MARINE REPORTS 1(2) (2022) : 110-121
DOI: 10.5281/zenodo.7394102

scopeScience

MARINE REPORTS

MARINE REPORTS
e-ISSN: 2822-5155
Journal homepage: https://scopesscience.com/index.php/marep/

Received: 06 November 2022; Received in revised form: 27 November 2022
Accepted: 03 December 2022; Available online: 03 December 2022
RESEARCH PAPER

Citation: Uyan, O., Koshio, S., Ishikawa, M. & Yokoyama, S. (2022). Effects of feeding frequency on growth performances and nutrient digestibility of juvenile Japanese flounder, *Paralichthys olivaceus* (Temminck & Schlegel), under varied dietary protein levels. *Marine Reports*, *1*(2), 110-121. https://doi.org/10.5281/zenodo.7394102

EFFECTS OF FEEDING FREQUENCY ON GROWTH PERFORMANCES AND NUTRIENT DIGESTIBILITY OF JUVENILE JAPANESE FLOUNDER, *Paralichthys olivaceus* (TEMMINCK & SCHLEGEL), UNDER VARIED DIETARY PROTEIN LEVELS

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Abstract

The effects of feeding frequency on growth, body composition and nutrient digestibility were studied for the juveniles of Japanese flounders (*Paralichthys olivaceus*) under four different dietary protein levels. Four isocaloric fishmeal-casein based diets containing different dietary protein levels from 46 to 65% were formulated and fed to juvenile flounder (initial mean weight 0.4 g) daily near satiation corresponding to three different feeding frequencies (1, 2 and 3 times/day) for 40 days. Results of feeding trial indicated that both dietary protein level and feeding frequency significantly affected the growth parameters. Regardless of the dietary protein level, the optimum growth performance was obtained from the fish fed 2 times/day. Under two times feeding a day, no significant difference was found between the fish fed diet containing 54 and 59% protein, and no further significant improvement on growth was observed when the dietary protein level increased from 59 to 65%. Protein digestibility was not affected by the feeding frequency or dietary protein level. Findings of this study suggest that feeding frequency twice per day was found to be the optimal, and the optimum dietary protein level

could be 54-59% for the superior growth performances of juvenile Japanese flounder from 0.4 to 18 g.

Keywords: Feeding frequency, dietary protein, digestibility, Japanese flounder, *Paralichthys olivaceus*

Introduction

The rapid growth and efficient feed utilization in cultured organisms can be achieved by optimizing dietary nutrient requirements and feeding frequency. Dietary nutrient deficiencies cause slower growth while excesses or imbalances of dietary nutrient cause water quality deterioration; subsequently leading to disease outbreaks and poor production performance (NRC, 1993). Optimization of feeding frequency is known to control feed intake, a key factor in intaking maximum nutrition (Jobling, 1994), and it affects not only the economies of aquaculture but also the degree of pollution to the environment. Optimization of feeding frequency depends on the size of fish, gut evacuation rate and water temperature. The optimum feeding frequency is also known to be species-specific (Ruohonen et al., 1998; Wang et al., 1998; Lee et al., 2000; Lambert & Dutil, 2001; Dwyer et al., 2002).

The Japanese flounder constitutes one of the most important reared fish species in Asia (Sánchez et al., 2008). Therefore, establishing of the nutritional requirements as well as optimum feeding conditions might be highly crucial on the economy of the flounder aquaculture. Currently available information on Japanese flounder reveals that optimum feeding frequency for the juvenile fish from 3.5 to 15 g was two or three times daily, depending on dietary energy level (Lee et al., 2000).

Because Japanese flounders have limited ability to utilize dietary carbohydrates and lipid, protein seems to be a most important nutrient in the diets (Kikuchi et al., 1992; Kikuchi et al., 2000; Kikuchi & Takeda, 2001). The optimum dietary protein level for Japanese flounders has been reported as 45% (Lee et al., 2002). Previous studies indicate that the possible reason for variation in the optimum protein level was due to the size of fish ranging between 3 to 55 g (Lee et al., 2000; Kikuchi et al., 2000; Kikuchi, 1999; Kikuchi & Furuta, 2000; Kim et al., 2002; Lee et al., 2002; Kim et al., 2003). Therefore, the optimum dietary protein requirement should be clarified based on the size of flounders. At present, information on nutrient digestibility and the optimum dietary protein level for Japanese flounders below 3 g is very limited (Yamamoto et al., 1998; Tani, 2002). Furthermore, there is still limited information on the interactive effects of feeding frequency and dietary protein level on growth performance and nutrient digestibility of this species. Thus, the present study was designed to evaluate the interactive effects of feeding frequency and dietary protein level on growth, body composition and nutrient digestibility in juvenile Japanese flounder with 0.4 g initial body weight.

Materials and Methods

Formulations of Test Diets

The ingredients and proximate composition of the experimental diets are shown in Table 1. Four isocaloric diets were formulated containing four different crude protein levels (46, 54, 59 or 65%) using casein and defatted brown fish meal as the protein sources, pollock liver oil, soybean lecithin and HUFA as the lipid sources, dextrin and α-starch as the carbohydrate sources. Lactoferrin was added to the formulated feeds for improving resistance of experimental fishes to pathogenic bacteria and reinforce fish against the environmental stress (Gallardo-Cigarro et al., 2000). Dietary protein level was increased by adjusting the proportion of the

brown fish meal at the expense of dextrin, α-starch and pollock liver oil. To determine the digestibility of dietary nutrients, 1% chromic oxide was added to each diet as an inert marker substituting the same amount of α-cellulose.

All dry ingredients were well mixed for 30 min in a food mixer. Then lipid sources were added and mixed for 15 min. Finally, water (35% of the dry weight of ingredients) was added, and resulting mash was mixed for 15 min. The pH of the diets was adjusted to 7.0–7.5 with 4 N sodium hydroxide. The mixture was cold extruded to form 1.2 – 2.2 mm diameter pellets, and pellets were then dried in a mechanical convection oven (DK 400, Yamato Scientific, Japan) at 70°C for 30-65 min to reduce the moisture content to 10%. The experimental diets were stored in a refrigerator until use.

Table 1. Formulation and proximate composition of the test diets for Japanese flounder.

	Test Diets			
	Diet 1	Diet 2	Diet 3	Diet 4
Ingredients				
Brown fishmeal (Defatted)[1]	33.0	40.0	47.6	55.0
Casein	20.0	20.0	20.0	20.0
Feed oil[2]	9.2	6.2	3.3	0.7
Soybean lechitin[3]	5.0	5.0	5.0	5.0
HUFA[4]	1.0	1.0	1.0	1.0
Dextrin	3.8	3.1	2.4	1.7
α-starch	3.8	3.1	2.4	1.7
Cholesterol[5]	1.0	1.0	1.0	1.0
Lactoferrin[6]	0.1	0.1	0.1	0.1
Vitamin mixture[7]	2.5	2.5	2.5	2.5
Mineral mixture[8]	2.5	2.5	2.5	2.5
Attractant mixture[9]	1.0	1.0	1.0	1.0
Binder[10]	1.0	1.0	1.0	1.0
α-cellulose	16.1	13.5	10.2	6.8
	100	100	100	100
Proximate composition (% in D.M.)				
Crude protein	46.4	53.9	59.1	65.1
Crude lipid	18.1	15.0	12.1	9.9
Crude ash	8.4	9.6	10.9	12.4
Crude fiber	18.6	14.6	12.5	8.8
N-free extract	8.5	6.9	5.4	3.8
Gross energy (kj/g)[12]	19.6	20.0	19.8	20.1

[1]Defatted by soxhlet (large size) with diethyl ether in the laboratory.
[2]Riken Vitamin, Tokyo, Japan.
[3]Cica-Reagent, Kanto Chemical Co., Inc., Tokyo, Japan.
[4]Poweash A, (Tuna oil ethylester mixture; composition (%): DHA; 40, EPA; 20, lecithin; 5, 14:0; 1.5, 16:0; 3.1, 16:1; 2.8, 18:0; 1.0, 18:1; 3.0, 18:2; 2.6, 18:3, 0.2, 18:4ω3; 3.0, 20:1; 1.4, 20:4ω3; 0.9, 20:5ω3; 22.9, 22:1+20:4ω6; 4.8, 22:4; 2.2, 22:5ω3; 3.4, 22:6ω3; 43.9, 24:1; 1.2, others; 2.1, Oriental Yeast Co. Ltd., Tokyo, Japan.
[5]Cholesterol (cholesterin), Nacalai Tesque.
[6]Morinaga Milk Industry Co. Ltd.
[7]Vitamin mixture (g kg^{-1} diet);, ρ-aminobenzoic acid 0.67; biotin, 0.01; inositol, 6.68; nicotinic acid, 1.30; Ca-pantothenate, 0.47; pyridoxine-HCl, 0.08; riboflovin, 0.33; thiamin – HCl, 0.10; menadione, 0.08; vitamin A-palmitate, 0.32; α-tocopherol, 0.67; cyanocobalamin, 0.46; calciferol, 0.02, ascorbyl-2-phosphate-Mg, 0.12; folic acid, 0.03 and choline chloride, 13.65

[8]Mineral mixture (g kg^{-1} diet); NaCl, 0.919; $MgSO_4$-$7H_2O$, 3.425; $NaHPO_4$-$2H_2O$, 2.18; KH_2PO_4, 5.995; $Ca(H_2PO_4)_2$-$2H_2O$, 3.395; Fe-citrate, 0.743; Ca-lactate, 8.175; $AlCl_3$-$6H_2O$, 0.004; $ZnSO_4$-$7H_2O$, 0.090; $CuCl_2$, 0.003; $MnSO_4$-$4H_2O$, 0.020; KI, 0.004 and $CoCl_2$, 0.025
[9]Attractant mixture (g kg^{-1} diet); DL-α-alanine, 3; Betaine monohydrate, 3; L(-)-proline, 2 and inosine 5-mono phosphate (IMP), 2.
[10]Berda, Bayer, Germany.
[11]N-free extract = 100 – (crude protein + crude lipid + crude ash +crude fiber)
[12]Gross energy is calculated based on: protein, 24 kj g^{-1}; lipid, 39 kj g^{-1}; carbohydrates (NFE) 17 kj g^{-1}.

Growth experiment

Juvenile Japanese flounder, 0.2 g/fish, were collected from a commercial hatchery (Matsumoto Suisan Co., Miyazaki, Japan) and transported to the Kamoike Marine Production Laboratory, Faculty of Fisheries, Kagoshima University. Collected fishes were maintained in experimental tanks for 10 days prior to the start of this study to acclimate well with the laboratory condition. Fishes were fed with a commercial formulated flounder diet (Higashimaru Foods, Kagoshima, Japan) during the acclimation phase.

A 3 (feeding frequency) X 4 (dietary protein level) factorial experiment was designed. Triplicate groups of fish (initial weight 0.4 g/fish) were randomly stocked into 36 polycarbonate 30 L circular tanks fillet with 25 L filtered sea water. The fish were shifted from 30 L tanks to 100 L tanks at Day 20. Twenty fish were placed in each tank and filtered seawater (33,5 ppt) was supplied at a flow rate of 1.5 L/min. The tanks were provided with continuous aeration to maintain dissolved oxygen level at saturation. Water temperature during the trial was 24.9 ± 1.4°C. All rearing tanks were maintained under natural light/dark regime (12:12 h).

Each diet was hand-fed to near the satiation level with one (08:00), two (08:00 - 18:00) or three (08:00 - 12:00 - 18:00 h) times a day for 40 days. To achieve maximum feed intake, the fish were allowed to feed until the time point of settling to the tank bottom. Then, they were allowed to feed again additional five min. After 20 min, uneaten feed particles were siphoned-out, freeze-dried and weighed. Feed intake was recorded and computed taking into account uneaten feed amounts, on a dry matter basis, every 10 days. Total fish weight in each tank was determined every 10 days, and at the end of the experiment. In sampling, all animals were placed on paper towels to remove excess water and then weighed using an electronic balance (model AEL 200, Shimadzu Co. Ltd., Kyoto, Japan).

At the end of the feeding trial, four fish were randomly removed from each tank and freeze dried for body composition analysis. Three fish from each tank were individually weighed and dissected to determine hepatosomatic index after exposing to over dose of eugenol. Initial fish samples were also analyzed for proximate composition.

Determination of digestibility

After termination of the growth trial, three replications of each group were pooled and randomly divided into two replicate groups of 15 fish. The feeding procedures and conditions were the same as those used in the growth trial. Fish were fed the diets containing chromium oxide for one week for acclimation. Each of the experimental diets containing 1% of chromic oxide was fed to fish for 15 days. Before feces collection, tanks were cleaned to remove uneaten diet. Feces were continuously collected by siphoning at 30 min intervals after feeding following 12 h, immediately washed with pure water, patted with tissue paper to dry and freeze dried.

Chemical analysis

Crude protein and total lipid in samples were determined by Kjeldahl method and by Bligh & Dyer, 1959 method, respectively. Ash, fiber and moisture contents of the diets were determined

by Association of Official Analytical Chemistry (AOAC) methods (AOAC, 1995). Chromium contents were analyzed according to the Furukawa & Tsukahara (1966). Nitrogen free extract (NFE) was estimated by the difference: NFE = 100 – (crude protein + crude lipid + crude ash +crude fiber). Gross energy in diets was calculated based on protein, 24 kj/g; lipid, 39 kj/g; carbohydrate (NFE), 17 kj/g (Jobling, 1993).

Statistical analysis

Data were subjected to one or two-way analysis of variance (package super-ANOVA, Abacus Concepts, Berkeley, California, USA). If significant ($P<0.05$) differences were found in the ANOVA, Duncan`s new multiple range test was used to rank the groups. Regression analysis was performed using the software package StatView^{tm} (Abacus Concepts, Berkeley, California, USA). ANOVA results of survival were performed by transformation method (arcsin).

Results

Growth and survival performance

All the experimental groups exhibited high survival (≈90%) and no significant differences were observed among the dietary treatments. As shown in Table 2, both feeding frequency and dietary protein level were significant factors ($P<0.05$) on final average fish weight, SGR, feed intake, and protein intake. Dietary protein level was a significant factor only for the FER and protein retention. On the other hand, there was no interaction between two factors on all the parameters showed in Table 2. There is a trend that final mean weight and SGR improved with increasing dietary protein level and feeding frequency.

Table 2. Growth performance of the experimental groups (Initial weight; 0.40±0.01 g/fish)[1]

Dietary Protein Level (%)	Feeding Frequency (times/day)[2]	Final average fish weight (g/fish)	SGR (%)[3]	Feed intake (g/fish)	Protein intake (g/fish)	FER[4]	Protein retention[5]
46	1	8.7	7.63	5.0	2.3	1.7	58.1
54	1	10.7	8.13	5.6	3.1	1.8	56.5
59	1	12.1	8.52	5.9	3.5	1.9	57.0
65	1	12.4	8.55	6.1	4.0	1.9	52.2
46	2	12.7	8.60	7.5	3.5	1.6	57.3
54	2	15.4	9.09	8.6	4.6	1.8	56.1
59	2	18.3	9.48	9.1	5.3	1.9	59.4
65	2	18.3	9.67	9.3	6.0	1.9	52.5
46	3	13.2	8.69	8.0	3.7	1.6	57.6
54	3	15.0	9.15	8.7	4.7	1.7	54.0
59	3	20.0	9.71	10.3	6.1	1.9	58.2
65	3	20.8	9.77	11.0	7.2	1.9	52.2
Pooled SEM		6.41	1.21	3.56	2.06	0.59	18.33
Two-way ANOVA							
Dietary protein level		0.0001	0.0001	0.0001	0.0001	0.0001	0.0031
Feeding frequency		0.0001	0.0001	0.0001	0.0001	NS	NS
Interaction		NS[6]	NS	NS	NS	NS	NS

[1] Means of three replicate groups

[2] one time a day; at 08:00, two times a day; at 08:00 and 18:00; three times a day; at 08:00, 12:00 and 18:00

[3]Specific growth rate (%/day) = {[ln(final weight) – ln(initial weight)] / duration} x 100

[4]Feed efficiency ratio = wet weight gain / dry feed intake

[5](Protein gain /protein intake) x 100

[6]NS = Non-significant at $P>0.05$

Mean final weight and SGR of the fish fed twice a day was significantly higher (P<0.05) than those of the fish fed once daily in each dietary protein group, but it was not significantly different from the fish fed three times a day. Under the optimum feeding frequency (two times a day) condition, growth (based on SGR) of the fish significantly (P<0.05) improved with increasing dietary protein level up to 59% protein. No further significant improvement on growth was observed when the dietary protein level increased from 59% to 65%. The difference of SGR`s of the fish fed diets containing 54 and 59% protein also did not differ significantly (P>0.05) from each other (Table 2).

Feed and protein intakes significantly increased with increasing dietary protein level and feeding frequency. There was a strong positive correlation between protein intake and protein gain in each feeding frequency. As indicated in Table 2, only dietary protein level was a significant factor on FER and FER which was improved with increasing protein level. Dietary protein level was only a significant factor on the protein retention, and the lowest protein retention was observed from the fish fed the diet containing 65% protein (Table 2).

Body composition and hepatosomatic index (HSI)

The chemical composition of whole body of flounder and HSI values are shown in Table 3. Both feeding frequency and dietary protein level significantly altered dry matter, crude protein, and crude lipid content (P<0.05) of the fish; only exception was for the ash content. The increase in dietary lipid with decreasing dietary protein led to a significant increase in whole body lipid, and dry matter (P<0.05). HSI values were not significantly altered by feeding frequency but by dietary protein level and there was a decreasing trend with increasing dietary protein. Whole body protein content of the fish significantly increased with increasing feeding frequency and dietary protein level (P<0.05). No interactive effect of feeding frequency and dietary protein level was observed on body composition.

Table 3. Whole body proximate composition of the Japanese flounder fed experimental diets (% in wet sample).[1]

Dietary Protein Levels (%)	Feeding Frequency (times/day)[2]	Dry matter	Crude protein	Crude lipid	Crude ash	HSI (%)[3]
Initial		*18.3*	*12.6*	*1.9*	*3.4*	-
46	1	24.7	15.8	4.6	3.2	2.75
54	1	24.3	16.4	3.6	3.5	1.84
59	1	23.7	16.9	2.7	3.5	1.49
65	1	23.1	17.2	2.2	3.5	1.36
46	2	26.0	16.2	5.4	3.3	3.37
54	2	25.3	17.1	4.2	3.3	2.55
59	2	25.1	17.7	3.4	3.3	1.65
65	2	23.4	17.6	2.3	3.4	1.10
46	3	26.4	16.5	5.7	3.4	3.08
54	3	25.8	17.2	4.8	3.3	2.02
59	3	25.1	18.0	3.5	3.5	1.59
65	3	24.4	18.2	2.6	3.4	1.37
Pooled SEM		2.52	1.8	3.0	1.2	2.64
Two-way ANOVA						
Dietary protein level		0.0001	0.0001	0.0001	NS	0.0001
Feeding frequency		0.0001	0.0001	0.0001	NS	NS
Interaction		NS[3]	NS	NS	NS	NS

[1]Means of three replicate groups

[2]One time a day; at 08:00, two times a day; at 08:00 and 18:00; three times a day; at 08:00, 12:00 and 18:00
[3]Hepatosomatic index = (liver weight/final body weight) x 100
[4]NS = Non-significant at P>0.05.

Nutrient digestibility

No significant effect of feeding frequency on apparent dry matter and nutrient digestibility was found, although digestibility coefficients on dry matter and lipid were significantly affected by dietary protein levels (Table 4). Dry matter and lipid digestibility significantly increased with increasing protein or decreasing lipid level. The lowest dry matter digestibility was observed in flounders fed the diet containing lowest protein (46%) and highest fiber (18.6%) levels. No interactive effect of feeding frequency and dietary protein level was observed on nutrient digestibility.

Table 4. Apparent digestibility coefficient (ADC, %) of dry matter, dietary nutrients and energy of Japanese flounder fed experimental diets.[1,2]

Dietary Protein Levels (%)	Feeding Frequency (times/day)[3]	Dry matter	Protein	Lipid
46	1	57.8	92.0	82.1
54	1	64.6	92.5	89.6
59	1	66.5	92.7	90.4
65	1	69.2	92.7	92.9
46	2	59.0	91.5	84.2
54	2	64.5	92.4	88.9
59	2	65.4	92.3	89.0
65	2	71.4	93.4	92.3
46	3	60.2	91.6	82.6
54	3	64.5	92.5	88.3
59	3	65.3	92.4	88.1
65	3	69.6	92.5	91.3
Pooled SEM		13.08	4.68	13.7
Two-way ANOVA				
Dietary protein level		0.0001	NS	0.0001
Feeding frequency		NS[4]	NS	NS
Interaction		NS	NS	NS

[1]Means of three replicate groups.
[2]ADC of dry matter = (1-%Cr_2O_3 in diet/%Cr_2O_3 in feces) X 100; ADC of dietary nutrients = (1-{(%dietary nutrient in feces/%Cr_2O_3 in feces) / (%dietary nutrient in diet/%Cr_2O_3 in diet)}) X 100
[3]One time a day; at 08:00, two times a day; at 08:00 and 18:00; three times a day; at 08:00, 12:00 and 18:00
[4]NS = Non-significant at P>0.05.

Discussion

Feed intake is one of the most important factors for achieving target growth rates and has a significant effect on efficiency of production. In the present study, the final average fish weight and SGR improved when feeding frequency increased from once a day to twice or thrice a day. This is probably because increased growth rate by increasing feeding frequency has been a result of increased food consumption (Table 3), which has also been demonstrated in some fish species such as puffer fish, yellowtail (Ishiwata, 1968; Ishiwata, 1969a, b), rainbow trout (Ruohonen et al., 1998), rock fish (Lee et. al., 2000) and sunshine bass (Webster et al., 2002). Further improvement in growth was not observed even the feeding frequency was increased

from two to three times a day, suggesting that optimum feeding frequency of juvenile Japanese flounder from 0.4 to 18 g is two times a day. Feed intake also increased with increasing dietary protein/decreasing lipid. As a highly palatable feed ingredient, fishmeal affects the feed intake of the fish (Alam et. al., 2003). In the present study, increasing dietary protein level with increasing fishmeal might have promoted feed intake because of increasing feed palatability.

At the optimum feeding frequency (2 times a day), no significant difference was found between the fish fed diet containing 54 and 59% protein, this indicating that optimum dietary protein level for optimum growth could be 54-59% under the present experimental conditions. In general, dietary protein requirements of flatfish such as turbot (Caceres-Martinez et. al., 1984; Person-Le Ruyet, 2002), halibut (Hjertnes & Opstvedt, 1989) and summer flounder (Daniels & Gallagher, 2000) are higher compared to some other marine carnivorous fish, and vary from 55 to 70 %. In Japanese flounders, the reported optimal dietary protein levels have also showed some variations ranging between 40 to 60 %, and seem to decrease with increasing fish size (Kikuchi et. al., 1992; Kikuchi, 1999; Kikuchi et. al., 2000; Kikuchi & Furuta, 2000; Lee et. al., 2000; Kim et. al., 2002; Lee et. al., 2002; Kim et. al., 2003). The study of Lee et. al. (2002) has indicated that a dietary protein level of 45% was good enough for optimum growth and efficient protein utilization of flounder growing between 22.7 g and 110 g. Bai et al. (2001) also suggested that the optimum dietary protein level for the maximum growth and survival of Japanese flounder larvae should be 60% or more. The protein requirement of fish depends on several factors such as size, amount of non-protein energy in the feed and amino acid balance (Jobling, 1995; Lovell, 1998; Guillaume et al., 1999; Thoman, 1999). It has been suggested for some fish species, protein utilization decrease with increasing fish size (Page & Andrews, 1973; Tibbets et. al., 2000; Watanabe et. al., 2000). The difference observed in optimal dietary protein of juvenile Japanese flounder could partly be attributed to the difference in fish size used in those studies.

It is unlikely that the need for high protein is related to imbalances in amino acid levels in present study since protein retention values of the fish fed the diet containing 46, 54 and 59% protein were not significantly different from each other, but significantly higher than those the fish fed diet containing 65% protein. Even though increasing protein intake with increasing dietary protein resulted in growth promotion, it seems that excess protein (65%) is not retained. This is probably because that protein beyond that actively needed for growth could not be utilized for growth, broken down and used for energy by juvenile Japanese flounder (McGoogan & Gatlin 1999). The high correlation between protein intake and protein gain observed in the present study also supports that growth of Japanese flounder highly depends on the dietary protein. It is reportedly known that this fish cannot efficiently utilize dietary lipid and carbohydrate as energy sources, therefore, protein sparing effect of these nutrients is quite poor (Kikuchi et al., 1992; Kikuchi et al., 2000; Kikuchi & Takeda, 2001). Hence, the lipid and carbohydrate levels of the test diets were satisfied to meet requirement level of Japanese flounder, and growth response could be attributed to dietary protein level.

Fish tend to increase their lipid deposition with increasing fat levels in diets in conjunction with decreasing protein intake (Sæther & Jobling, 200; Lee et al., 2002; Alam et al., 2003). Whole body lipid deposition and HSI values showed that although Japanese flounder could digest the dietary lipid with 80 to 90 % even at high dietary levels, fish could not utilize it for protein gain (Table 4), and consequently, excess lipid was stored in body, mainly in the liver. These findings also illustrate that Japanese flounder is able to digest dietary lipid in high degree when the dietary lipid level is around 10% and it decreases with increasing dietary lipid level from 10 to 18%. This is good in agreement with Lee et al. (2000), Oku and Ogata (2000), Tani (2002) and Alam et al. (2002). Growth of young Japanese flounders appears highly protein-dependent, and

protein could not be spared by increasing dietary lipid and carbohydrates, particularly, when the fish were small.

Our results on digestibility are in agreement with those reported by Tani (2002)**,** who described that apparent protein digestibility of Japanese flounders fed diets containing 34 to 65% protein was above 90%. Yamamoto et al. (1998) also reported higher dietary protein digestibility (around 91%) for juvenile Japanese flounder. In the present study, lipid and dry matter digestibility increased with decreasing dietary lipid level. This has also been observed in the study of Tani (2002) using juvenile Japanese flounders. Decreasing lipid digestibility with increasing dietary lipid level could be attributed to limited ability of lipid utilization of Japanese flounders as explained by Kikuchi et al. (2000) and Lee et al. (2000). Furthermore, increasing dietary cellulose concentration with increasing dietary lipid might reduce the lipid and dry matter digestibility because cellulose is an indigestible material.

Nutrient digestibility was not affected by feeding frequency in the present study, which is in agreement with data from rainbow trout (Cho & Kaushik, 1990). Bureau et al. (2002) stated that the lack of effects of feeding frequency on digestibility was not surprising since the rate of passage of feed was determined not by the feeding frequency but rather by fish itself, based on its needs and the chemical characteristics of the feed.

Conclusion

The present study indicated that nutrient digestibility was not affected by feeding frequency, twice daily feeding was optimal, and the optimum dietary protein level could be 54-59% for the best growth performances of juvenile Japanese flounder from 0.4 to 18 g under the present experimental conditions. Since protein is the most important and valuable dietary ingredient in aquafeeds, and growth in early life periods of Japanese flounder is highly dependent on protein, researches on size-dependent absolute protein requirements on Japanese flounder should be expected.

Acknowledgements

The scholarship received by the first author from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Monbukagakusho) is gratefully acknowledged.

Ethical approval

The experiment was performed under the approval of the Committee on Animal Ethics, Kagoshima University, Japan, as a partial fulfilment of the graduate studies of first author.

Informed consent

Not available.

Data availability statement

The authors declare that data are available from authors upon reasonable request.

Conflicts of interest

There is no conflict of interests for publishing of this study.

Funding organizations

This study was financially supported by Kagoshima University, United Graduate School of Agricultural Sciences (RENDAI), Department of Sciences of Marine Resources, Kagoshima, Japan, as a part of the graduate studies of first author.

Contribution of authors

All authors in this study have equally contributed in terms of conceptualization, data curation,

formal analysis, writing original draft, funding acquisition, investigation, methodology, resources, validation, and visualization, and finalizing paper.

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