim, K., Wang, X. & Bai, S. (2003) Reevaluation of the dietary protein requirements of Japanese flounder *Paralichthys olivaceus*. *J. World Aquacult. Soc.*, **34**, 133-139.

Kolkovski, S., Koven, W. & Tandler, A. (1997) The mode of action of *Artemia* in enhancing utilization of microdiet by gilthead seabream *Sparus aurata* larvae. *Aquaculture*, **155,** 193-205.

Kolkovski, S. (2013) Microdiets as alternative to live feeds for fish larvae in aquaculture: improving the efficiency of feed particle utilization. *In Advances in aquaculture hatchery technology* (ed. by G. Allan & G. Burnell), 203-222.

Li, W., Ai, Q., Mai, K., Xu, W., Luo, Y. & Zhang, Y. (2013) Effects of dietary amino acid patterns on growth and protein metabolism of large yellow croaker (*Larimichthys crocea*) larvae. *Aquaculture*, **406-407,** 1-8.

López-Alvarado, J. & Kanazawa, A. (1994a) Effect of dietary arginine levels on growth of red sea bream larvae fed diets supplemented with crystalline amino acids. *Fish. Sci.*, **60,** 435-439.

López-Alvarado, J. & Kanazawa, A. (1994b) Effect of dietary crystalline amino acids on the larval performance of the Japanese flounder (*Paralichthys olivaceus*). *Special Publication-European Aquaculture Society*, **22,** 261-272.

Mangalik, A. (1986) Dietary energy requirements for channel catfish. Ph.D. dissertation, Auburn University, AL, USA.

MFAFF (2016) Statistical Yearbook 2015. Ministry for food, agriculture, forestry and fisheries. Korea.

Péres, A., Cahu, C.L., Zambonino Infante, J.L., Le Gall, M.M. & Quazuguel, P. (1996). A mylase and trypsin responses to intake of dietary carbohydrate and protein depend on the development stage in sea bass (*Dicentrarchus labrax*) larvae. *Fish Physiol. Biochem*., **15,** 237-242.

Person-Le Ruyet, J.P., Alexandre, J.C., Thebaud, L. & Mugnier, C. (1993) Marine fish larvae feeding: formulated diets or live prey? *J. World Aquacult. Soc*., **24,** 211-224.

Robbins, K.R., Norton, H.R. & Baker, D.H. (1979) Estimation of nutrient requirements from growth data. *J. Nutr*., **109,** 1710-1714.

Saavedra, M., Beltran, M., Pousão-Ferreira, P., Dinis, M.T., Blasco, J. & Conceição, L.E.C. (2007) Evaluation of bioavailability of individual amino acids in *Diplodus puntazzo* larvae: Towards the ideal dietary amino acid profile. *Aquaculture*, **263**, 192-198.

Saavedra, M., Candeias-Mendes, A., Castanho, S., Teixera,B., Mendes, R. & Pousão-Ferreira, P. (2015) Amino acid profiles of meagre (*Argyrosomus regius*) larvae: Towards the formulation of an amino acid balanced diet. *Aquaculture*, **448,** 315-320.

Saleh, R., Betancor, M.B., Roo, J., Hernandez-Cruz, C.M., Moyano, F. & Izquierdo, M. (2013) Optimum soybean lecithin contents in microdiets for gilthead seabream (*Sparus aurata*) larvae. *Aquacult. Nutr*., **19,** 585-597.

Sweilum, M.A., Abdella, M.M. & El-Din, S.A.S. (2005) Effect of dietary protein-energy levels and fish initial sizes on growth rate, development and production of Nile tilapia, *Oreochromis niloticus* L. *Aquacult. Res*., **36**, 1414-1421.

Takeuchi, T., Wang, Q., Furuita, H., Hirota, T., Ishida, S. & Hayasawa, H. (2003) Development of microparticle diets for Japanese flounder *Paralichthys olivaceus* larvae. *Fish. Sci*., **69,** 547-554.

Takeuchi, T. & Haga, Y. (2015) Development of microparticulate diets with special reference to Pacific bluefin tuna, abalone, and Japanese spiny lobster: a review. *Fish. Sci*., **81,** 591-600.

Tang, B.G., Chen, G. & Wu, Z.H. (2010) Application of a microdiet in cobia *Rachycentron canadum* (Linnaeus, 1766) larvae rearing. *Aquacult. Res*., **41,** 315-320.

Teshima, S.I., Koshio, S., Ishikawa, M., Alam,M.S. & Hernadez, L.H.H. (2004) Effects of protein and lipid sources on the growth and survival of red sea bream *Pagrus major* and Japanese flounder *Paralichthys olivaceus* receiving micro-bound diets during larval and early juvenile state. *Aquacult. Nutr*., **10,** 279-287.

Yigit, M., Koshio, S., Teshima, S. & Ishikawa, M. (2004) Dietary protein and energy requirements of juvenile Japanese flounder, *Paralichthys olivaceus*. *J. App. Sci*., **4,** 486-492.

Yúfera, M., Fernández-Díaz, C., Pascual, E., Sarasquete, M.C., Moyano, F.J., Díaz, M., Alarcón, F.J., García-Gallego,M. & Parra, G. (2000) Towards an inert diet for first-feeding gilthead seabream *Sparus aurata* L. larvae. *Aquacult. Nutr*., **6,** 143-152.

Wang, Q., Takeuchi, T., Hirota,T., Ishida, S., Miyakawa, H. & Hayasawa, H. (2004) Application of microparticle diets for Japanese flounder *Paralichthys olivaceus* larvae. *Fish. Sci*., **70,** 611-619.