CHAPTER V

DIETARY PROTEIN REQUIREMENT OF SOUTHERN FLOUNDER (*Paralichthys lethostigma*)[1]

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ABSTRACT

A 12-week feeding trial was conducted to determine the protein requirement of southern flounder, *Paralichthys lethostigma*. Diets varied crude protein (CP) levels (35, 40, 45, 50, 55, and 60%), while dietary lipid levels were held constant at 14% and dextrin was included to maintain diets isocaloric, with all diets providing 370 kcal available energy/100g diet.

Weight gain (percent of initial weight) ranged from 292-394% and was not significantly affected by dietary protein levels. Feed efficiency ratio values (g gain/g fed) ranged between 0.40-0.54 ($P > 0.05$). Protein efficiency ratio (g weight gained/g dietary protein fed) did not differ significantly ($P > 0.05$) between groups (0.9-1.27).

The protein requirement was determined by relating percent increase in weight gain with dietary protein levels utilizing two statistical methodologies: least squares regression (protein requirement: 50.3% CP) and a four-parameter logistic growth curve (50.8 % CP).
KEYWORDS. Southern flounder, *Paralichthys lethostigma*, Protein requirement, weight gain

INTRODUCTION

Southern flounder (*Paralichthys lethostigma*) supports active commercial and recreational fisheries from North Carolina to Texas (Benetti et al. 1999b). Since 1997 however, the species has experienced a significant weakening in landings (NMFS, 1999) although market demand remains strong. The latter has stimulated interest in the potential of southern flounder for intensive aquaculture and has encouraged production-related research with the species (Schwarz et al. 2002). Significant success has been attained in spawning and broodstock selection (Smith et al. 1999b; Luckenbach et al. 2002), as well as in pond rearing and weaning (Jenkins and Smith 1999). The species expresses an adaptable, euryhaline nature, survives over a broad range of temperatures (Daniels et al. 1996; Smith et al. 1999a, 1999b; Taylor et al. 2000; Watanabe and Carroll 2001) and deployment of monosex female lines would further enhance production characteristics of this species (Luckenbach et al. 2003). Research to date therefore, indicates that southern flounder represents an excellent candidate for intensive coastal and inland aquaculture (Smith and Denson 1999).

A fundamental requirement to optimizing growth rates of cultured teleosts is an accurate assessment of their dietary protein requirement. Since protein represents the most costly component of fish feeds, relatively small adjustments to dietary protein levels can result in significant operating gains (Bassompierre et al. 1997). This factor is of particular importance to recirculating aquaculture systems where capital investment is high and profit margins may be nominal. However, while significant information is available upon the protein requirements of Japanese flounder *Paralichthys olivaceus* (Kim et al. 2002; Kim et al. 2003; Lee et al. 2000; Lee et al. 2002; Kikuchi et al. 1992; Kikuchi et al. 2000; Bai-Sungchul et al. 2001), similar information for southern flounder is scant. Daniels and Gallagher (2000) reported no effect upon weight gain in juvenile summer flounder *Paralichthys dentatus* fed diets containing 37-51% dietary protein. However, a diet containing 56% protein significantly elevated weight gains. The latter feed however, differed in energy (4.7-4.9 kcal/g) and lipid content on a dry weight basis, when compared to the other test diets. Nevertheless, Daniels and Gallagher (2000) concluded that summer flounder likely expressed similar demands for dietary protein as other flatfish species. Irrespective of established dietary protein requirements for southern and Japanese flounder however, a need persists to determine optimal dietary protein levels for all candidate aquaculture species and preferably, during different stages of a production cycle. Accordingly, the objective of the present study was to determine the protein requirement for juvenile southern flounder that supported optimal growth.

MATERIALS AND METHODS

Animals and husbandry

Southern flounder eggs were obtained from the University of North Carolina at Wilmington Marine Finfish program. Eggs were hatched and larvae reared at the Virginia Seafood Agricultural Research and Extension Center in Hampton, Virginia. Juvenile flounder were subsequently transferred to the Virginia Tech Aquaculture Center, quarantined and acclimated to conditions of recirculation prior to stocking in a custom-made, 40 L aquaria-based recirculating aquaculture system (RAS). The RAS employed a KMT-based (Kaldnes Inc., Providence, RI, USA) fluidized-bed biofilter for conversion of ammonia to nitrate, a bead filter (Aquaculture Technologies Inc., Metaire, LA, USA) used to eliminate solids (uneaten feed, fecal material, mucus and other fish waste), a protein skimmer for removal of small particulate and dissolved material and a UV sterilizer for disinfection. The aquaria and sump water were oxygenated using diffusion air lines connected to a 1 hp Sweetwater remote drive regenerative blower (Aquatic Ecosystems, Apopka, FL, USA). Water temperature and dissolved oxygen (DO) were monitored daily using an YSI-85 Series dissolved oxygen meter (YSI Inc., Yellow Springs, OH, USA). Total ammonia nitrogen (TAN) was monitored daily by spectrophotometric analysis and nitrite and nitrate levels were quantified once weekly (Hach Inc., Loveland, CO, USA). Throughout quarantine and during experimentation, photoperiod was maintained at a 12 h photophase-scotophase using an automated timer. Light was derived from banks of commercial fluorescent tubes positioned 1.8 m above the experimental system. Following acclimation, fish were sorted by weight (14-35 g) when approximately 180 days old and subsequently randomly distributed into twenty-one 40-L glass aquaria (n=12 per tank). Aquaria were then randomly assigned to one of seven dietary groups (triplicate tanks, n=36 fish per treatment). Water temperature was maintained through ambient air heating/cooling and salinity held at 6 ppt through the addition of synthetic sea salt (Crystal Sea Marine Mix®, Marine Enterprises International, Inc., Baltimore, MD, USA).

Diets and feeding

Six experimental and one control diet, varying in protein content (35-60% crude protein on a dry weight basis) were manufactured (Table 1). A mixture of low temperature menhaden fishmeal (Special Select®, Omega Protein, Hammond, LA, USA) and casein (50:50 w/w) were utilized as the protein sources in the dietary formulations. The control diet was formulated to provide 50% crude protein solely from fishmeal. This feed was employed as a means to verify the ability of southern flounder to utilize dietary casein without detrimental impacts to growth performance. Dietary lipid levels were held constant at 14% dry weight basis and dextrin (US Biochemical Company, Cleveland, OH, USA) was included to ensure that all diets were isocaloric (370 kcal available energy/100 g diet; Table 1). In order to establish feeding rhythmicity, feed palatability and appetite, fish were fed to apparent satiation for 2 wk using the control diet (Table 1). At trial start, fish were fed 3% body weight d^{-1} of the respective diets (Table 1). Diets were hand fed twice daily at 09.00 and 16.00 h. Hand feeding, while labor intensive and time-consuming, was employed instead of automatic feeders in order to monitor feeding behavior and health status of the experimental animals and to guard against feed accumulation and wastage. The experiment was of 12 wk duration.

Analytical procedures

During the trial, fish were group weighed every 2 wk to enable adjustment of feeding rate to account for weight gain. All fish were individually weighed and measured at the beginning and at the end of the study. At trial termination, feed (g gain/g fed) and protein (g gain/g protein fed) efficiency ratios and net protein utilization (initial nitrogen-final nitrogen/total nitrogen consumed) were calculated for each treatment. Visceral somatic index (VSI), hepatosomatic index (HSI) and relative gut length (RGL) were recorded for 3 fish randomly taken from each tank (n=9 per treatment). Prior to dissection, these fish were bled via caudal venipuncture and blood examined for packed cell volume (PCV) and plasma protein concentrations. An additional 3 animals (n=9 per treatment) were used for whole-body proximate analyses. Total lipid was determined gravimetrically (Folch et al. 1957), and crude protein and moisture following standard methods (AOAC 1984). Muscle tissue was separated into interior and fin-ray samples. Fin-ray samples were derived from the musculature lying below the dorsal fins but above the lateral line based upon myomere separation as described by Gaylord et al. (2003). These muscle samples were taken from 3 fish per tank (n=9 per treatment) and likewise examined for lipid, protein and moisture content.

Statistical analysis

All data were subjected to one-way analysis of variance and means were compared by Student's *t*-test with differences considered significant at the $P \leq 0.05$ level (SAS Inc., Cary, NC, USA). The protein requirement was determined using least squares regression (Robins 1986) and a four-parameter logistic growth curve (SAS Inc., Cary, NC, USA). One tank (55% CP diet) was removed from all analyses due to significantly lower performance as assessed by Student's *t*-test.

RESULTS

Water quality parameters were as follows: temperature 26.8 ± 1.94° C, salinity 6.0 ± 2.0 ppt, DO 6.58 ± 0.32 mg/L, TAN 0.37 ± 0.14 mg/L, and nitrite 0.57 ± 0.77 mg/L.

Survival was excellent, ranging from 83-100% for all dietary treatments. No differences were observed between dietary treatments with respect to the growth performance of experimental animals at any stage during the trial. The control diet therefore, verified that the inclusion of casein was without effect upon growth performance in southern flounder. Percent weight gain as an increase from initial weight ranged from 292-394% and was not significantly affected by dietary protein levels (Table 2). Feed (FER) and protein efficiency ratios (PER) did not differ between groups ($P > 0.05$), ranging from 0.40-0.54 and 0.90-1.27 for FER and PER, respectively. Moreover, net protein utilization values (NPU) were likewise identical across all treatments with values ranging from 0.52 to 0.79 (Table 2).

An examination of relative gut length (RGL) and visceral somatic index (VSI) did not reveal differences ($P > 0.05$) between dietary treatment groups (Table 3). Hepatosomatic indices (HSI) did not differ significantly ($P > 0.05$) between treatments; however, southern flounder receiving 45% dietary protein expressed larger liver size (Table 3). Hepatic lipid levels were similar across dietary treatments ($P > 0.05$; Table 3) and ranged from 22.1-34.3%. No apparent relationship existed between HSI and liver lipid content. No differences ($P > 0.05$) were observed in blood parameters including total plasma protein and PCV (Table 4).

Table 5 summarizes the data relating to whole-body composition. Whole-body crude protein levels ranged from 62-69% (dry weight basis), lipid from 18-24% (dry weight basis) and moisture from 72-74%. Fish fed the experimental diets were identical with respect to whole-body protein and moisture analysis ($P > 0.05$). However, whole body lipid content was significantly lower in fish provided with the diet containing 60% crude protein ($P \leq 0.05$). Body lipid also was lower in flounder fed 55% crude protein when compared against fish fed upon the diet containing 35% crude protein ($P \leq 0.05$). An examination of interior and fin-ray muscle composition revealed no differences in lipid content with respect to diet or muscle origin with values ranging from 1.77-4.58% total lipid (dry weight basis) for interior musculature and 2.75-4.58% for fin-ray musculature (Table 6). However, differences ($P \leq 0.05$) were distinguished in protein content of interior muscle samples, with fish fed the diet containing 50% protein diet having significantly higher muscle protein levels (93.7% crude protein {CP}, dry weight basis) compared to fish fed the remaining diets with exception of fish fed the diet containing 55% protein (92.3% CP, dry weight basis). Protein levels in the fin-ray muscle samples did not differ significantly, ranging from 85-93% CP on a dry weight basis.

The protein requirement determined by relating percent increase in weight gain with dietary protein levels utilizing least squares regression was 50.3% CP, while that determined with the four-parameter logistic growth curve was 50.8 % CP (dry weight basis).

DISCUSSION

Techniques that permit the intensive cultivation of southern flounder and other flatfish species in recirculating aquaculture systems are well established (Schwarz et al. 2002). Moreover, the biotechnical demands for southern flounder with regard to induced ovulation, spermiation and spawning, larval rearing and weaning and their salinity tolerance and thermal optima for growth have been described (Jenkins and Smith 1999; Watanabe and Carroll 2001; Benetti et al. 2001a, 2001b). Although success also has been gained in juvenile production and grow-out, this has been accomplished utilizing diets formulated for other species. However, for the culture of southern flounder to become optimized, definitive, species-specific dietary formulations must be developed. As protein represents the most expensive component of fishmeal-based aquafeeds, a prerequisite for cost-effective formulations is the determination of accurate protein requirements.

It is generally accepted that marine flatfish require dietary protein levels that range between 40-60% (Guillaume et al. 1991). For the Bothidae, protein requirements fall in a narrower range of 45-55% crude protein (Kikuchi et al. 1992; Lee et al. 2000, Lee et al. 2002; Daniels and Gallagher 2000; Kim et al. 2002; Hebb et al. 2003). The protein requirements for a number of other carnivorous marine species such as haddock (*Melanogrammus aeglefinus*), rockfish (*Sebastes schlegeli*), olive flounder (*Paralichthys olivaceus*) and spotted sand bass (*Paralabrax maculatofasciatus*), also have been determined to fall in the 45-55% range (Kim et al. 2001a, 2001b; Kim et al. 2002; Alvarez-González et al. 2001).

In the present study, the protein requirement for southern flounder, based upon weight gain, was determined by two different statistical methodologies and yielded very similar results. When least squares regression analysis was fitted to the data, a p-value of 0.012 was obtained and the protein requirement value was determined to be 50.3 % CP (dry weight basis) (Zeitoun et al. 1976). Utilizing a second statistical methodology to verify the requirement value obtained with the least squares regression method, a four-parameter logistic growth curve, a p-value of 0.046 and a requirement of 50.8% CP (dry weight basis) was obtained. This methodology produced a mean square error lower than the least squares regression. Both methods were in agreement (least squares regression Pseudo-R^2 = 95%; four-parameter logistic growth curve Pseudo-R^2 = 97%).

In contrast to the findings of the present trial with southern flounder, Daniels and Gallagher

(2000) reported that summer flounder *P. dentatus*, fed graded protein levels (37-56%) performed optimally at 56% crude protein. The divergence between the findings of the present trial with those of summer flounder is likely a consequence of differences in dietary lipid and energy levels. In the current study, dietary lipid and calorific values were maintained constant across all dietary treatments. Available evidence thus indicates that bothids have a high requirement for dietary protein. Accordingly, commercially available flounder diets, which incorporate 55% protein on a dry weight basis, are over-formulated. It is clear from the results of the present study that comparable growth in juvenile southern flounder was attained with significantly lower dietary protein levels. From an economic and environmental perspective therefore, reduction of dietary protein levels by 10-15% not only could significantly reduce the nitrogen and phosphorus loading in effluents, but also could lead to the manufacture of more cost-effective diets, thereby increasing economic viability of commercial production of this species.

It is noteworthy that southern flounder were able to utilize casein as a dietary protein source without impact upon overall performance. This ability provides flexibility with regard to experimental diet formulations while indicating that this species may be amenable to the incorporation of alternate proteins within their diet. This area of research has considerable significance with respect to the development of less costly dietary formulations and the development of strategies to further reduce the environmental impact potential of aquaculture. An interesting trend observed in the present study was decreasing PER values with increasing dietary protein level. This observation has been recorded previously in other species (Al Hafedh 1999; Yang et al. 2003; Lee et al. 2002; Kim et al. 2001a, 2001b; Kim et al. 2002), and has been attributed to excess dietary protein being utilized as metabolic energy (Alvarez- González et al. 2001; Lee et al. 2002; Yang et al. 2003).

An apparent relationship existed between dietary carbohydrate and protein concentration and HSI and hepatic lipid levels. HSI values numerically decreased in fish fed diets containing greatest protein content but with a correspondingly low carbohydrate level. In contrast, high HSIs were observed in fish fed diets containing 45 and 50 % crude protein. A number of studies have demonstrated that increased dietary lipid and carbohydrate can act to spare dietary protein (Cho and Kaushik 1990; Rasmussen et al. 2000; Kikuchi and Takeda 2001; Lee et al. 2002). However, results presented herein suggest that at optimal dietary protein levels (50%), carbohydrate is not utilized as an energy source. Rather, the enhanced hepatic size, relative to body weight, most likely reflects storage of carbohydrate as fat or glycogen**.** In the present study, glycogen content of the liver was not determined, however the importance of examining hepatic glycogen and lipid content in studies that include a carbohydrate source in the diet, is acknowledged. Research on this matter needs to be addressed with southern flounder to determine if carbohydrates could improve feed efficiencies, growth and furthermore promote a protein-sparing effect that could influence protein requirements and therefore, commercial dietary formulations.

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TABLES

Table 1. Ingredients and composition of the 6 experimental diets fed to southern flounder for 12 weeks.

DIET		1	2	3	4	5	6
		Ingredients g/100g					
Protein %	Control (50)	35	40	45	50	55	60
Fishmeal[1]	70.2	24.6	28.1	31.6	35.1	38.6	42.1
Casein[2,3]	0.0	18.3	21.0	23.6	26.2	28.8	31.4
Dextrin[3]	11.0	26.0	21.0	16.0	11.0	6.0	1.0
Lipid	7.9	11.7	11.4	11.0	10.7	10.4	10.0
Mineral Mix[4]	4.0	4.0	4.0	4.0	4.0	4.0	4.0
Vitamin[4]	3.0	3.0	3.0	3.0	3.0	3.0	3.0
CMC[3,5]	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Cellufill[3]	2.8	11.4	10.6	9.8	9.0	8.2	7.4
		Proximate composition					
Energy[6] (kcal/100g)	370	370	370	370	370	370	370
Protein[7]	51.1	35.1	38.3	45.6	48.9	54.3	60.1
Lipid[7]	14.9	14.4	12.4	13.1	14.3	12.2	13.7

[1] Special Select® menhaden fishmeal (Omega Protein, Hammond, LA): 71.2% crude protein, 8.63% lipid (dry weight basis).

[2] Casein, 95.4% crude protein, 1% lipid (dry weight basis)

[3] US Biochemical Corporation, Cleveland, OH

[4] Mineral and vitamin mix, see Moon and Gatlin (1991)

[5] Carboxymethyl cellulose.

[6] Calculated

[7] Analyzed

Table 2. Weight gain, feed efficiency and protein efficiency ratio (means ± standard error) of southern flounder fed diets containing different dietary protein levels for 12 weeks.

% Dietary protein	Weight gain[1]	Feed efficiency[2]	PER[3]	NPU[4]
35	292 ± 11.4	0.45 ± 0.04	1.27 ± 0.10	0.78 ± 0.07
40	295 ± 49.1	0.40 ± 0.08	1.06 ± 0.20	0.64 ± 0.13
45	350± 14.0	0.48 ± 0.03	1.05 ± 0.06	0.79 ± 0.09
50	377 ± 28.7	0.51 ± 0.02	1.04 ± 0.03	0.52 ± 0.02
55	370 ± 22.0	0.47 ± 0.02	0.87 ± 0.04	0.61 ± 0.07
60	394 ± 24.7	0.54 ± 0.02	0.90 ± 0.03	0.56 ± 0.07

[1]Weight gain (% initial weight)

[2]Feed efficiency (g gain/g fed)

[3]PER (g gain/g protein fed)

[4]NPU (Initial nitrogen - final nitrogen)/total nitrogen consumed)

Table 3. Visceral somatic index (VSI[1]), hepatosomatic index (HSI[2]), relative gut length (RGL[3]), lipid content of livers[4] (means ± standard error) of southern flounder fed diets containing different dietary protein levels for 12 weeks.

% Dietary protein	VSI	HSI	RGL	Liver lipids
35	4.03 ± 0.16	1.82 ± 0.11	101.78 ± 4.09	27.07 ± 1.92
40	3.70 ± 0.16	1.56 ± 0.12	90.56 ± 4.03	27.68 ± 1.92
45	4.46 ± 0.56	2.05 ± 0.30	108.00 ± 5.89	29.24 ± 3.21
50	3.58 ± 0.16	1.64 ± 0.11	94.89 ± 5.02	34.32 ± 3.58
55	3.80 ± 0.29	1.69 ± 0.18	96.33 ± 6.83	25.15 ± 6.06
60	3.40 ± 0.19	1.33 ± 0.12	94.56 ± 5.49	22.12 ± 4.13

[1]Visceral somatic index (Viscera weight X 100/body weight)

[2]Hepatosomatic index (Liver weight X 100/body weight)

[3]Relative gut lengths (mm)

[4]Liver lipid values corrected by dry matter

Table 4. Blood parameters (means ± standard error) of southern flounder fed diets containing different dietary protein levels for 12 weeks.

% Dietary protein	PCV[1]	Plasma protein (g/100 ml)
35	40 ± 2.79	6.30 ± 0.25
40	43 ± 3.12	5.98 ± 0.21
45	35 ± 1.49	5.97 ± 0.26
50	37 ± 2.28	6.13 ± 0.21
55	40 ± 2.08	5.95 ± 0.21
60	37 ± 2.39	5.58 ± 0.15

[1] Packed cell volume (%)

Table 5. Whole body lipid and protein (means ± standard error) of southern flounder fed diets containing different dietary protein levels for 12 weeks.

% Dietary protein	Crude protein (%)[1]	Lipid (%)[2]	Moisture
35	62.9± 1.76	24.0 ± 2.15[a]	72.8 ± 1.01
40	61.9 ± 2.09	23.4 ± 1.71[ac]	72.8 ± 0.18
45	68.7 ± 2.73	23.2 ± 1.13[ac]	72.7 ± 0.74
50	67.8 ± 4.37	23.0 ± 0.42[ac]	74.4 ± 1.71
55	65.5 ± 1.44	18.9 ± 1.56[bc]	72.8 ± 0.30
60	65.4 ± 0.63	18.2 ± 0.61[b]	73.3 ± 0.42

[1]Whole body crude protein values corrected by dry matter.

[2]Whole body lipid values corrected by dry matter; Means within a column with different letters are significantly different ($P \leq 0.05$)

Table 6. Protein and lipid content of inner and fin-ray muscle (means ± standard error) of southern flounder fed diets containing different dietary protein levels for 12 weeks.

% Dietary protein	Crude protein (%)[1]		Lipid (%)[2]	
	Inner muscle	Fin-ray muscle	Inner muscle	Fin-ray muscle
35	91.0 ± 0.53[bc]	85.2 ± 5.51	3.94 ± 0.80	4.58 ± 1.84
40	91.2 ± 1.02[bc]	86.2 ± 5.48	4.00 ± 0.12	4.54 ± 1.52
45	90.0 ± 0.36[c]	85.3 ± 5.68	4.59 ± 0.34	5.23 ± 1.78
50	93.7 ± 0.58[a]	91.6 ± 1.16	3.76 ± 0.30	3.54 ± 0.26
55	92.3 ± 0.99[ab]	93.0 ± 1.56	3.02 ± 0.44	2.66 ± 0.51
60	91.4 ± 0.47[bc]	87.6 ± 4.89	2.75 ± 0.20	3.03 ± 0.91

[1]Protein values corrected by dry matter. Means within a column with different letters are significantly different ($P \leq 0.05$)

[2] Lipid muscle values corrected by dry matter