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Proceedings of the Twentieth U.S.-Japan Symposium on Aquaculture Nutrition

Panel Chairmen:

James P. McVey, United States
Kenji Takagi, Japan

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Preface

The United States and Japanese counterpart panels on aquaculture were formed in 1969 under the United States-Japan Cooperative Program in Natural Resources (UJNR). The panels currently include specialists drawn from the federal departments most concerned with aquaculture. Charged with exploring and developing bilateral cooperation, the panels have focused their efforts on exchanging information related to aquaculture that could be of benefit to both countries.

The UJNR was begun during the Third Cabinet-Level Meeting of the Joint United States-Japan Committee on Trade and Economic Affairs in January 1964. In addition to aquaculture, current subjects in the program include desalination of seawater, toxic micro-organisms, air pollution, energy, forage crops, national park management, mycoplasmosis, wind and seismic effects, protein resources, forestry, and several joint panels and committees in marine resources research, development, and utilization.

Accomplishments include increased communication and cooperation among technical specialists; exchanges of information, data, and research findings; annual meetings of the panels, a policy-coordinative body; administrative staff meetings; exchanges of equipment, materials, and samples; several major technical conferences; and beneficial effects on international relations.

James P. McVey - United States
Kenji Takagi - Japan

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A REVIEW OF SOME STUDIES OF FOOD REQUIREMENT AND FOOD WEBS IN THE FISH STOCK ASSESSMENT

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ABSTRACT

The stock assessments studied are mainly about single species in Japan, but some scientists are studying about multi-species. These stock assessments are based on population dynamics or system dynamics. In this process, the necessity of studies of food webs and transfer efficiency between the same species or another species is increased. A few scientists study these parameters in the field, but mainly these parameters are studied by nutritional or bioenergetics studies in laboratories.

Introduction

The main purpose for fisheries management is how the limited fish resources should be divided among competing fishermen. Fisheries management is dependent on the sciences of economics, sociology, and biology. Fisheries management is also based on planning, organizing, leading, and controlling fisheries.

Fisheries management is based on stock assessments that are studied by population dynamics or system dynamics. Stock assessment studies are about the quality and quantity of fisheries. Stock assessments are studied mainly about the single species in Japan, but some scientists are studying about the multi-species. Stock assessments about multi-species are mainly based on the studies of bioenergetics or food webs about individuals and populations.

Quantity and Quality

Many acronyms are used to describe the quantity of fisheries management and are described as follows:

1. MSY (maximum sustainable yield)
   a. Surplus production model
   b. Reproduction model (Beverton-Holt model, Richer model, Growth model)
2. MEY (Maximum net Economic Yield)
3. TAC (Total Allowable Catch)
4. ABC (Acceptable Biological Catch)
5. OY (Optimum Yield)
6. DAH (Domestic Annual Harvest)
7. DAP (Domestic Annual Processing)
8. JVP (Joint Venture Processing)
9. TALFF (Total Allowable Level of Foreign Fishing)

The formula is TALFF=OY-DAH-Reserves.

The primary goal of fisheries management in Japan is MSY. A lot of effort and cost combine to make an accurate MSY. But, MSY is the outstanding goal to management of the fisheries.

Quality of fisheries management is described as follows:

1. Catch males or females only after spawning (do not catch spawning fish).
2. Condition is the most valuable component of price.
3. The most effective use of the energy is to catch fish at the maximum period of the food coefficient, and the plateau of the growth rate, or to catch low-trophic-level fish.

Individual and Population

The non-biological environments, such as temperature, light intensity, and quality of water (oxygen, salinity, pH, etc.) are not only studied in the laboratory, but they are also studied in the field. There are many studies about biological environments such as quality and quantity of food in lakes, bays, and near the shore. But little research has been done in the open sea. The information or parameters mainly studied in the laboratories are the nutritional studies or the bioenergetical studies.

From these studies, there are many bioenergetics models. Bioenergetics modeling has its own strengths and weaknesses. A typical goal of the bioenergetic approach is to estimate the pattern and amount of consumption by a fish or a population of fishes over some interval of time with a specified change in mass. Given initial mass, final mass, parameter values, and the temperature regime, the amount of food consumption and change in mass per day can be calculated. Given initial mass, parameter values, temperature regime, and proportion of maximum consumption per day, final mass attained by a fish can be estimated for some time interval. Also, which food is converted to new tissue. Transfer coefficient from one trophic level to the higher trophic level is estimated mainly in calories. Each annual has a number of food choices: primary producers; herbivores; carnivores; and scavengers. Herbivores can become carnivores within their life cycles, so the food web itself changes in time. The categories of function (producer, herbivores, carnivore, and scavenger) do not change, but they do necessarily correspond with groups of animals, because the functions can change within a life cycle. The secondary "production" is about one-tenth of the primary "production," but the third level consumer is about one-fifth of the secondary production. Growth efficiency declines with age, and population efficiency increases with age (Cushing, 1975).

Conclusion

Fisheries management is done to benefit a lot of people (commercial fishermen, processors, foreign fishermen, sport fishermen, distributors, consumers, etc.). Conservation, economic performance, social equality, administrative feasibility, and political acceptability are aspects of fishery management. Fisheries management is treating the natural resources so that they can be recycled. The continuous use or sustainability of fisheries resources is the most important goal for the fisheries management. There are also many opinions about the effect of fisheries or game fishing on the environment. Therefore, we have to assess these effects based on science not on emotion. In this situation, scientific study about multi-species stock assessment is necessary. Even to make the stock assessment for a single species, we need a lot of parameters and information about a particular fish and its biological- and non-biological environments. These data are not generally available from the field. A lot of information comes from the nutritional studies or bioenergetics studies in the laboratories. We have few parameters and information about the fisheries management of multi-species. But we would like to take one step toward multi-species fisheries management.

Literature Cited

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INTERACTIONS OF DIETARY ENERGY AND PROTEIN IN DIETS FOR STRIPED BASS AND ITS HYBRIDS (*MORONE* SP.)

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ABSTRACT

A series of studies was conducted to evaluate the interactions of dietary energy and protein in diets for striped bass and its hybrids. In the preliminary feeding trial, hybrid striped bass (50-60g) were trained to feed on semi-purified diets based on casein and egg-albumin protein. Diets were fed at three energy levels (4.5, 3.5, and 2.7 kcal ME/g) with four levels of protein (25, 30, 35, and 40%) at each caloric level. At each of the four protein levels, fish fed diets with 4.5 kcal ME/g gained significantly less weight than those fed lower amounts of energy. Food consumption was probably restricted at this high energy intake. At the 3.5 kcal/g energy level, fish fed 25% protein achieved significantly less growth compared to 30, 35, or 40% protein levels. In the second and third feeding trials using hybrid striped bass (15-20g) and striped bass (1g), respectively, fish were fed diets based on commercial feed ingredients containing 25, 30, 35, or 40% protein at either 2.5 or 3.5 kcal ME/g. Hybrid striped bass receiving 35 or 40% protein and 3.5 kcal ME/g grew significantly ($p \leq 0.05$) better than those fish receiving any of the diets with 2.5 kcal ME/g. There was no significant improvement in growth at the 3.5 kcal ME/g after the protein level reached 30% of the diet. Results for striped bass were similar, fish fed diets with 3.5 kcal ME/g grew significantly ($P \leq 0.05$) better at each protein level compared to their 2.5 kcal ME/g counterparts. Likewise there was no significant difference in growth for striped bass fed 30, 35, or 40% protein at the higher calorie level. In both feeding trials, Protein Efficiency Ratio (PER) and Feed Conversion Ratios improved at the higher (3.5 kcal/g) calorie level. Best values for PER and Feed Conversion were at 35% protein and 3.5 kcal/g. Based on the results of these studies, the minimum amount of dietary protein to support growth in striped bass was around 30%, while the optimum level was nearer 35% of the diet. The preferred energy level was 3.5 kcal ME/g (based on Physiological Fuel Factors), and the protein energy ratio giving the best results was 100 mg protein/kcal. Therefore, pond trials using (209 ± 9g) hybrid striped bass were conducted using these data to construct diets based upon fish meal. Weight gain doubled in 6 weeks. At the end of 12 weeks the average weight per fish was 563 ± 9 g (1.24 lbs), which is a marketable size. Feed conversion was 1.6 feed intake/g gain. Specific growth rates averaged 3.7 ± 0.05 g/day.

Introduction

Protein requirements of fish are a function of the quantitative requirements for each of the essential amino acids and the protein-to-energy ratio (mg protein/kcal) of the diet (Cowey and Sargent, 1979; Millikin, 1982a). Wilson (1985) reviewed the amino acid requirements of fish and demonstrated that the same 10 amino acids (arginine, histidine, isoleucine, lysine, leucine, methionine, phenylalanine, threonine, tryptophan, and valine) are required for all species studied. There is evidence, however, that quantitative amino acid requirements vary from species to species (Hidalgo, et al., 1987; Arai et al., 1972; Wilson, 1985).

That fish have optimum dietary protein energy ratios is well documented. Optimum ratios range from 73 mg protein/kcal for channel catfish to 140 mg protein/kcal for American eels (Ringrose 1971, Lee and Putnam 1973, Garling and Wilson 1976). Protein-to-energy ratios have important influences on protein requirements; diets too high in energy will

cause fish to stop feeding before enough protein is consumed to meet the needs of the animal, unless the diet is excessively high in protein. Diets too low in energy will cause dietary protein to be used to meet energy needs. In both cases, poor growth, increased feed costs, or both are likely to occur.

Total protein requirements have been investigated in a few temperate water species and appear to range from 35 to 45% of the total diet. Using the classical growth and food conversion approach, Anderson et al. (1981) reported that the dietary protein requirements for large mouth bass, *Micropterus salmoides*, was near 40%. Moore et al. (1988) calculated the protein requirements of juvenile white sturgeon (*Acipenser transmontanus*) with four separate statistical manipulations of percent body weight increase and concluded that the optimal level was 40%. Hung et al. (1987), however, reported that purified diet containing 50% protein resulted in significantly better growth in *A. transmontanus*. Millikin (1982b) reported that best growth of striped bass (*Morone saxatilis*) was obtained on a diet containing 55% protein, while Berger and Halver (1987) reported a value of 52% protein. The purposes of the studies reported here were to evaluate the effect of total dietary energy content on the protein need of striped bass (*Morone saxatilis*) and its hybrids and to estimate appropriate protein energy ratios and dietary protein levels for these fish.

Materials and Methods

All feeding trials were conducted in a closed freshwater system containing a total of 12,000 L of water. The system was equipped with gravel biological filters and 24-200 L culture tanks. All hybrid striped bass were obtained from the North Carolina Demonstration Aquaculture facility in Aurora, NC, or from Virginia State University. Crosses were made, larvae reared, and fingerlings adapted to fishmeal-based chow at these facilities. Striped bass were obtained from the North Carolina Wildlife Resources Commission.

Preliminary Studies

Hybrid striped bass [striped bass (female) x white perch (male), weight 50-60 g] were trained to consume semi-purified diets over a 10-day period by step-wise (10% per day) replacement of fishmeal chow with the semi-purified diet. Replacement mixtures were fed as 1/16" pellets at 7% of body weight/day. The feeding rate was determined based on the maximum consumption rate of fishmeal chow by the fish. After fish were trained to consume the semi-purified diet, ten fish were placed in each of two replications for each of the diet formulations shown in Table 1. Casein and egg white were used as protein sources to produce appropriate amino acid balance. Diets contained four levels of protein (25, 30, 35, and 40%) each at three energy levels (2.7, 3.5, and 4.5 kcal/g). Energy levels were calculated using the physiological fuel factors 4.0 kcal/g (1 kcal = 4.184 kilojoules) for protein, 4.0 kcal/g for carbohydrate, and 9 kcal/g lipid (Kleiber, 1975). Although the caloric values of nutrients in fish are a matter of some discussion, these caloric values have been used extensively (Garling and Wilson, 1976, 1977; Lee and Putnam, 1973; Millikin, 1982b; Yu, et al., 1977; Furuichi and Yone, 1980). Fish were fed diets as pellets by hand three times a day for 10 weeks. At the end of the feeding trials, fish were weighed (± 0.1 g) and fork length determined (mm FL). Triplicate determinations were made on five individual fish from each replication for moisture, lipid, protein, and ash (Gallagher, et al., 1984). Moisture was measured by freeze-drying (-50°C) to a constant weight. Lipid content was determined by Soxhlet extraction with chloroform/methanol (2/1), and protein was measured by the biuret method. Ash was determined by combustion at 600°C for 6 hours. Temperature was monitored using high-low thermometers. Mean low temperature was 20.0 ± 2.0°C and mean high temperature was 23.0 ± 2.5°C. Ammonia levels were measured daily and remained at less than 1 ppm, nitrate levels were at less than 4 ppm throughout the experimental period, and O_2 levels were at 7-8 ppm.

Protein Energy Studies

Diet formulations based on results from the preliminary study were prepared using commercial ingredients (Table 2). Striped bass (female) x white bass (male) hybrids (15-20 g) were used in the first feeding trial. Twenty fish were placed in each of three replications and fed experimental diets (1/16" pellet, 7% body weight) for 6 weeks. Four protein levels (25, 30, 35, and 40%) at two energy levels (2.5 kcal/g and 3.5 kcal/g) were utilized. In addition, a control group with two replications was fed Zeigler trout chow (Ziegler Bros. Inc, Gardner, PA, crude protein 38.2%, lipid 8.2%, fiber 3%, and ash 9.9%) at the same feeding level. Fish were weighed and measured at the end of 6 weeks, and body composition was determined as described above. Mean low temperature was 21±1.0°C, and mean high temperature was

Table 1. Semi-Purified Diet Formulations[1] for Hybrid Striped Bass (Striped Bass x White Perch) Feeding Trials

Diet #		1	2	3	4	5	6	7	8	9	10	11	12
Energy													
Kcal/g		4.50	4.50	4.50	4.50	3.50	3.50	3.50	3.50	2.70	2.70	2.70	2.70
Protein	%	25	30	35	40	25	30	35	40	25	30	35	40
Casein	%	26.00	31.20	36.50	41.60	26.00	31.20	36.50	41.60	26.00	31.20	36.50	41.60
Egg White	%	3.50	4.00	4.60	5.30	3.50	4.00	4.60	5.30	3.50	4.00	4.60	5.30
Corn Starch	%	28.30	19.20	9.00	0.00	28.30	19.20	9.00	0.00	28.30	19.20	9.00	0.00
Cellulose	%	12.90	16.30	20.90	23.80	24.70	28.10	32.40	35.60	31.90	37.30	41.60	44.80
Vitamin Premix[2]	%	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Vitamin C	%	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Choline chloride 60%	%	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Salt (plain)	%	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
USFW #3 mineral mix	%	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05	0.05
Cod liver oil	%	26.00	26.00	26.00	26.00	14.20	14.20	14.20	14.20	5.00	5.00	5.00	5.00
CMC (Carboxyl methylcellulose)	%	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00	2.00

[1]Diet ingredients and diet preparation by Zeigler Brothers, Inc., Gardner, PA.

[2]Vitamins in g/kg - pantothenate 26.5, pyridoxine HCl 7.7, riboflavin 13.2, niacinamide 55.1, folic acid 2.2, thiamine mononitrate 8.8,menadione 2.76; in mg/kg - biotin 88.2, cobalamin 5.5; vitamin E, 88,200 i.u., vitamin D 110,250 i.u., vitamin A 1,653,750 U.S.P.

Table 2. Test Diet Formulations[a] Based on Commercial Ingredients for Feeding Trials With Striped Bass and Striped Bass Hybrids (Striped Bass x White Bass)

Ingredient or nutrient (%)	#1	#2	#3	#4	#5	#6	#7	#8
Fish meal	22.0	29.0	34.0	40.0	20.0	26.0	34.0	40.0
Blood meal	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Poultry/meat meal	10.0	14.0	17.0	20.0	10.0	13.0	15.0	20.0
Wheat middling	20.0	9.0	2.0	2.0	35.0	27.0	17.0	7.0
Fish oil	5.0	5.0	5.0	2.0	10.0	10.0	10.0	10.0
Cellulose	35.1	35.1	34.1	28.1	17.1	16.1	16.1	15.1
Vitamin Premix[b]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Mineral Mix[c]	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Ascorbic Acid	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
Choline Chloride	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
g Protein	25.5	30.8	35.0	40.1	26.4	30.9	35.8	41.2
g Fat	9.2	9.8	10.2	8.1	14.6	15.2	15.7	16.4
g CHOH	16.4	10.0	6.0	5.5	26.3	21.7	15.8	10.2
g ASH	5.9	7.2	7.9	9.2	6.2	7.2	8.2	9.5
Kcal/100g	250	251	255	255	343	347	348	352

[a]Diets prepared by Zeigler Brothers, Inc., Gardner, PA.

[b]Vitamins in g/kg - pantothenate 26.5, pyridoxine HCl 7.7, riboflavin 13.2, niacinamide 55.1, folic acid 2.2, thiamine mononitrate 8.8, menadione 2.76; in mg/kg - biotin 88.2, cabolamin 5.5; vitamin E, 88,200 i.u., vitamin D 110,250 i.u., vitamin A 1,653,750 U.S.P.

[c]American Institute of Nutrition, 1976.

23 ± 1.0°C. Ammonia levels were less than 0.2 ppm, nitrate less than 2.6 ppm, nitrite less than 0.06 ppm, and oxygen 8 ppm during the experiment.

The second trial was conducted using striped bass (*Morone saxatilis*) weighing approximately 1 g. Fish were randomly placed into three replications of 20 fish each. The same test diets as shown in Table 2 were used at a #2 crumble size (0.85-1.18mm). Fish were fed and handled as described above. Mean low temperature was 22 ± 0.58°C, and mean high temperature was 23 ± 1.0°C. Ammonia levels were less than 0.1 ppm, nitrate less than 1.0 ppm, nitrite less than 0.02 ppm, and oxygen 8 ppm throughout the experimental period.

Body composition data and changes in weight and length were analyzed statistically using one-way analysis of variance (ANOVA). The least significant difference (LSD) method of mean separation was used when there was a significant F-test (Snedecor and Cochran, 1967). In all tests the significance level was set at = 0.05. Although other methods have been used to determine protein requirements, we chose ANOVA with LSD because of the conservative nature of the test, and because Moore et al. (1988) demonstrated that analysis of variance, broken line regression analysis, quadratic regression analysis, and second-order polynomial regression analysis all lead to similar conclusions.

Pond Trials

The results of the laboratory trials were used to formulate a ration for pond feeding (Table 3). Two 0.1 hectare ponds were stocked with 350 hybrid striped bass with a mean weight of 209 ± 9g. Fish were fed in the morning and evening at a rate of 4% of their body weight/day or until feeding activity ceased, whichever was less. At the end of 6 weeks, samples of 50 fish from each pond were weighed and daily feed allowances readjusted. At the end of 12 weeks, ponds were drained and all fish were weighed. Mean average water temperature was 25.9°C and ranged from 23-30°C. Dissolved oxygen ranged from 3.8 to 8.2 ppm. During a 1-week period in July, water temperatures rose to 28-30°, reducing feeding activity.

Results and Discussion

Preliminary Study

Hybrid striped bass fed diets containing 4.5 kcal/g generally gained significantly ($p \leq 0.05$)

Table 3. Diet Formulations for Pond Trial Using Hybrid Striped Bass

Ingredient	%
Fish meal (Herring)	47.0
Fish oil (Menhaden)	5.9
Wheat middlings	12.5
Dextrin	12.5
Cellulose (filler)	18.9
USFW Trace Mineral Mix	0.1
Choline Chloride	0.2
Ascorbic Acid (ASPP)	0.3
Vitamin Mix #30	0.6
Calcium di Phosphate	2.0
Nutrient Composition	
Digestible Energy (Kcal/g)	3.0
Protein (%)	34.7
Lipid (%)	10.2
Ash (%)	7.7

less weight and length than those receiving diets with lower caloric levels (Table 4). There were no significant differences in weight gain or length gain between groups fed different protein levels at 2.7 kcal/g. However, when caloric density of the diet was increased to 3.5 kcal/g, the fish fed diets with 25% protein achieved significantly less growth compared to those fed either 30, 35, or 40% protein. Best growth was obtained at 3.5 kcal/g and 2.7 kcal/g with a 35% protein level.

With the exception of crude lipid content, there were no significant differences in body composition (Table 5). Fish fed diets with 4.5 kcal/g had higher lipid content compared to lipid content of fish from other energy levels. There were no significant differences in weight gain of fish fed diets containing 2.7 kcal/g or 3.5 kcal at any protein level, with one exception. Fish fed diets with diets containing 25% protein and 3.5 kcal/g gained significantly less weight than all other diets with either 2.7 or 3.5 kcal/g.

Results from the first study indicated that appropriate protein levels for hybrid striped bass were between 30 and 35%. However, the appropriate energy level was unclear. Energy levels of 4.5 kcal/g were too high, and this energy level was dropped from future experiments. In addition, to improve feed intake, further trials were conducted using commercially based diet formulations.

Protein Energy Studies

In the first feeding trial, hybrid striped bass fed diets with 3.5 kcal/g showed no significant improvement in growth (change in weight or length) after the protein level in the diet reached 30%. There were no significant differences in growth of fish (change in weight or length) among any of the protein levels of diets with 2.5 kcal/g. When growth of fish fed diets containing 2.5 or 3.5 kcal/g was compared at each protein level, there were no differences between 2.5 kcal/g and 3.5 kcal/g at 25 or 30% protein. However, at both the 35 and 40% protein level, fish fed diets containing 3.5 kcal/g grew significantly better than those fed 2.5 kcal/g (Table 6).

Fish fed Zeigler trout chow exhibited significantly better weight gain than fish fed test diets. The same trend was shown with change in length, with only those fish fed diets with protein levels of 40% and 3.5 kcal/g achieving growth that was not statistically less than fish fed Zeigler trout chow. There are several reasons that may account for this difference. First, it may be attributed to sources of protein in the formulation. Zeigler trout chow is principally fishmeal, whereas the test diets contained blood meal and poultry meat meal in addition to fishmeal. Secondly, Zeigler trout chow is 38.2% protein and only one of the test diets contained that much protein. However, it should not be assumed that this weight gain reflects increased muscle protein. Indeed, body composition data (Table 7) indicated that fish fed trout chow diets also had significantly higher body fat content than fish fed test diets.

In his channel catfish model for protein and fat dynamics, Cacho (1990) showed that under most circumstances percent of gain as fat decreases as dietary protein increases. This effect is shown as protein increased in the test diets at both 2.5 and 3.5 kcal/g. But under some circumstances (i.e., in larger fish), increasing protein above the requirement results in increased fat deposition. In some cases, fat deposition may be a desired characteristic (i.e., overwintering), but feeding protein is an expensive way to achieve fat deposition.

A third reason for higher weight and lipid gain may have been ration size. Cacho (1990) showed that percent body fat increases with increasing ration size, with the highest lipid level being achieved at satiation. These results may be accounted for by the relatively high feeding rate used in these studies (7%) compared to studies using feeding rates of 2.5% of body weight by Berger and Halver (1987) or Millikin (1982a, 1982b), which results in more efficient feed conversion and higher PER's. The fishmeal chow may have been more palatable and resulted in less wastage of the diet, effectively increasing ration size for these fish.

When the test diets were compared to one another, feed conversion and PER's were better for fish fed diets with 3.5 kcal/g and 30% or more protein. These fish tended to have significantly higher lipid content than those fed lower energy levels (2.5kcal/g). It should be noted that none of the lipid values is out of line with those found in wild populations (Gallagher, et al., 1989) or those reported by Berger and Halver (1987). However, increased lipid content at the expense of body protein can adversely effect a standard product, which is usually the goal in culture operations.

In the second feeding trial, striped bass fed diets with 3.5 kcal/g showed no significant increase in weight and length after protein levels reached 30% of the diet (Table 8). However, when energy levels were 2.5 kcal/g, only fish receiving 40% protein diets grew significantly better than fish on other diets. When growth at 2.5 or 3.5 kcal/g was compared at each protein level, fish fed 3.5 kcal/g of diet gained significantly more weight and length at all protein levels. Food Conversion Ratios and PER's were similar to

Table 4. Growth and Survival of Hybrid Striped Bass (Striped Bass x White Perch) Fed Semi-purified Diets for 10 Weeks. Means (± SD) Within a Row Sharing a Letter in Common Are Not Significantly Different (Analysis of Variance and Least Significant Difference Tests), $P > 0.05$.

	Diet											
	1	2	3	4	5	6	7	8	9	10	11	12
% Protein	25	30	35	40	25	30	35	40	25	30	35	40
Kcal/g	4.5	4.5	4.5	4.5	3.5	3.5	3.5	3.5	2.7	2.7	2.7	2.7
Survival %	85 (21)	70 (14)	85 (7)	90 (14)	80 (14)	85 (7)	95 (7)	90 (14)	95 (7)	95 (7)	95 (7)	95 (7)
Mean Weight Gain (g)	1.47^{ab} (2.07)	-1.24^{a} (3.83)	6.21^{bcd} (1.77)	4.17^{abc} (1.88)	0.09^{ab} (1.20)	10.46^{cde} (0.11)	15.05^{e} (0.72)	8.90^{cde} (4.76)	10.11^{de} (1.76)	12.43^{de} (0.76)	15.73^{e} (1.15)	11.06^{de} (1.97)
Mean Fork Length Gain (cm)	0.25^{a} (0.22)	0.77^{b} (0.06)	0.70^{b} (0.18)	0.61^{b} (0.01)	0.53^{ab} (0.11)	0.95^{cde} (0.01)	1.28^{e} (0.08)	0.66^{cd} (0.37)	0.51^{ab} (0.61)	1.10^{de} (0.08)	1.35^{de} (0.16)	0.93^{de} (0.13)

Table 5. Body Composition of Hybrid Striped Bass (Striped Bass x White Perch) Fed Semi-purified Diets for 10 Weeks. Means (± SD) Within a Row Sharing a Letter in Common Are NotSignificantly Different (Analysis of Variance and Least Significant Difference Tests), P > 0.05.

	Diet											
	1	2	3	4	5	6	7	8	9	10	11	12
% Protein	25	30	35	40	25	30	35	40	25	30	35	40
Kcal/g	4.5	4.5	4.5	4.5	3.5	3.5	3.5	3.5	2.7	2.7	2.7	2.7
Moisture (%)	67.83[a] (1.42)	67.08[a] (3.26)	68.09[a] (1.17)	67.53[a] (1.74)	67.67[a] (2.06)	66.69[a] (1.84)	66.07[a] (1.30)	67.14[a] (3.82)	66.30[a] (0.81)	66.69[a] (2.11)	67.64 (2.19)	67.54[a] (1.06)
Protein (%, moisture and lipid free)	58.23[a] (10.21)	54.37[a] (6.68)	57.65[a] (3.37)	51.43[a] (8.86)	57.60[a] (6.47)	58.32[a] (5.71)	58.87[a] (4.86)	59.06[a] (5.85)	58.87[a] (4.86)	59.06[a]	53.54[a]	55.90[a]
Protein (%, wet weight)	11.98[a] (2.10)	11.73[a] (2.66)	12.10[a] (0.53)	10.93[a] (1.88)	11.08[a] (1.77)	11.30[a] (1.91)	12.19[a] (2.88)	12.59[a] (1.30)	12.67[a] (1.28)	13.12[a] (1.78)	12.05[a] (1.64)	12.62[a] (1.26)
Lipid (%, dry weight)	35.99[bc] (4.96)	35.31[bc] (6.11)	34.59[bc] (3.71)	34.37[bc] (3.79)	35.18[bc] (2.67)	41.50[a] (9.34)	36.88[ab] (3.68)	33.59[b] (3.82)	36.20[b] (1.76)	33.87[bc] (4.32)	31.80[bc] (2.20)	31.14[c] (2.76)
Lipid (%, wet weight)	11.56[bc] (1.37)	11.73[bc] (2.66)	11.04[bc] (1.53)	11.21[bc] (1.79)	11.38[bc] (1.25)	13.84[a] (3.19)	12.55[ab] (3.68)	11.07[bc] (1.58)	12.20[abc] (0.65)	11.90[bc] (1.78)	10.41[c] (1.40)	10.12[c] (1.21)
Ash (%, moisture and lipid free)	22.62[a] (1.39)	22.55[a] (1.42)	21.52[a] (2.24)	21.23[a] (1.84)	22.40[a] (1.46)	19.21[a] (1.28)	21.24[a] (1.71)	23.35[a] (8.92)	21.14[a] (0.79)	20.33[a] (0.86)	21.31[a] (1.40)	20.20[a] (1.26)
Ash (%, wet weight)	4.67[a] (0.54)	4.78[a] (0.57)	4.50[a] (0.56)	4.52[a] (0.52)	4.68[a] (0.42)	3.75[a] (0.67)	4.52[a] (0.72)	5.09[a] (1.93)	4.54[a] (0.20)	4.52[a] (0.35)	5.83[a] (3.40)	4.48[a] (0.32)

Table 6. Growth and Survival of Hybrid Striped Bass (Striped Bass x White Bass) Fed Artificial Diets Formulated From Commercial Ingredients for 7 Weeks. Means (± SD) Within a Row Sharing a Letter in Common Are Not Significantly Different (Analysis of Variance and Least Significant Difference), P >0.05.

		Diet Formulations								
		1	2	3	4	5	6	7	8	
Variable	% Protein	25	30	35	40	25	30	35	40	Trout Chow
	Kcal/g	2.5	2.5	2.5	2.5	3.5	3.5	3.5	3.5	
Survival %		100 (0.00)	100 (0.00)	100 (0.00)	100 (0.00)	85 (0.00)	95 (0.00)	100 (0.00)	100 (0.00)	100 (0.00)
Mean Weight Gain (g)		6.52[ab] (0.96)	7.01[ab] (2.10)	4.78[a] (2.08)	7.67[ab] (3.10)	7.23[ab] (0.70)	10.22[bc] (2.00)	10.37[bc] (0.44)	12.45[c] (9.7)	17.94[d] (2.57)
Mean Fork Length Gain (cm)		2.01[a] (0.29)	2.27[abc] (0.15)	1.76[a] (0.35)	2.23[abc] (0.28)	2.07[ab] (0.06)	2.64[cd] (0.12)	2.58[bcd] (0.18)	2.98[de] (0.32)	3.27e (0.12)
Food Conversion Ratio (dry weight fed/wet weight gain)		9.3	8.0	12.6	7.8	7.5	5.1	5.1	4.4	3.3
Protein Efficiency Ratio (weight weight gain/g protein fed)		.42	.44	.25	.38	.51	.63	.55	.53	.78

Table 7. Body Composition of Hybrid Striped Bass (Striped Bass x White Bass) Fed Artificial Diets Formulated From Commercial Ingredients for 7 Weeks. Means (± SD) Within a Row Sharing a Letter in Common Are Not Significantly Different (Analysis of Variance and Least Significant Difference), P >0.05.

		Diet Formulations								
		1	2	3	4	5	6	7	8	
	% Protein	25	30	35	40	25	30	35	40	Trout
Variable	Kcal/g	2.5	2.5	2.5	2.5	3.5	3.5	3.5	3.5	Chow
Moisture (%)		69.93^{cde} (1.92)	70.22^{de} (1.21)	69.29^{cd} (0.87)	70.64^{e} (1.42)	68.34^{bc} (0.98)	70.63^{c} (1.43)	67.75^{b} (1.00)	69.03^{c} (0.77)	66.54^{a} (0.97)
Protein (%, moisture and lipid free)		54.10^{abc} (1.56)	55.30^{bcd} (1.89)	57.48^{d} (2.34)	57.72^{d} (2.36)	52.36^{a} (3.23)	54.43^{abc} (4.08)	53.65^{ab} (2.81)	56.31^{cd} (2.78)	53.92^{ab} (2.84)
Protein (%, wet weight)		11.14^{abc} (0.59)	11.68^{bc} (0.55)	13.46^{d} (2.28)	12.04^{c} (0.55)	10.86^{ab} (0.65)	10.55^{a} (1.77)	11.26^{abc} (0.91)	11.61^{bc} (0.49)	11.34^{abc} (0.89)
Lipid (%, dry weight)		31.41^{bc} (2.78)	28.43^{a} (3.50)	29.57^{ab} (2.56)	28.79^{a} (3.40)	34.38^{d} (2.64)	34.18^{d} (1.45)	34.97^{de} (2.57)	33.33^{cd} (2.39)	37.19^{e} (2.22)
Lipid (%, wet weight)		9.48^{bc} (1.33)	8.49^{a} (1.28)	9.15^{ab} (0.92)	8.50^{a} (1.34)	10.90^{de} (1.11)	10.00^{bcd} (0.68)	11.29^{e} (1.01)	10.33^{cde} (0.92)	12.45^{f} (0.90)
Ash (%, Moisture and lipid free)		18.33^{b} (1.07)	19.14^{c} (0.67)	19.31^{c} (0.76)	19.11^{e} (0.68)	19.30^{c} (0.79)	18.72^{bc} (0.80)	19.11^{c} (1.08)	18.60^{bc} (0.57)	17.02^{a} (1.06)
Ash (%, wet weight)		3.78^{ab} (1.31)	4.07^{b} (0.19)	4.52^{c} (0.70)	3.99^{b} (0.16)	4.01^{b} (0.23)	3.62^{a} (0.27)	4.01^{b} (0.36)	3.84^{ab} (0.17)	3.58^{a} (0.28)

Table 8. Growth and Survival of Striped Bass (*Morone saxatilis*) Fed Experimental Diets for 10 Weeks. Means (± SD) Within a Row Sharing a Letter in Common Are Not Significantly Different (Analysis of Variance and Least Significant Difference), P >0.05.

		Diet Formulations							
		1	2	3	4	5	6	7	8
	% Protein	25	30	35	40	25	30	35	40
Variable	Kcal/g	2.5	2.5	2.5	2.5	3.5	3.5	3.5	3.5
Survival %		90 (0.00)	80 (2.80)	85 (21.00)	65 (49.00)	95 (7.00)	95 (0.00)	90 (0.00)	95 (0.00)
Mean Weight Gain (g)		1.42[a] (0.45)	2.48[b] (0.05)	1.26[a] (0.22)	3.57[cd] (1.17)	2.66[bc] (0.29)	3.66[cd] (0.00)	3.86[d] (0.46)	4.05[d] (0.73)
Mean Fork Length Gain (cm)		1.27[a] (0.21)	2.01[b] (0.23)	1.29[a] (0.29)	2.39[cd] (1.01)	2.13[bc] (0.13)	2.64[cde] (0.16)	2.66[e] (0.14)	2.75[e] (0.26)
Food Conversion Ratio (dry weight fed/wet weight gain)		9.6	6.3	12.0	5.1	5.2	4.1	3.4	4.0
Protein Efficiency Ratio (wet weight gain/g protein fed)		0.41	0.52	0.24	0.49	0.73	0.80	0.81	0.60

Table 9. Body Composition of Striped Bass (Morone saxatilis) Fed Experimental Diets for 10 Weeks. Means (± SD) Within a Row Sharing a Letter in Common Are Not Significantly Different (Analysis of Variance and Least Significant Difference), P >0.05.

	Diet Formulations							
	1	2	3	4	5	6	7	8
% Protein	25	30	35	40	25	30	35	40
Variable Kcal/g	2.5	2.5	2.5	2.5	3.5	3.5	3.5	3.5
Moisture (%)	69.82[a] (3.83)	70.07[a] (1.33)	70.43[a] (1.52)	66.57[a] (2.73)	67.43[a] (1.91)	67.50[a] (0.85)	66.78[a] (1.37)	66.29[a] (1.87)
Protein (%, Moisture and lipid free)	56.24[a] (1.63)	57.58[a] (2.96)	59.25[a] (0.94)	57.86[a] (1.75)	56.26[a] (3.87)	56.39[a] (3.41)	53.60[a] (2.11)	54.29[a] (2.14)
Protein (%, wet weight)	12.12[a] (1.94)	12.39[a] (1.00)	12.53[a] (0.72)	13.57[a] (0.88)	12.15[a] (0.96)	11.99[a] (1.24)	11.12[a] (0.84)	11.26[a] (1.04)
Lipid (%, dry weight)	28.65[ab] (5.39)	28.08[a] (2.95)	28.47[a] (1.22)	29.61[abc] (2.29)	33.57[bcd] (3.05)	34.66[cd] (3.82)	37.53[d] (2.82)	38.54[d] (1.66)
Lipid (%, wet weight)	8.66[a] (1.99)	8.43[a] (1.11)	8.43[a] (0.67)	9.91[ab] (1.00)	10.98[bc] (1.51)	11.26[bc] (1.21)	12.49[c] (1.31)	12.98[c] (0.78)
Ash (% Moisture and lipid free)	17.14[c] (1.63)	16.88[bc] (0.46)	16.90[c] (0.28)	16.91[c] (0.69)	16.72[bc] (0.51)	16.44[bc] (0.19)	15.95[ab] (0.46)	15.86[ab] (0.16)
Ash (%, wet weight)	3.70[a] (0.60)	3.63[a] (0.09)	3.57[a] (0.17)	3.98[a] (0.49)	3.61[a] (0.25)	3.49[a] (0.26)	3.31[a] (0.18)	3.29[a] (0.24)

those found for hybrid striped bass. Both feed conversion and PER were improved when the diets contained 3.5 kcal/g. The best values for PER and feed conversion were obtained when the diet contained 35% protein and 3.5 kcal/g. The PER dropped when the protein level was increased to 40% of the diet. Increased energy level resulted in increased lipid content, as in the previous trials, but, in this trial, there were no significant changes in protein content (Table 9).

Results of these studies emphasize the importance of the protein-to-energy ratio in the diet of fish. In each feeding trial, the amount of energy clearly affected the response of the fish to the protein level. By feeding each protein level at different energy levels, it was determined that 3.5 kcal (based on the physiological fuel factors) was the preferred energy level and that 30% protein is the minimal protein necessary for striped bass and its hybrids. Based on results from PER and food conversion ratios, we suggest that the protein requirement is around 35% of the diet when the energy level is 3.5 kcal/g. Caloric levels also influenced total body composition of the fish, with higher energy levels resulting in increased lipid content. The type of energy added may have been important also. For the purposes of this study, caloric content of diets was increased primarily by increasing lipid content. Therefore, increases in lipid content are not surprising.

The range of specific growth rates for hybrid striped bass (0.10-0.25g/day) fed diets was comparable to those calculated for Millikin (1983) (0.07-0.11g/day) and Millikin (1982b) (0.13-0.16g/day). The range in percent body weight gain for hybrid striped bass (0.66-1.73%) and striped bass (1.8-5.79%) was also similar to the ranges (0.28-2.19%) reported by Berger and Halver (1987) for striped bass. However, the protein levels determined to give best growth in this study were 13-30% below those suggested by these authors. One reason for this discrepancy in 0-year striped bass is very likely the variations in dietary energy levels. Millikin (1982b) found that striped bass obtained significantly higher weight gains on 55% formulated protein diet compared to 34 or 44% protein diets. Caloric density of these diets was 4.15 kcal/g diet. In this study, 4.5 kcal/g of diet was shown to reduce growth compared to 3.5 or 2.5 kcal/g and to give results that demonstrated no significant differences among wide variations in protein content of the diet. Therefore, energy levels may have been high enough in Millikin's diets to restrict food intake, so that not enough protein was taken in at the lower protein levels. Thus, the best weight gain was at the highest protein level, just as it was at the 4.5 kcal/g level in the preliminary study reported here. This reasoning is supported by the work of Page and Andrews (1973) who demonstrated that diets high in protein and energy resulted in decreased food consumption. Berger and Halver (1987), feeding at 2.5% of the body weight, found that 52% protein produced maximum increase in percent body weight gain per day in striped bass. Restricted ration size may have influenced growth in these fish. As these authors note, this value is higher than those found for many carnivorous species.

The effect of increasing growth at lower protein (30%) levels by increasing energy (from 2.5 to 3.5 kcal/g) is also supported by other studies. Shiau and Huang (1990) showed that energy level can have significant effects on protein requirements. In earlier studies, the investigators (Shiau and Huang, 1990) had estimated that the protein requirement of hybrid fingerling tilapia (*Oreochromis miloticus* X *O. aureus*) was around 24% of the diet. However, they were able to reduce the amount of protein needed to 21% by increasing energy levels from 230 kcal/g to 310 kcal/g (4.5 kcal/g for protein, 8.51 kcal/g for lipid, 3.49 kcal/g for CHOH). No improvement in weight gain was made by increasing energy level above 310 kcal/g, thus demonstrating that providing energy from lipids or carbohydrates can spare costly protein. Clark et al. (1990) found that there was no significant decrease in growth of red tilapia fed 20% protein compared to 25 or 30%, if energy was high enough. However, total gross energy in these diets ranged from 4.0 - 4.14 kcal/g.

Watanabe et al. (1990) showed that higher levels of protein (32%) inhibited growth in red tilapia compared to lower levels (28%). The reason for this small but significant decrease in growth is not fully understood but may be due to increased liver fat deposition at higher protein levels. The dietary energy levels in this study were not available.

Parazao (1990) described the growth of rabbitfish (*Siganua guttatus*) fed three levels of protein (25, 35, 45%) and 2 levels of energy (3.2 and 3.8 kcal/g). These warm water herbivores gained significantly more weight on diets with higher energy and higher protein. However, body composition indicated that weight gain on the diets was due to significant increase in body fat rather than growth due to protein. Since the fish used in these studies were small (0.93 g initial weight), it is problematic as to whether this increase in fat was beneficial. Protein efficiency ratios (PER's) were highest in diets with lowest protein (25%). Based on weight gain and PER, the authors recommended 35% protein and 3.8 kcal/g.

Table 10. Performance Parameters for Pond-Reared Hybrid Striped Bass Fed Fishmeal Diets for 12 Weeks Between June and September

	Pond 1	Pond 2	Mean
Initial mean weight	209g (0.46 lb)	209g (0.45 lb)	209±9g (0.45±0.02 lb)
6 wk			
Mean weight	409g (0.90 lb)	431g (0.95 lb)	422±9g (0.93±0.03 lb)
Mean weight gain	222g (0.49 lb)	204g (0.45 lb)	213±9g (0.47±0.02 lb)
Specific growth rate (g/day)	5.37	5.00	519±0.26
Feed conversion (g intake/g gain)	1.3	1.4	1.4±0.07
12 wk			
Mean weight	567g (1.25 lb)	554g (1.22 lb)	563±9g (1.24±0.02 lb)
Mean weight gain	354g (0.78 lb)	350g (0.77 lb)	354±5 (0.78±0.01 lb)
Growth rate (g/day)	3.7	3.6	3.7±0.05
Feed conversion (g intake/g gain)	1.6	1.6	1.6

Pond Trials

Results of laboratory trials were confirmed by growth rates and feed conversions found in ponds (Table 10). Fish reached marketable size in 12 weeks. Growth rates and feed conversions were higher than those normally expected under pond conditions. Therefore, we conclude that hybrid striped bass can grow well on diets with 35% protein, 10% lipid, and digestible energy of 3.3 kcal/g. Higher levels of protein are probably not beneficial and increase the cost of production. For example, if fishmeal costs $600/ton and a diet was formulated at 45% protein, depending upon costs of other ingredients, such a formulation could cost $100 to $150/ton more than a 35% protein diet.

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QUANTITATIVE AMINO ACID REQUIREMENTS OF FINGERLING COHO SALMON (*ONCORHYNCHUS KISUTCH*)

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ABSTRACT

Ten amino acids have been shown to be dietary essentials for the coho salmon. We conducted a series of growth studies with fingerling coho salmon to determine the quantitative requirements of the 10 essential amino acids. Each amino acid study was performed using a purified diet with 30% crude protein level. Crystalline L-amino acids without any intact proteins were used as a nitrogen source in the diets, being mixed to provide the amino acid profile of the eyed-egg protein of coho salmon. The amino acid requirements were determined graphically utilizing a first-order regression line based on data of daily specific growth rate; that is: Arg, 3.2g; His, 0.9g; Ile, 1.2g; Leu, 3.4g; Lys, 3.8g; Met, 2.7g; Phe, 4.5g; Thr, 2.0g; Trp, 0.5g; Val, 2.2g/100g dietary protein, and Arg, 42mg; His, 10mg; Ile, 9mg; Leu, 33mg; Lys, 38mg; Met, 30mg; Phe, 45mg; Thr, 20mg; Trp, 5mg; Val, 20mg/100g fish/day. The essential amino acid ratios of the determined requirements more closely resembled those of the whole body protein of coho salmon, in spite of using the amino acid profile of the eyed-egg protein to formulate the test diets. Thus, the essential amino acid profile of the whole body protein appears to serve as an ideal pattern in designing test diets of fish for which quantitative amino acid requirements have not been determined.

Introduction

In a previous paper (Arai et al., 1982), one of the authors reported that 10 amino acids (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine) are dietary essential for the coho salmon (*Oncorhynchus kisutch*). Although the quantitative requirements of arginine and histidine were already determined by Klein and Halver (1970), the requirement values of the other essential amino acids still remained unknown. Therefore, in the last 10 years we have conducted a series of the studies to quantitate the 10 essential amino acids for fingerling coho salmon. Some of the results will be briefly summarized in the present paper.

Materials and Methods

The test animals used in this study were fingerling coho salmon whose eyed-eggs were obtained from a private farm in the United States. After being hatched out in our laboratory, the fry were raised at 15±0.5°C on a commercially prepared trout diet until the start of the preliminary experiment. Approximately 50 to 66 fish (initial mean body weight ranging from 1.2g to 3.5g) were distributed randomly into each of the polyvinyl chloride tanks (50x20x30cm, depth 20cm), and they were acclimatized to the experimental conditions for 2 weeks while being fed the basal diet. Water quality and temperature were controlled by aeration and continuous addition of precooled well water (15 ± 0.5°C) at a rate of 1.5-2.0 l/min to each tank. The fish were fed their respective test diets by hand to satiation twice daily, 6 days per week, for a period of 4 or 6 weeks. The fish were weighed at the beginning of each study and then biweekly during the experimental period after being anesthetized in a 0.01% MS-222 solution.

The formulation of the basal diet is presented in Table 1. Each amino acid study was conducted using a purified diet, with 30% crude protein level. Crystalline L-amino acids were used as a nitrogen source to simulate the essential amino acid ratios of the eyed-egg protein of coho salmon (Arai, 1981); i.e., the basal amino acid composition was as follows: L-

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Table 1. Composition of Basal Diet

Ingredients	%
Amino acid on study	Variable
L-Alanine	Variable
Amino acid mix[1]	Variable
Crude protein	30.0
Potato starch	27.0
Vitamin mix[2]	4.5
Mineral mix[3]	4.0
Corn oil	6.0
Pollack liver oil	3.0
Binder[4]	8.0
Cellulose flour	Variable
Water	100ml

The pH of test diet was adjusted to pH 6.5-7.0 with 6N NaOH.
[1]Crystalline L-amino acids were mixed to simulate the amino acid profile of the eyed-egg protein of coho salmon (Arai, 1981).
[2] The composition of vitamin mixture was equal to that reported by Halver (1957).
[3] U.S.P. XII Salt mixture no. 2 with trace elements (Halver, 1957).
[4] Carboxymethylcellulose sodium salt.

arginine, 2.00; L-histidine, 1.00; L-isoleucine, 1.89; L-leucine, 3.06; L-lysine.HCl, 3.74; L-methionine, 1.03; L-cystine, 0.62; L-phenylalanine, 1.69; L-tyrosine, 1.51; L-threonine, 1.85; L-tryptophan, 0.24; L-valine, 2.30; L-alanine, 3.66; L-aspartic acid, 3.10; L-glutamic acid, 4.09; glycine, 1.63; and L-proline, 1.96 (g/100g diet). L-serine was not added to the amino acid mixture due to financial reasons. All the crystalline L-amino acids employed here were products manufactured by Ajinomoto Co., Inc., and Nippon Rikagakuyakuhin Co., Ltd., in Japan. In each study, the basal diet was supplemented with graded levels of the amino acid on test to supply a test range with 0% to suitable levels as shown in Tables 2-11. For instance, in the case of the test diets to determine the arginine requirement (Table 2), the arginine level was designed to range from 0% to 3.50% of the dry diet, or from 0% to 11.7% of the dietary protein. The test diets were maintained isonitrogenous by adjusting the L-alanine levels. The ingredients were mixed thoroughly with an equal weight of water. The pH of the test diets was adjusted to pH 6.5-7.0 with 6N NaOH. The prepared diets were stored at -20°C until feeding. The test diets were given to the fish as a moist and noodle-like diet, by being extruded with a 50ml plastic syringe.

The requirements of the 10 essential amino acids were estimated using a first-order regression line based on data of daily specific growth rate (%): [l.n(final mean body weight)-ln(initial mean body weight)/(days of feeding test)]x100. As previously mentioned, only a single group was assigned to each test diet, so that iii the present paper differences in growth performance by dietary treatment were not substantially evaluated by multiple comparison of treatment means.

Results and Discussion

Figure 1 shows an example of typical growth response, and the data in Figure 1 indicate that the arginine requirement of fingerling coho salmon is 0.96% of the dry diet or 3.20% of the dietary protein. Tables 2-11 show performance of fingerling coho salmon fed the experimental diets in each study. Table 12 presents a summary of the quantitative amino acid requirements expressed as a percentage of the dietary protein and as mg/100g fish/day, with the requirement values of arginine and histidine reported by Klein and Halver (1970). As shown in Table 12, the values of arginine and histidine requirements that we determined are almost one-half of those reported by Klein and Halver (1970). Even in the same species, dietary requirements of nutrients are affected by a variety of factors such as dietary ingredients, the size and age of test animals, and the experimental design. The last factor seems to be the most possible reason for the discrepancy in the values of the arginine and histidine requirements (Fig. 2). In each amino acid study, the crystalline amino acids were used as the nitrogen source without any intact proteins so that we were able to set up a control group fed the test diet which was completely free of the amino acid being tested; i.e., its dietary level was 0%. As the arginine requirement in our study was estimated by including the datum of the arginine-free group, the slope of the regression line was greater than that of Klein and Halver (1970). Consequently, the point of interception of the regression line with the horizontal line, which demonstrated the average of daily specific growth rates reaching the plateau, was located at a lower level of dietary arginine, showing that the arginine requirement is 3.2% of the dietary protein (Fig. 2). On the other hand, Klein and Halver (1970)

Table 2. Performance of Coho Salmon Fed the Arginine Test Diet for 6 Weeks (n=50)

Diet No.		1	2	3	4	5	6	7	8	9	10
L-Arginine Level		0	0.30	0.60	0.90	1.20	1.50	2.00	2.50	3.00	3.50
	Initial	3.49	3.49	3.59	3.51	3.57	3.58	3.55	3.56	3.55	3.57
Mean	SD	0.30	0.35	0.34	0.32	0.34	0.34	0.36	0.34	0.32	0.35
body (g)	Final	3.20	3.86	4.59	4.85	5.07	5.21	5.28	5.28	5.33	5.41
weight	SD	0.31	0.37	0.50	0.66	0.79	0.92	0.76	0.83	0.79	0.84
DSGR(%)[1]		-0.21	0.24	0.58	0.77	0.83	0.89	0.94	0.94	0.97	0.99
Feed consumption[2]		2.98	3.94	4.44	4.31	4.62	4.20	4.29	4.07	4.20	4.11
Feed efficiency[3]		-	6.1	13.2	17.6	17.9	21.0	21.7	22.6	22.8	23.7
PER[4]		-	0.20	0.44	0.59	0.60	0.70	0.72	0.75	0.76	0.79
Protein intake[5]		0.895	1.183	1.331	1.294	1.385	1.260	1.288	1.221	1.260	1.233
L-Arg intake[6]		0	0.012	0.027	0.039	0.055	0.063	0.086	0.102	0.126	0.144
Mortality (%)		0	0	0	0	0	0	0	2.0	0	0

[1] Daily specific growth rate.
[2] % of wet biomass/day.
[3] (Wet weight gain/dry food intake)x100.
[4] Protein efficiency ratio.
[5] % of wet biomass/day : (feed consumption)x(dietary crude protein level).
[6] % of wet biomass/day : (feed consumption)x(dietary level of amino acid being tested).

Table 3. Performance of Coho Salmon Fed the Histidine Test Diet for 4 Weeks (n=50)

Diet No.		1	2	3	4	5	6	7	8	9
L-Histidine Level		0	0.13	0.25	0.50	0.75	1.00	1.25	1.50	2.00
	Initial	2.13	2.09	2.13	2.12	2.12	2.08	2.02	2.06	2.16
Mean	SD	0.26	0.24	0.23	0.27	0.26	0.22	0.23	0.26	0.28
body (g)	Final	2.08	2.63	3.15	3.18	3.20	3.13	3.11	3.14	3.37
weight	SD	0.27	0.30	0.41	0.44	0.46	0.40	0.35	0.44	0.47
DSGR (%)[1]		-0.08	0.82	1.40	1.45	1.47	1.46	1.54	1.50	1.59
Feed consumption[2]		2.80	3.81	3.88	4.08	4.13	4.32	4.43	4.04	4.35
Feed efficiency[3]		-	21.3	35.3	35.1	35.3	33.5	34.0	36.6	36.1
PER[4]		-	0.71	1.18	1.17	1.18	1.12	1.13	1.22	1.20
Protein intake[5]		0.841	1.142	1.164	1.225	1.238	1.295	1.330	1.211	1.304
L-His intake[6]		0	0.005	0.010	0.020	0.031	0.043	0.055	0.061	0.087
Mortality (%)		0	0	0	0	0	0	0	0	0

[1-6] See Table 2.

Table 4. Performance of Coho Salmon Fed the Isoleucine Test Diet for 6 Weeks (n=60)

Diet No.		1	2	3	4	5	6	7	8	9
L-Isoleucine Level (Leu=3.06)		0	0.31	0.63	0.94	1.26	1.57	1.89	2.22	2.53
	Initial	1.72	1.72	1.75	1.76	1.74	1.73	1.71	1.75	1.70
Mean	SD	0.17	0.14	0.26	0.31	0.25	0.28	0.25	0.28	0.28
body (g)	Final	1.79	2.95	3.12	3.19	3.32	3.17	3.13	3.22	2.94
weight	SD	0.17	0.30	0.71	0.78	0.73	0.71	0.74	0.70	0.65
DSGR (%)[1]		0.09	1.28	1.38	1.42	1.54	1.44	1.44	1.45	1.30
Feed consumption[2]		1.62	2.48	3.29	3.26	3.39	3.39	3.37	3.34	3.55
Feed efficiency[3]		6.2	50.7	41.0	42.2	43.9	41.2	41.5	42.3	35.9
PER[4]		0.21	1.69	1.37	1.41	1.46	1.37	1.38	1.41	1.20
Protein intake[5]		0.485	0.745	0.986	0.979	1.017	1.017	1.012	1.003	1.064
L-Ile intake[6]		0	0.008	0.021	0.031	0.043	0.053	0.064	0.074	0.090
Mortality (%)		0	0	0	0	0	0	0	1.7	0

[1-6] See Table 2.

Table 5. Performance of Coho Salmon Fed the Leucine Test Diet for 4 Weeks (n=60)

Diet No.		1	2	3	4	5	6	7	8	9
L-Leucine Level (Ile=1.89)		0	0.51	1.02	1.53	2.04	2.55	3.06	3.57	4.08
	Initial	1.25	1.21	1.22	1.24	1.23	1.26	1.23	1.26	1.25
Mean	SD	0.14	0.14	0.13	0.13	0.13	0.13	0.13	0.15	0.16
body (g)	Final	1.31	1.55	2.05	2.00	2.06	2.11	2.00	2.14	2.06
weight	SD	0.16	0.21	0.32	0.29	0.28	0.34	0.27	0.33	0.32
DSGR (%)[1]		0.17	0.88	1.85	1.71	1.84	1.84	1.74	1.89	1.78
Feed consumption[2]		1.98	2.57	3.34	3.49	3.37	3.20	3.34	3.33	3.35
Feed efficiency[3]		8.7	33.8	54.3	48.1	53.2	55.9	51.1	55.6	51.8
PER[4]		0.29	1.12	1.81	1.60	1.77	1.87	1.71	1.85	1.73
Protein intake[5]		0.953	0.771	1.001	1.048	1.011	0.960	1.002	0.999	1.005
L-Leu intake[6]		0	0.013	0.034	0.053	0.069	0.082	0.102	0.119	0.137
Mortality (%)		0	0	0	0	0	0	0	0	0

[1-6] See Table 2.

Table 6. Performance of Coho Salmon Fed the Lysine Test Diet for 4 Weeks (n=66)

Diet No.		1	2	3	4	5	6	7	8	9
L-Lys Level		0	0.50	1.00	1.50	2.00	2.50	3.00	3.50	4.00
	Initial	1.77	1.78	1.78	1.72	1.74	1.75	1.75	1.71	1.73
Mean	SD	0.19	0.20	0.20	0.19	0.19	0.17	0.16	0.16	0.18
body (g)	Final	1.77	2.20	2.45	2.50	2.49	2.55	2.50	2.57	2.58
weight	SD	0.23	0.32	0.41	0.41	0.43	0.48	0.41	0.36	0.43
DSGR (%)[1]		0	0.76	1.14	1.33	1.28	1.34	1.27	1.45	1.43
Feed consumption[2]		2.23	2.72	3.44	3.59	3.68	3.78	3.69	3.52	3.73
Feed efficiency[3]		-	27.9	32.8	35.9	34.4	36.0	34.4	40.3	38.2
PER[4]		-	0.93	1.09	1.20	1.15	1.20	1.15	1.34	1.27
Protein intake[5]		0	0.82	1.03	1.08	1.10	1.13	1.11	1.06	1.12
L-Lysine intake[6]		0	0.014	0.034	0.054	0.074	0.095	0.111	0.123	0.149
Mortality (%)		0	0	0	3.0	1.5	1.5	0	1.5	1.5

[1-6]See Table 2.

Table 7. Performance of Coho Salmon Fed the Methionine Test Diet for 4 Weeks (n=55)

Diet No.		1	2	3	4	5	6	7	8	9
L-Met Level (Cys=0)		0	0.26	0.52	0.78	1.03	1.29	1.55	1.81	2.07
	Initial	1.17	1.18	1.16	1.17	1.17	1.19	1.17	1.18	1.16
Mean	SD	0.10	0.10	0.09	0.10	0.08	0.09	0.08	0.08	0.09
body (g)	Final	1.18	1.50	1.67	1.74	1.81	1.92	1.87	1.90	1.80
weight	SD	0.10	0.14	0.13	0.18	0.21	0.20	1.21	0.21	0.23
DSGR (%)[1]		0.03	0.86	1.30	1.42	1.56	1.71	1.67	1.70	1.57
Feed consumption[2]		1.74	2.61	3.58	3.74	3.84	4.07	3.82	3.91	3.84
Feed efficiency[3]		1.3	33.2	35.8	37.3	39.9	41.5	43.0	42.9	40.0
PER[4]		0.04	1.11	1.19	1.24	1.33	1.38	1.43	1.43	1.33
Protein intake[5]		0.522	0.783	1.074	1.122	1.152	1.221	1.146	1.173	1.152
L-Met intake[6]		0	0.007	0.019	0.029	0.040	0.053	0.059	0.071	0.079
Mortality (%)		0	0	0	0	0	0	0	0	0

[1-6]See Table 2.

Table 8. Performance of Coho Salmon Fed the PhenylalanineTest Diet for 4 Weeks (n=55)

Diet No.		1	2	3	4	5	6	7	8	9
L-Phe Level (Tyr=0)		0	0.42	0.84	1.26	1.69	2.10	2.52	2.94	3.36
	Initial	1.23	1.22	1.21	1.22	1.21	1.22	1.22	1.21	1.19
Mean	SD	0.11	0.10	0.10	0.10	0.10	0.09	0.09	0.09	0.09
body (g)	Final	1.25	1.40	1.65	1.94	1.95	2.01	1.99	1.94	1.90
weight	SD	0.10	0.12	0.17	0.18	0.18	0.24	1.23	0.20	0.20
DSGR (%)[1]		0.06	0.49	1.11	1.65	1.70	1.78	1.75	1.68	1.67
Feed consumption[2]		2.08	2.51	2.95	3.58	3.62	3.96	3.63	3.66	3.47
Feed efficiency[3]		2.1	19.0	37.9	45.3	46.1	43.9	47.7	45.1	47.0
PER[4]		0.07	0.63	1.26	1.51	1.54	1.46	1.59	1.50	1.57
Protein intake[5]		0.624	0.753	0.885	1.074	1.086	1.188	1.089	1.098	1.041
L-Phe intake[6]		0	0.011	0.025	0.045	0.061	0.083	0.091	0.107	0.117
Mortality (%)		0	0	0	0	0	0	0	0	0

[1-6] See Table 2.

Table 9. Performance of Coho Salmon Fed the Threonine Test Diet for 4 Weeks (n=60)

Diet No.		1	2	3	4	5	6	7	8	9
L-Threonine Level		0	0.15	0.31	0.62	0.93	1.24	1.55	1.86	2.17
	Initial	1.42	1.38	1.39	1.37	1.37	1.37	1.38	1.41	1.37
Mean	SD	0.14	0.13	0.13	0.13	0.13	0.14	0.12	0.13	0.13
body (g)	Final	1.41	1.62	1.87	2.08	2.07	2.11	2.16	2.18	2.04
weight	SD	0.14	0.15	0.17	0.21	0.24	0.25	0.26	0.23	0.22
DSGR (%)[1]		-0.03	0.57	1.06	1.49	1.47	1.54	1.60	1.56	1.42
Feed consumption[2]		2.14	2.33	2.48	3.32	3.71	3.77	4.01	3.69	3.86
Feed efficiency[3]		-	24.4	42.7	43.8	38.7	40.4	39.1	41.8	36.1
PER[4]		-	0.81	1.42	1.46	1.29	1.35	1.31	1.39	1.20
Protein intake[5]		0.641	0.700	1.744	0.996	1.114	1.130	1.204	1.107	1.157
L-Thr intake[6]		0	0.004	0.008	0.021	0.035	0.047	0.062	0.069	0.084
Mortality (%)		0	0	0	0	0	0	0	0	0

[1-6] See Table 2.

Table 10. Performance of Coho Salmon Fed the Tryptophan Test Diet for 4 Weeks (n=60)

Diet No.		1	2	3	4	5	6	7	8	9
L-Tryptophan Level		0	0.06	0.12	0.24	0.36	0.48	0.60	0.72	0.84
	Initial	1.66	1.66	1.66	1.64	1.66	1.69	1.68	1.66	1.69
Mean	SD	0.20	0.18	0.19	0.21	0.18	0.19	0.21	0.18	0.19
body (g)	Final	1.63	1.93	2.49	2.62	2.68	2.69	2.63	2.69	2.69
weight	SD	0.21	0.20	0.29	0.37	0.35	0.34	0.35	0.30	0.34
DSGR (%)[1]		-0.07	0.54	1.45	1.67	1.71	1.66	1.60	1.72	1.66
Feed consumption[2]		2.18	2.47	3.30	3.74	4.00	3.97	3.74	4.09	3.99
Feed efficiency[3]		-	21.3	43.2	44.2	42.2	41.2	42.0	41.0	40.9
PER[4]		-	0.71	1.44	1.47	1.40	1.37	1.40	1.37	1.36
Protein intake[5]		0.304	0.742	0.990	0.122	1.200	1.192	1.122	1.227	1.196
L-Trp intake[6]		0	0.0015	0.004	0.009	0.014	0.019	0.022	0.029	0.034
Mortality (%)		0	0	0	0	0	0	0	0	0

[1-6] See Table 2.

Table 11. Performance of Coho Salmon Fed the Valine Test Diet for 4 Weeks (n=60)

Diet No.		1	2	3	4	5	6	7	8	9
L-Valine Level		0	0.19	0.38	0.57	0.77	1.15	1.53	1.91	2.30
	Initial	1.40	1.40	1.38	1.40	1.37	1.38	1.37	1.38	1.38
Mean	SD	0.11	0.12	0.12	0.13	0.13	0.12	0.13	0.12	0.13
body (g)	Final	1.38	1.58	1.75	2.11	2.10	2.19	2.16	2.27	2.19
weight	SD	0.13	0.16	0.16	0.24	0.21	0.25	0.21	0.25	0.24
DSGR (%)[1]		-0.04	0.43	0.85	1.47	1.53	1.65	1.62	1.77	1.65
Feed consumption[2]		2.09	2.16	2.84	3.17	3.46	3.90	3.64	3.74	3.67
Feed efficiency[3]		-	20.0	29.8	48.2	43.6	41.6	43.9	46.4	44.2
PER[4]			0.67	0.99	1.61	1.41	1.39	1.46	1.55	1.47
Protein intake[5]		0.627	0.647	0.851	0.950	1.039	1.170	1.091	1.121	1.101
L-Val intake[6]		0	0.004	0.011	0.018	0.027	0.045	0.056	0.071	0.084
Mortality (%)		0	0	0	0	0	0	0	0	0

[1-6] See Table 2.

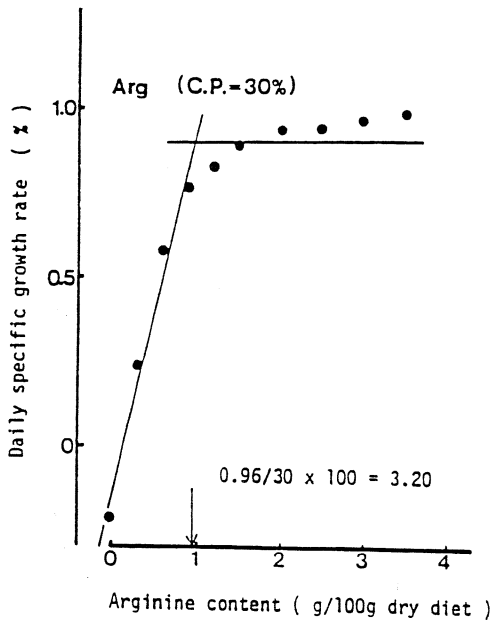

Figure 1. Daily specific growth rate of fingerling coho salmon fed test diets with varying arginine levels

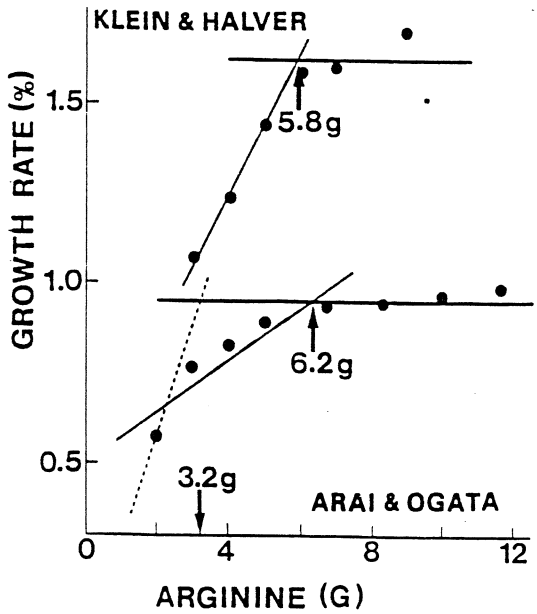

Figure 2. Comparison of data on performance of fingerling coho salmon that were fed test diets to determine the arginine requirement. In the data by Arai and Ogata, the dotted regression line was calculated including the data of 0% and 1.0% arginine levels, the solid regression line being done excluding those data. The arginine levels are expressed as the dietary protein.

Table 12. Amino Acid Requirements of Coho Salmon

Amino Acid	Klein & Halver	Arai & Ogata	
	% of the Dietary Protein	% of the Dietary Protein	mg/100g Fish/Day
Arg	5.8	3.2	42
His	1.8	0.9	10
Ile		1.2	9
Leu		3.4	33
Lys		3.8	38
Met[1]		2.7	30
Phe[2]		4.5	45
Thr		2.0	20
Trp		0.5	5
Val		2.2	20
Total		24.4	252

[1] In the absence of cystine.
[2] In the absence of tyrosine.

Table 13. Essential Amino Acid Ratios[1] of the Requirement Values, the Eyed-Egg Protein, and the Whole Body Protein of Coho Salmon

Amino Acid	Requirement	Eggs[2]	Whole Body[2]
Arg	131	104	115
His	37	48	58
Ile	49	93	71
Leu	139	146	144
Lys	156	145	166
Met + Cys	111	83	92
Phe + Tyr	184	158	140
Thr	82	94	98
Trp	20	14	27
Val	90	113	83

[1] A/E ratio = $\frac{\text{each essential amino acid (g)} \times 1000}{\text{total essential amino acid (g)}}$

[2] Arai (1981).

determined the arginine requirement using the test diets with intact proteins (casein and gelatin) as the nitrogen sources, in addition to amino acid mixture. This means that the test diets contained basically 3.0% arginine originating from these proteins even without any supplementation of crystalline arginine. Thus, the arginine level in their test diets ranged from 3.0% to 9.0% of the dietary protein, while those in our study were between 0% and 11.7%. If the arginine requirement is re-estimated using the data at and beyond the 2.0% dietary arginine levels, that is, excluding the data of 0% and 1.0% arginine levels, the slope of the regression line becomes gentle. The point of interception of the regression line shifts to a higher level of arginine (Fig. 2), indicating that the arginine requirement of coho salmon is 6.2% of the dietary protein, which appears to be comparable to the value reported by Klein and Halver (1970). Thus, the difference in values of the arginine requirement between their study and ours may largely be attributable to the design of test diets. When datum of the test amino acid-free group is excluded in the calculation of each amino acid requirement (except for the case of arginine where data of the arginine levels of both 0% and 1% were omitted as described above), the requirement values are found as follows: arginine, 6.2%; histidine, 2.2%; isoleucine, 3.2%; leucine, 5.7%; lysine, 6.3%; methionine, 3.8%; phenylalanine, 5.1%; threonine, 2.8%; tryptophan, 0.7%; valine, 3.2% of the dietary protein, and arginine, 81mg; histidine, 24mg; isoleucine, 24mg; leucine, 55mg; lysine, 65mg; methionine, 42mg; phenylalanine, 51mg; threonine, 28mg; tryptophan, 6mg; valine, 29mg/100g fish/day. The values in Table 12 may mean that marginal levels of the amino acids are required for the allowable growth of fingerling coho salmon, while those demonstrated above would be the levels needed for their standard growth.

Although the initial mean body weight (3.5g) of fish used for the determination of arginine requirement in this study was approximately equal to that (2.9g) of Klein and Halver (1970), the growth rates were inferior to those of fish reported by them as shown in Figure 2, even though the test groups given diets with the same level of dietary arginine were compared. Murai et al. (1982b) reported that supplementation of only a small amount of casein to an amino acid-based diet drastically improved efficiency of crystalline amino acid utilization in diets in carp (*Cyprinus carpio*) and in channel catfish (*Ictalurus punctatus*). They concluded that casein itself may contain some still unknown factor(s) that improve utilization of crystalline amino acids. Thus, the superior performance of fish in the Klein and Halver

Table 14. Correlation Coeffcients of the A/E Ratios of Requirement vs. the Whole Body A/E Ratios and Egg A/E Ratios

	Eyed-Egg	Fry-7 Weeks
Requirement	0.899	0.932

(1970) study must be due to using the intact proteins in addition to the amino acid mixture as the dietary nitrogen sources.

Since Arai (1981) introduced the concept of essential amino acid ratio (A/E ratio) into the field of fish nutrition, the concept has been emphasized as a useful index to evaluate nutritional qualities of diets and nutritional conditions of test animals (Murai et al., 1982a; Ogata et al., 1983; Wilson and Poe, 1985; Murai et al., 1986; Gatlin 1987; Moon and Gatlin, 1991). The A/E ratios are calculated by the following equation: [(amount of each essential amino acid)/ (total amount of essential amino acids including cystine and tyrosine)] x 1000. Table 13 summarizes the A/E ratios of the requirement values in Table 12, the eyed-egg protein of coho salmon, and the whole body protein of 7-week fry after hatching (Arai, 1981). The A/E ratios between the requirement values and the whole body protein seem to be in much better agreement than those between the requirement values and the eyed-egg protein. Indeed, if the A/E ratios of the requirement values are regressed against the whole body A/E ratios or the eyed-egg A/E ratios, a higher correlation is found in the former than in the latter (Table 14). Thus, in spite of using the amino acid profile of the eyed-egg protein to formulate the basal diet in this study, the amino acid profile of the determined requirements more closely resembled the amino acid profile of the whole body protein. This result confirms the idea that the essential amino acid profile of the whole body protein would be useful to design test diets for those species where their requirement data are not available.

Acknowledgments

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THE NUTRITIVE VALUE OF ALTERNATIVE PROTEIN SOURCES IN THE DIETS OF CULTURED SALMONIDS

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ABSTRACT

The overall goal of this collaborative project was to identify, develop, and evaluate the suitability of new or underutilized plant or animal protein sources as replacements for high-quality fish meal in diets of post-juvenile salmonids. The specific objectives were: (1) to determine the levels of either poultry by-product meal or processed rapeseed protein concentrate in feeds that can provide high nutritive value and palatability; (2) to develop an in vitro assay to quantitate protein digestibility of salmonid diets; (3) to determine the processing conditions required to thermally inactivate selected fish pathogens in model systems, in acid-preserved silage, and in enzymatically hydrolyzed fish by-products; (4) to evaluate the protein quality and digestibility of fish meal products prepared by various methods; and (5) to measure the effects of fermentation and co-drying on the nutritive value of feeds containing combinations of the alternate protein sources. Results indicate that alternate protein sources can successfully replace a significant portion of traditional high-quality fish meal in feeds for salmonids.

Introduction

Aquaculture feeds represent a growing market for marine by-products, primarily fish meal and fish oil. In 1988, approximately 700,000 tons of fish meal were used in fish feed worldwide. By the year 2000, the consumption of fish meal by the aquaculture industry is expected to double to 1.5 million tons,

which would represent approximately 20-25% of total world-wide production of fish meal (Barlow, 1989).

Here in the western region of the United States and Canada, salmonid aquaculture is an expanding industry, providing a consistent year-round supply of high quality fish for the consumer. In 1988, estimated demand for fish meal for trout and salmon feeds in North America was 44,000 tons, which represented approximately 6% of world-wide use of fish meal for fish feeds (Barlow, 1989). This market for salmonid feeds is concentrated in the Pacific Northwest, especially Idaho, California, Oregon, Washington, and British Columbia. More of the feed is used by commercial growers of trout than of salmon.

However, a major concern among fisheries scientists and the aquaculture industry is that the demand for high-quality fish meal, which comprises about 20-45% of most practical salmonid diets, will soon exceed the supply. Fish meal is currently the major component and single most costly ingredient in feeds for salmonids. Future supplies and prices are unstable due to variable supplies of South American fish meal and competition for existing fish meal supplies from poultry, swine, pet food, and fish feed producers. The fluctuations in fish meal prices make it necessary to examine alternative sources of high-quality protein and their suitability in the diets of post-juvenile salmonids.

The goal of this research project is to identify and develop alternate sources of animal and plant proteins and to assess the nutritional value of these protein sources, which could partially or completely replace high-quality fish meal in salmonid diets. This research is sponsored by the Western Regional Aquaculture Consortium (WRAC), which was designed as one of five regional centers under the U. S. Department of Agriculture, where funding would be available to support collaborative research and to develop projects in aquaculture. WRAC is authorized under Title 14 (Subtitle L) of the Food and Security Act of 1985. The concept of cooperative regional research was developed to stimulate and facilitate inter-state and inter-institutional cooperation on research of a regional or national character and to plan and coordinate research to achieve replication but avoid duplication in research efforts. WRAC encompasses 12 states in the western region: Alaska, Arizona, California, Colorado, Idaho, Montana, Nebraska, New Mexico, Oregon, Utah, Washington, and Wyoming.

Acceptable Replacement Levels of Alternate Protein Sources

The first objective was to determine the acceptable dietary levels for poultry by-product meal (PBM) and specially processed rapeseed protein concentrate (RPC) as a fish meal substitute in feeds for salmonids. PBM, which is obtained by rendering poultry processing waste exclusive of feathers, contains approximately 60-65% protein. It has been studied as a partial protein replacement in the diets of channel catfish (*Ictalurus punctatus*) by Brown, Strange, and Robbins (1985), rainbow trout (*Oncorhynchus mykiss*) by Alexis, Papaparaskeva-Papoutsoglou, and Theochari (1985), chinook salmon (*Oncorhynchus tshawytscha*) by Fowler (1981, 1982), coho salmon (*Oncorhynchus kisutch*) by Higgs et al. (1979), and European eels (*Anguilla anguilla*) by Gallagher and Degani (1988). Generally, when protein from PBM replaced a high percentage of fish meal protein, growth was compromised.

Canola seed is the name of a new variety of rapeseed that has been bred specifically to contain low levels of anti-nutrients such as glucosinolates in the meal and erucic acid in the oil. Canola meal contains approximately 38-40% protein. Higgs et al. (1983) and Leatherland, Hilton, and Slinger (1987) showed that residual levels of glucosinolate hydrolytic products had the greatest effect on the meal's nutritive value for salmonids because of negative effects on thyroid function. Higher levels of canola meal in salmonid diets may be possible if 3,5,3' triiodo-L-thyronine (T-3) is administered concurrently to reinstate normal thyroid function (Higgs et al., 1983; Fagerlund et al., 1987). Canola seed is the major oilseed crop grown in Canada (Higgs et al., 1982). Relative to canola meal, RPC contains lower levels of glucosinolates (anti-thyroid compounds), fiber, N-free extract (carbohydrate), and phenolic compounds (Higgs et al., 1988).

The experimental conditions for all feeding trials were as follows. First, all trials were 6 to 12 weeks in duration. This enabled the fish to double or triple their initial body weight and thereby permitted detection of possible differences in body composition between groups using standard methods. Second, careful records were maintained of daily food intake, mortality, and water temperature, and fish of a given species were selected for uniform initial size. Third, triplicate groups were assigned to each treatment. The

results of the studies indicated that PBM could replace ≤ 37% and ≤ 40% of the herring meal protein in diets for rainbow trout (*Oncorhynchus mykiss*) and coho salmon (*Oncorhynchus kisutch*) reared in fresh water, respectively. Further, it was possible to successfully replace two-thirds of the herring meal protein in a dry basal diet for rainbow trout with protein from undephytinized or dephytinized RPC. Undephytinized RPC comprised about 38% of the dietary protein and fish meal constituted only 11% of the dry diet without adversely affecting fish performance (Higgs et al., 1991).

In Vitro Assay for Protein Digestibility

There is currently a need for a simple and reliable in vitro method for measuring digestibility of protein in diets for fish. Use of the current AOAC (1990) mammalian proteolytic enzyme method (sec. 982.30) for estimating protein digestibility in fish is restrictive because: (1) mammalian enzymes have different intrinsic characteristics from those of salmonids; and (2) pH changes occurring as peptide hydrolysis proceeds appear to suppress both rate and degree of proteolysis, thereby inaccurately indicating protein digestibility. Therefore, one goal was to develop an in vitro assay for protein digestibility that would predict in vivo digestibility of proteins by salmonids.

The in vitro assay method employed digestive enzymes from the pyloric ceca of salmonid fish (2 mg isolated as described by Grabner, 1985) and a feed sample containing 100 mg protein (assuming 16% nitrogen and samples were free of non-protein nitrogen) in a 15 ml initial volume at pH 8.0. The degree of hydrolysis (DH) of sample protein at 15° C after 500 minutes was measured using a pH-stat titration system interfaced to a Mac IIcx computer with a Molytek data logger and Parameter Manager Plus software.

Specific questions addressed were: (1) could mathematical curve fitting be used to forecast the final DH, thereby reducing the assay time at 15°C; (2) was the DH obtained with pyloric cecal enzymes influenced by the biological age of the trout source; (3) did diet history of trout before sacrifice influence the DH obtained with pyloric cecal enzymes? It was found that: (1) an exponential curve fitting program could be applied to most samples so that a shorter assay time of 150 min could be used to predict the DH at 500 min; (2) the biological age of trout appeared to have an effect on the DH obtained with pyloric cecal enzymes from these fish: i.e., the DH obtained with enzymes from juvenile fish was less than that obtained from mature fish; and (3) feeding of diets containing either herring meal, PBM, or a herring meal and PBM combination several weeks prior to sacrifice of trout did not affect the DH of casein when pyloric cecal enzymes from these fish were used.

Thermal Inactivation of Selected Fish Pathogens

The third objective was to determine the processing conditions required to thermally inactivate selected fish pathogens in model systems, in acid-preserved silage, and in enzymatically hydrolyzed fish by-products. A potential problem with ensiled fisheries by-products in dry or moist diets is the transmission of disease to aquacultured fish. Even though many ingredients included in formulated moist diets are pasteurized, recent investigations have shown that currently used procedures for manufacturing fish diets do not completely eliminate fish or human pathogens, and relatively large populations of bacteria remain in these materials (Moffit-Westover, 1986). In the past, microbial contamination of fish feeds caused epizootics among hatchery-reared salmonids of the Pacific Northwest. Besides the threat that contaminated feed poses to the health of fish, bacterial growth can decrease the nutritional value of a feed by depleting the level of micronutrients. Contaminated feed, particularly moist feeds, also have a decreased shelf-life.

The organisms chosen for the investigations included two viruses and three bacteria. The two viruses were infectious hematopoietic necrosis virus (IHNV), a rhabdovirus considered likely to be sensitive to heat and low pH; and infectious pancreatic necrosis virus (IPNV), a non-enveloped birnavirus that was likely to be resistant to these conditions. The three bacteria studied were: *Aeromonas salmonicida*, a Gram negative bacterium which causes furunculosis and was selected as a representative of the bacteria that cause septicemias (blood borne problems caused by systemic bacteria) in fish; *Renibacterium salmoninarum*, a Gram positive organism which is responsible for bacterial kidney disease; and *Mycobacterium chelonei*, a group of (acid-tolerant) bacteria that cause "fish tuberculosis", the disease which

originally prompted the pasteurization of fish products incorporated into fish food.

It was found that *A. salmonicida*, *M. chelonei*, and IHNV were all sensitive to heat and pH values that would be common in pasteurization procedures used for fish silage. Introduction of these three pathogens at different steps in silage production indicated that the silage had a rapid toxic effect on IHNV; *A. salmonicida* survived for several days in fish viscera, but only a few minutes in silage; *M. chelonei* also survived for several days in the fish viscera but for only 90 min in fish silage. The preservatives butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), which are sometimes added to fish silage, had a toxic effect on *A. salmonicida* and *M. chelonei* in media that was not observed in the fish viscera.

The more resistant IPNV appeared unaffected by low pH, the fish silage, or the BHA and BHT. IPNV was tested in two different simulations of pasteurization procedures, and although initial titers were reduced by more than 3 logs in both experiments, a low level of virus survived in media at neutral pH. The time to totally eliminate IPNV in pasteurization simulations is being investigated further.

Due to the tendency of *R. salmoninarum* to autoagglutinate and the resulting problems in achieving a homogeneous suspension of dispersed cells, and to difficulties in the culture of the organism on artificial medium, thermal inactivation curves were complicated. However, it was determined that currently used pasteurization protocols used in fish feed manufacture do inactivate *R. salmoninarum*.

Fish Meal Quality

The fourth objective was to evaluate protein quality and digestibility of fish meal products prepared by various methods. Currently, there is no single standard procedure employed for grading fish meal. Hence, there are a variety of methods that include: total volatile nitrogen, ammonia nitrogen, pepsin digestibility, levels of biogenic amines, free fatty acids, peroxide value, anisidine value, digestibility coefficients in mink, levels of ash, gizzard erosion in chicks, odor, level of protein, etc. Studies were performed with fish meals not currently used in aquaculture because of perceived quality problems and which contained biogenic amines acutely toxic to poultry. These fish meals were found not to be acutely toxic to rainbow trout in short term feeding trials and to have equivalent nutritional value and protein quality as standard high quality fish meal. These results emphasize the need for quality indices that are known to directly reflect nutritive value of fish meal for farmed fish and shrimp species.

Lactic Acid Bacteria Fermented Silage

The fifth objective was to design a procedure for preparing lactic acid bacteria fermented silage using fish viscera, a commercial preparation of lactic acid bacteria (*Lactobacillus plantarum* and *Streptococcus faecium*), a carbohydrate source (dextrose), and a mold inhibitor (calcium propionate). This protocol was successful in allowing production of lactic acid, which decreased the pH to 4.5 on the first day of ensilation. The liquid silage was combined with PBM and co-dried on a double drum drier; the dried product (approximately 1:1, ensiled product:PBM, weight:weight) was then incorporated into compaction pelleted dry feeds for coho salmon. Several feeding trials with coho salmon have indicated that diets containing the lactic acid co-dried silage as the major protein source were less palatable, but of equivalent digestibility and nutritional value as the diets containing herring meal as the protein source.

In summary, this project has focused on assessing the nutritional value of protein sources other than traditional fish meal in salmonid diets. As part of this collaborative research, efforts have been directed at developing an in vitro method for predicting protein digestibility that correlates with the results of in vivo measurements. Moreover, conditions have been identified that inhibit pathogen survival in alternative protein sources and feeds containing these sources. Collectively, this project will contribute to public information regarding high quality, least-cost protein ingredients for formulated feeds, resulting in more economical salmonid production than at present.

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AMINO ACID REQUIREMENTS OF CHUM SALMON FRY AND SUPPLEMENTATION OF AMINO ACID TO DIET

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ABSTRACT

The quantitative requirements of the 10 essential amino acids for growth of chum salmon fry, *Oncorhynchus keta*, were determined by using a purified test diet with 40% crude protein containing crystalline amino acids and a small amount of casein as nitrogen sources. Weight gain showed the following requirements as a percentage of dietary protein: arginine, 6.47; histidine, 1.64; isoleucine, 2.37; leucine, 3.80; lysine, 4.96; methionine, with cystine (1.20% of protein), 3.02; phenylalanine with tyrosine (3.50% of protein), 6.27; threonine, 3.04; tryptophan, 0.73; valine, 3.04. The regression lines between A/E ratios (1000 x each essential amino acid content/total amino acid content including cystine and tyrosine) of the requirements of chum salmon and some other species were calculated. High correlations were noted with salmonids and channel catfish. The finding might be related to arginine metabolism in fishes.

Because of the decrease of fish meal production in Japan, alternative protein sources for fish diet are needed for the fish farming industry. Crystalline amino acids can be used as supplemental materials to adjust the essential amino acid balance in diets containing poorer protein sources. Some studies on these supplements clearly indicate feeding performances improved. Moreover, the useful physiological effects of crystalline amino acids supplemented to the diets are discussed.

I. Essential Amino Acid Requirements of Chum Salmon Fry and the Comparison Between Fishes

Introduction

It has been shown by growth assay that fishes such as salmonids (Halver et al. 1957; Halver and Shanks 1960; Shanks et al. 1962; Arai et al. 1980), channel catfish (Dupree and Halver 1970), European and Japanese eels (Arai et al. 1972), common carp (Nose et al. 1974), red sea bream (Yone 1976), and tilapia (Mazid et al. 1978) require 10 essential amino acids that are essential for normal growth. In addition, 14C labeling studies have shown that plaice and sole (Cowey et al. 1970) and sea bass (Metailler et al. 1973, cited in Wilson 1989) require the same 10 amino acids. Moreover, all quantitative requirements for these essential amino acids have been determined on chinook salmon (Halver et al. 1958, 1959; Halver 1965; Delong et al. 1962; Chance et al. 1964; Klein and Halver 1970), coho salmon (Arai 1980; Ogata and Arai 1981; Arai et al. 1981; Arai and Ogata 1993), common carp (Nose 1979), channel catfish (Harding et al. 1977; Wilson et al. 1977, 1978, 1980; Robinson et al. 1980, 1981), Japanese eel (Arai cited in Nose 1979), Nile tilapia (Santiago and Lovell 1988), and catla (Ravi and Devaraj 1991). We have reported that the amino acid requirements for fry of chum salmon *Oncorhynchus keta*, which is one of the most important species in recent fish enhancement projects in Japan (Akiyama et al. 1985a, 1985b; Akiyama 1987). In this paper, the relationships between the requirements for chum salmon and A/E ratios (1000 x each essential amino acid content/total essential amino acid content including cystine and tyrosine) of amino acid requirements of several fishes are discussed.

Experimental Methods

The experimental diets were formulated from purified ingredients (Table 1). The amino acid com-

position simulated those from whole body of chum salmon fry (Table 2). In each experiment, the essential amino acid for each requirement was supplemented at graded levels. The crude protein level was adjusted to approximately 40% by isonitrogenous substitutions with L-alanine, which satisfies the protein requirement of chum salmon fry (Akiyama et al. 1981). These ingredients were mixed with the same weight of water, neutralized by 25% NaOH, and stocked in a freezer until used. This was fed to the fish as a soft and noodle-like diet, using a 50ml disposable syringe.

All chum salmon eggs were transferred from the Chitose Branch of the Hokkaido Salmon Hatchery, which is located on Ishikari River of Japan. These eggs were hatched in our laboratory, and the fry were used for the experiments when they attained a mean body weight of more than 1g. These fry were held in polyvinyl-chloride tanks (20x50x30cm), and underwent a 1-week conditioning period. The 4-week feeding experiments were conducted using running well water of 1.4L/min at 14-16°C. All groups were fed the respective diets to satiation twice daily, 6 days a week. The fish were individually weighed at the beginning and end of the experimental period after being anesthetized in a 0.01% MS-222 solution.

Data on growth, feed efficiency, and so on were analyzed for significance ($P<0.05$) using Duncan's multiple-range test (Duncan 1955).

Table 1. Composition of the Test Diet (%)

Ingredient	%
Vitamin-free casein	5.0
Amino acid mix	39.54-42.62
Fish oil	12.0
Dextrin	20.0
Vitamin mix	4.5
Mineral mix	4.0
CMC	8.0
Cellulose	4.08-7.00

Table 2. Amino Acid Components of Dry Basal Diet

Amino acids*	Supplied by 5g casein	Supplied by crystalline amino acids	Total	40% whole body protein of chum salmon fry
L-Arginine	0.17	2.23	2.40	2.40
L-Histidine	0.13	1.27	1.40	1.40
L-Isoleucine	0.23	1.37	1.60	1.60
L-Leucine	0.44	2.48	2.92	2.92
L-Lysine	0.38	3.10	3.48	3.48
L-Methionine	0.12	1.08	1.20	1.20
L-Cystine	0.03	0.45	0.48	0.48
L-Phenylalanine	0.23	1.45	1.68	1.68
L-Tyrosine	0.26	1.14	1.40	1.40
L-Threonine	0.24	1.64	1.88	1.88
L-Tryptophan	0.05	0.55	0.60	0.60
L-Valine	0.28	1.56	1.84	1.84
L-Alanine	0.14	variable	variable	2.48
L-Aspartic acid	0.41	3.51	3.92	3.92
L-Glutamic acid	1.28	5.00	6.28	6.28
L-Glycine	0.09	2.63	2.72	2.72
L-Proline	0.51	1.41	1.92	1.92
L-Serine	0.34	1.46	1.80	1.80

*Expressed as g/100g diet.

Table 3. Effects of Dietary Leucine Levels on Growth, Feed Efficiency, and Feed and Leucine Consumption of Chum Salmon Fry[1]

Leucine		Initial mean body weight (g)	Weight gain (%)	Feed[3] efficiency (%)	Daily feed consumption on dry basis	Daily leucine consumption (mg/100g body wt.)
% in diet	% in protein					
0.44	1.1	1.20	40.6a[2]	28.9a[2]	4.17	18.3
0.80	2.0	1.19	89.3b	47.6b	4.63	37.0
1.20	3.0	1.19	157.5c	67.2c	4.68	56.2
1.60	4.0	1.18	197.1e	67.2c	5.29	84.6
2.00	5.0	1.19	188.3de	65.4c	5.29	105.8
2.40	6.0	1.20	190.1de	67.6c	5.15	123.6
2.80	7.0	1.22	177.8d	67.4c	4.96	138.9
3.20	8.0	1.19	181.7d	67.2c	5.06	161.9
SEM (df=8)			26.77	1.00		

[1] Average value of duplicate tanks containing 35 fish reared for 4 weeks at 16.5°C.
[2] Values with different superscripts in the same column are significantly different ($p < 0.05$).
[3] Expressed as (wet weight gain / dry feed intake) X 100.

Feeding Experiments and the Results

In the feeding experiment for leucine requirement, the effects of dietary leucine levels on weight gain, feed efficiency, body protein increment, hematocrit, plasma protein, and free amino acids in whole body extract were investigated (Akiyama 1987). Increasing the dietary leucine level up to 1.6% on dry basis significantly improved weight gain, but no significant difference was detected above 1.6 % (Table 3). The breakpoints were calculated using the regression equations to determine the requirements as leucine percent both in diet and dietary protein (Fig. 1). The leucine requirement was found to be 1.52% in diet and 3.80% in protein. The breakpoint of percent of body protein increment (3.79%) was exactly the same as that of weight gain. However, feed efficiency, ratio of protein deposited/protein ingested, hematocrit value, and plasma protein reached plateaus at lower leucine levels than that of weight gain (Table 3; Table 4). The free leucine in whole body extract showed relatively low levels in the range of 1.1-2.0% in dietary protein, but the free leucine level became higher in more than 3.0%, as well as the ratios of total essential amino acid content/total non-essential amino acid content (TEAA/TNEAA) (Table 5). Comparing the other branched-chain amino acids, both isoleucine and valine percentage in total essential amino acids increased in leucine-deficient fish and revealed relatively constant low levels in the fish fed the diets containing higher leucine levels. Similar phenomena were reported on muscle tissue of the common carp (Nose et al. 1978), serum of channel catfish (Wilson et al. 1980), and plasma of cat (Hargrove et al. 1983). Although Hambraeus et al. (1976) and Robinson et al. (1984) suggested that leucine promotes the tissue uptake of branched-chain amino acids as well as their intracellular metabolism, the evident mechanism is not elucidated.

Thus, some of these data did not confirm the requirement based on growth assay. However, a growth study has been applied in most experiments determining the essential amino acid requirements of fish. Therefore, the other requirements for chum salmon fry were determined based on growth data (Table 6).

Characteristics of Requirements for Chum Salmon and Comparison With the Other Fishes

The essential amino acid requirements for eight fishes are listed in Table 7. Since each of these studies

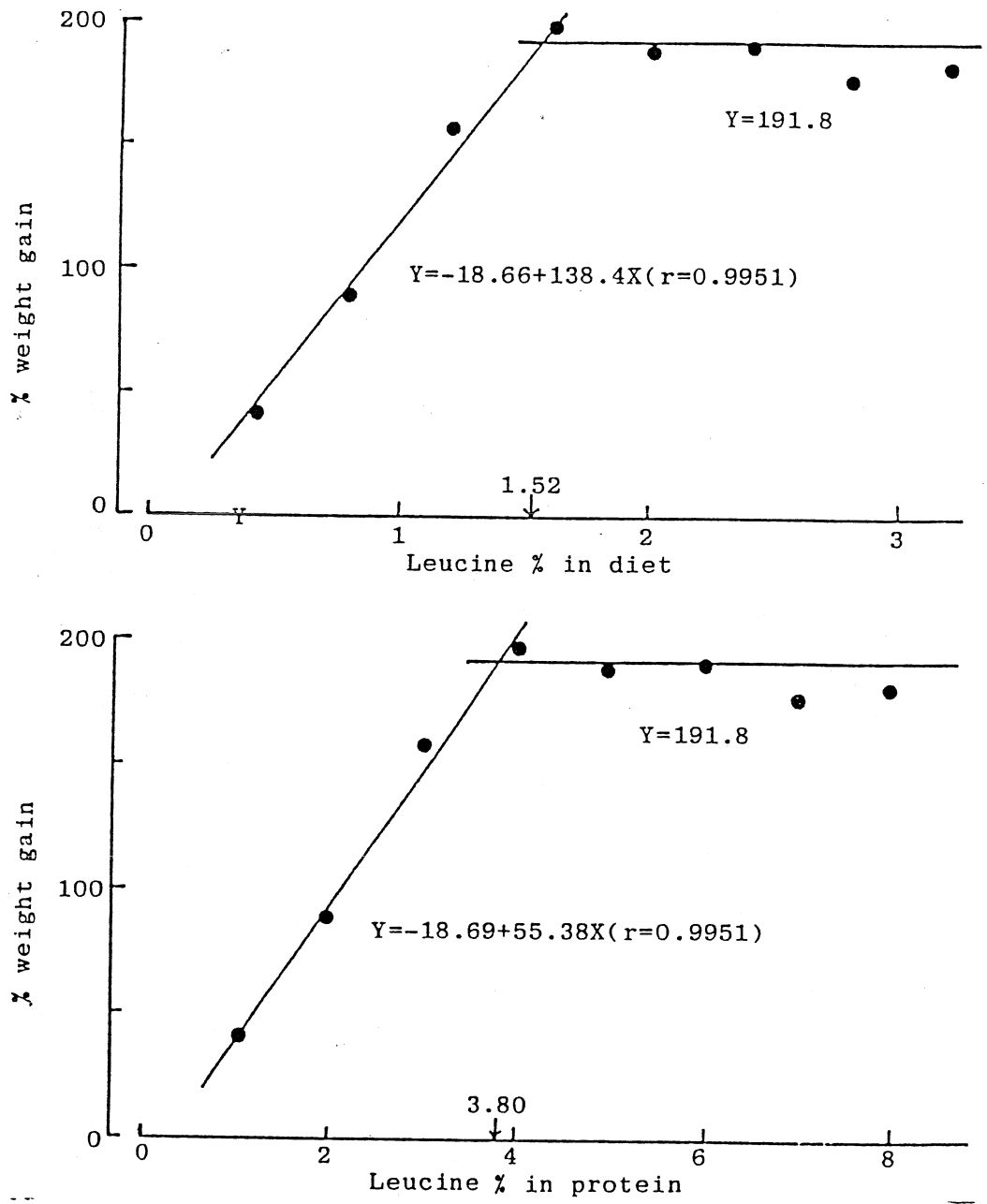

Figure 1. The relationship between dietary leucine level and weight gain of chum salmon fry; average value of duplicate tanks of 35 fish each fed for 4 weeks at 16.5°C (Akiyama et al. 1987)

Table 4. Effects of Leucine Levels in Dietary Protein on Hematocrit, Plasma Protein and Body Protein of Chum Salmon Fry*

Leucine % in protein	Blood Ht	Blood Plasma protein (g/dl)	Body protein increment (%)	Body protein deposited (%)	Protein ingested
1.1	32.8a	2.41a	30.7a	4.07a	
2.0	39.4ab	3.40b	84.9b	8.41b	
3.0	46.7c	4.07c	167.0c	13.23c	
4.0	50.0c	4.06c	210.4ef	13.27c	
5.0	47.6c	3.87bc	197.7de	12.81c	
6.0	46.1bc	3.83bc	206.1ef	13.57c	
7.0	46.9c	3.83bc	192.3d	13.52c	
8.0	45.4bc	4.02c	194.2d	13.33c	
SEM (df=8)	2.09	0.154	8.93	0.608	

* Average value of duplicate tanks. Values with different superscripts in the same column are significantly different ($p<0.05$).

Table 5. Effects of Leucine Levels in Dietary Protein on Free Amino Acids in Whole Body Extract of Chum Salmon Fry*

Leucine % in protein	Leucine (mg/100g dry) sample	Leu/TEAA (%)	Ile/TEAA (%)	Val/TEAA (%)	Branched amino acids	TEAA (%)	TEAA/TNEAA
1.1	55.7a	7.11a	4.61c	10.05d		21.8ab	0.86a
2.0	68.4ab	8.23ab	4.81c	9.15cd		22.2b	0.88a
3.0	111.7abc	9.19bc	4.46bc	8.49bc		22.1b	1.03ab
4.0	121.4abc	9.04bc	4.22ab	7.63ab		20.9ab	1.13ab
5.0	164.6c	10.29c	4.18ab	7.03a		21.5ab	1.27b
6.0	108.2abc	8.98bc	4.12ab	6.88a		20.0a	1.09ab
7.0	94.4abc	8.74abc	4.13ab	7.27a		20.1a	0.99ab
8.0	140.0bc	9.41bc	4.04a	7.00a		20.4ab	1.14ab
SEM (df=8)	60.75	1.527	0.312	0.965		1.531	0.258

* Average value of duplicate tanks. Values with different superscripts in the same column are significantly different ($p < 0.05$).

TEAA: Total essential amino acids.

TNEAA: Total non-essential amino acids.

was conducted using different nitrogen sources and applying a different amount of dietary crude protein, it seems difficult to directly compare these data. It is indicated that both fulfilling requirement level and keeping optimum amino acid balance are very important to elevate a protein nutritional value (Ariyoshi 1983). Arai (1981) reported that coho salmon fry, which were fed the crystalline amino acid supplemented-casein diet having the pattern of A/E ratios computed from amino acid compositions of the whole body, showed better growth and feed efficiency than those of the fish fed the diet containing only casein or fish meal as nitrogen source. These facts may suggest that A/E ratio is a useful and significant expression for essential amino acid requirements. Both A/E ratios of requirements and whole body protein of chum salmon fry are presented in Table 8. The A/E ratio of requirement for chum salmon is considerably higher on arginine and lower on histidine and tryptophan than those in whole body protein, although it is claimed that the dietary requirements for carp (Cowey and Tacon 1983, cited in Wilson 1989) and channnel catfish (Wilson and Poe 1985) correspond well with the essential amino acid patterns in the whole body.

Comparing dietary requirements for chum salmon with those of several fishes in terms of A/E ratio, it is recognized that the requirements for salmonids are very similar to each other. Moreover, these eight fishes may be divided broadly into two groups (Table 9). The arginine requirements are especially high for salmonids and catfish, compared to cyprinids, tilapia, and eel. Conversely, threonine and tryptophan requirements are relatively low in the former. The enzymes preceding arginase in the urea cycle

Table 6. Amino Acid Requirements of Chum Salmon Fry

Amino acids	% in diet	% in protein
Arginine	2.59	6.47
Histidine	0.66	1.64
Isoleucine	0.95	2.37
Leucine	1.52	3.80
Lysine	1.99	4.96
Methionine	0.73	1.82
(Cystine)	(0.48)	(1.20)
Phenylalanine	1.11	2.77
(Tyrosine)	(1.40)	(3.50)
Threonine	1.22	3.04
Tryptophan	0.29	0.73
Valine	1.22	3.04
Total	14.16	35.34

Table 7. Amino Acid Requirements in Dietary Protein (%)

Amino acids	Chum salmon	Chinook salmon	Coho salmon	Channel catfish	Common carp	Catla	Nile tilapia	Japenese eel
Arg	6.5	6.0	3.2	4.3	4.3	4.8	4.2	4.5
His	1.6	1.8	0.9	1.5	2.1	2.5	1.7	2.1
Ile	2.4	2.2	1.2	2.6	2.5	2.4	3.1	4.0
Leu	3.8	3.9	3.4	3.5	3.3	3.7	3.4	5.3
Lys	5.0	5.0	3.8	5.1	5.7	6.2	5.1	5.3
Met+Cys	3.0	4.0	2.7	2.3	3.1	3.4	3.2	3.2
Phe+Tyr	6.3	5.1	4.5	5.0	6.5	6.2	5.5	5.8
Thr	3.0	2.2	2.0	2.2	3.9	5.0	3.8	4.0
Trp	0.7	0.5	0.5	0.5	0.8	1.0	1.0	1.1
Val	3.0	3.2	2.2	3.0	3.6	3.6	2.8	4.0
Total	35.3	33.9	24.4	30.0	35.8	38.8	33.8	39.3

Table 8. A/E Ratios of Amino Acid Requirements and in Whole Body Protein of Chum Salmon Fry

Amino acids	A/E ratio of requirements	A/E ratio in whole body protein
Arginine	183	115
Histidine	46	67
Isoleucine	67	77
Leucine	108	140
Lysine	140	167
Methionine	52	57
(Cystine)	(34)	23
Phenylalanine	78	80
(Tyrosine)	99	67
Threonine	86	90
Tryptophan	21	29
Valine	86	88
Total	1000	1000

offer a route for the synthesis of arginine which is essential for the synthesis of protein, creatine, and arginine phosphate (Brown and Cohen 1960). Brown and Cohen (1960) supposed that, even though arginase has been present in teleosts, the deletion of remaining enzymes of the urea cycle occurred in the evolvement of Actinopterygii to Holostei and Teleostei, and teleostean fish lost the urea cycle to synthesize urea and arginine. Whereas, Huggins et al. (1969) found all five enzymes of the urea cycle in teleosts. Read (1971) reported that the rate of urea excretion is low compared with that of ammonia, although relatively high activities of all five of the urea cycle enzymes were found in the liver of the teleost *Opsanus tau*. These studies may suggest that the urea cycle in teleosts is not efficient as the pathway for urea synthesis, even if the urea cycle is present. In the commercially usable fishes, Wilson (1973) has reported that arginine synthetase is not detected in either liver or kidney tissues of channnel catfish which require a high level of dietary arginine as shown in Table 9. In salmonids, Chiu et al. (1986) have concluded that the urea cycle of rainbow trout provides for potential arginine synthesis. They also mentioned that further studies are needed to determine whether net synthesis of arginine occurs via this pathway. In recently fed sockeye salmon, the ammonia excretion rises but urea excretion remains steady (Brett and Zala 1975). Rainbow trout undoubtedly possess arginase activity in the liver and kidney (Onishi and Maruyama 1969,1970; Chiu et al. 1986). All of the salmonids require dietary arginine for normal growth. These facts indicate that the activities of enzymes in the urea cycle are not enough for arginine synthesis. A/E ratio requirements of cyprinids, eel, and tilapia are similar to those of the whole body of the fishes, whereas the extrusive ratio of arginine is recognized in salmonids and channel catfish. This may suggest that the considerable amount of arginine required by these fishes could be used not only for protein synthesis but also for conversion to other functional substances, such as creatine or degrading to proline, etc.

The correlations between A/E ratios of dietary requirements for chum salmon and the other fishes are shown in Table 10. The regression coefficients of chinook salmon and coho salmon are close to 1 and indicate the similarity of requirements between salmonids. Also, the coefficient of highly arginine requiring channel catfish, which belongs to the neighbor superorder Ostariophysi of Protacanthopterygii which contains salmonids, was relatively high. The lowest regression coefficient and correlation coefficients was shown in the Japanese eel which belongs to a different cohort than the other fishes in the phyletic line suggested by Greenwood et al. (cited in Iwai 1973; see Fig. 2).

II. Supplemental Effects of Dietary Amino Acids

Use of Crystalline Amino Acids

The practical use of industrial crystalline amino acids for livestock and cultured fishes, i.e.: (I) adjustment of the amino acid balance in diets, (II) use as a feeding stimulant, and (III) the physiological effects on the animals are considered. The effect of crystalline amino acids as a feeding stimulant has been limited to experiments on purified or semipurified diets. These components have not been used in practical diets containing ingredients such as fish meal. Therefore, the possibilities for practical use are discussed only in sections (I) and (III) in this paper.

Amino Acid Balance Adjustment in Practical Diet

Approximately 62% of the sardine landed at the main fishing ports of Japan in 1989 was used for the

Table 9. A/E Ratios of Amino Acid Requirements

	SALMONIDAE			AMEIURIDAE	CYPRINIDAE		CICHLIDAE	ANGUILLIDAE
Amino acids	Chum salmon	Chinook salmon	Coho salmon	Channel catfish	Common carp	Catla	Nile tilapia	Japanese eel
Arg	183	177	131	143	120	125	124	115
His	46	53	37	50	59	64	51	53
Ile	67	65	49	87	70	61	92	102
Leu	108	115	139	117	92	96	100	135
Lys	140	147	156	170	159	162	151	135
Met+Cys	86	118	111	77	87	87	95	81
Phe+Tyr	177	150	184	167	182	161	164	148
Thr	86	65	82	73	109	128	111	102
Trp	21	15	20	17	22	25	30	28
Val	86	94	77	100	101	92	83	102
Total	1000	999	999	1001	1001	1001	1001	1001

Table 10. The Relationships Between A/E Ratios of the Essential Amino Acid Requirement for Chum Salmon Fry and Those of the Other Fishes by Regression Analysis

Species	Regression Coefficient	Correlation Coefficient
SALMONIDAE		
Chum salmon	1	1
Chinook salmon	0.91	0.95
Coho salmon	0.92	0.90
AMEIURIDAE		
Channel catfish	0.87	0.93
CYPRINIDAE		
Common carp	0.78	0.88
Catla	0.72	0.86
CICHLIDAE		
Nile tilapia	0.69	0.90
ANGUILLIDAE		
Japanese eel	0.59	0.84

production of fish meal and fish oil. Now in Japan, however, the catch of sardine is speculated to rapidly decrease, based on the lowered catch of younger fish. According to an official estimate, the fish meal production in 1991 would decrease to two-thirds of the highest production (735 thousand metric tons) in 1988. The search for the alternative protein sources to complement the shortage of fish meal is one of the urgent problems in the Japanese fish farming industry. Therefore, plant proteins and by-products from food industries have been given attention again. However, these types of protein have several weak points, one of which is unbalance of essential amino acids. For instance, most plant materials such as soybean meal are deficient in sulfuric amino acids. Wheat flour, brewers grain, and tofu cake have to be supplemented with lysine to improve nutritional value. It is well known that excessive or deficient levels of amino acids may cause ill effects. There are also some negative interactions between amino acids. Tryptophan-deficient fish develop scoliosis (Halver and Shanks 1960; Shanks et al. 1962; Kitamura 1969; Kloppel and post 1975; Ogata and Arai 1981; Poston

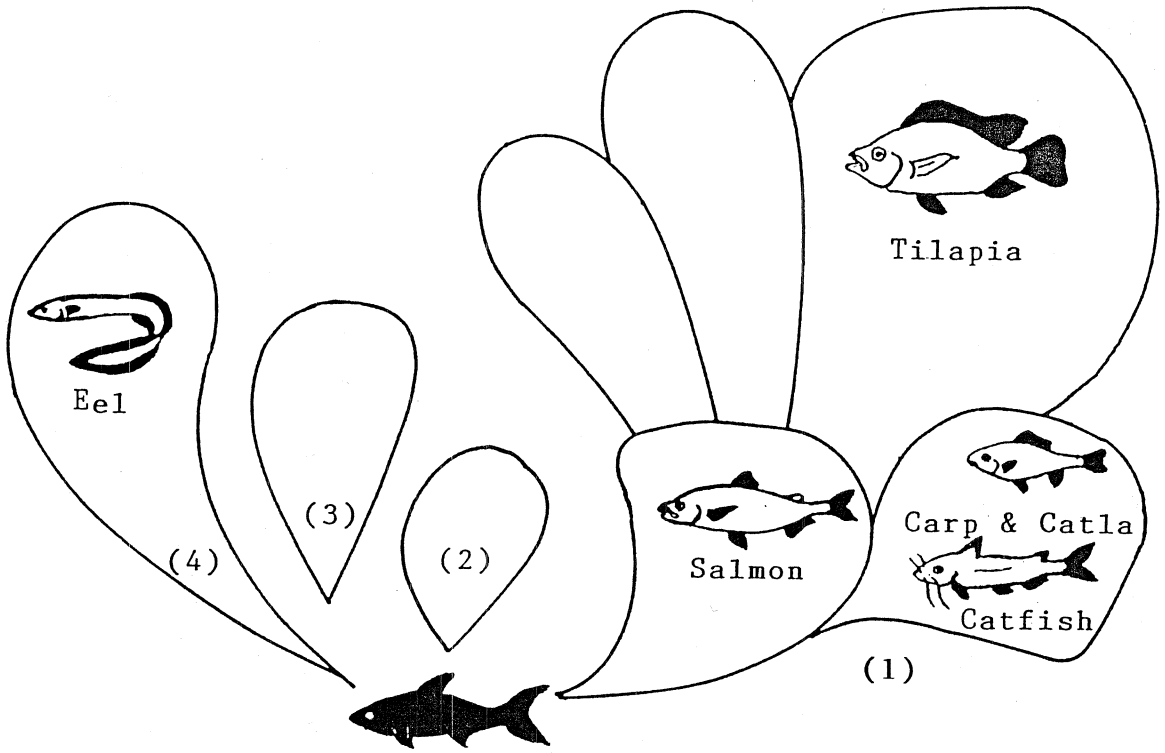

Figure 2. Phyletic lines of teleosts (by Greenwood et al., cited in Iwai 1973), classified as follows: (1) Euteleostei; (2) Clupeocephala; (3) Archaeophylaces; and (4) Taeniopaedia

and Rumsey 1983; Walton et al. 1984; Akiyama et al. 1985b,1986a,1986b,1989; Akiyama 1992). Cataracts occur in methionine-deficient rainbow trout (Walton et al. 1982; Rumsey et al. 1983) and in tryptophan-deficient salmonids (Poston and Rumsey 1983; Akiyama et al. 1986a,1986b,1986c). Antagonism between lysine and arginine (Kaushik and Faconneau 1984), or branched-chain amino acids (Chance et al. 1964; Nose 1979) is also confirmed in fishes. These studies indicate that fulfilling the amino acid requirements and maintaining the amino acid balance in a proper A/E ratio must be considered when we produce a fish diet including much plant protein. When 60% of fish meal protein in the diet is substituted with soybean protein simulating the A/E ratio of amino acids of fish meal, rainbow trout show the same weight gain and protein digestibility as those of fish meal alone (Yamamoto and Akiyama 1991).

For the livestock industry, lysine, methionine, and tryptophan are commercially sold as additives for practical diets. On the other hand, in the fish farming industry the supplementation of amino acids to diets has not been popular because the amino acid balance of fish meal is most ideal for fish growth, at least so far. Therefore, the studies on crystalline amino acid utilization for fish diets must be further expected, as the use of alternative protein sources increase.

Physiological Effects of Amino Acids

Amino acids are not only materials for protein synthesis but they are also the precursors of physiologically functional substances. Arginine is converted to a creatine or ornitine. Phenylalanine and tyrosine are well known as the precursors of thyroxine, dopamine, norepinephrine, epinephrine, melanine, etc. Also, tryptophan is converted to many functional substances including neurotransmitters and vitamin.

In this paper, we would like to describe tryptophan as one example. Tryptophan has three main pathways, but the most important one is to contribute to protein synthesis as an essential amino acid (Fig. 3). Another is the pathway through kynurenine to be finally catabolized to CO_2 and H_2O by the cleavage of an indole ring. During the process, some known functional metabolites are produced such as xanthurenic acid, a molt-inhibiting hormone of crustaceans (Naya et al. 1988; Naya and Sonobe 1990), and niacin and nicotinamide adenine denucleotide which is not efficiently converted from tryptophan in salmonids (Poston and Combs 1980). Another pathway is the serotonin (5-HT) pathway, resulting in a shortening of the side-chain of tryptophan. Especially, 5-HT is known to function as a brain neurotransmitter or modulator in mammals, and it is also chemically or immunohistochemically detected in fish brains (Vivien-Roels and Meiniel 1983; Ekstrom and Veen 1984; James and Wigham 1984; Akiyama et al. 1989). Tryptophan per se is likely to function to increase gamma-globulin, promote protein synthesis in the liver, and enhance resistance against disease (Ariyoshi 1983). Moreover, tryptophan has some physiological effects through intermediating 5-HT, such as anti-stress (Oomagari et al. 1983), promotion of growth hormone and prolactin secretion (Uemura and Kobayashi 1981; James and Wigham 1984), appetite depression (Fernstrom 1985), and suppression of aggressive behavior (Ueki and Shibata 1988). Tryptophan has been already used as the appetite suppressant to restrict food consumption and as a sedative to control people's stress. In livestock, it is used as a sedative for broilers and layers during rearing and for swine during transportation to the butchery. Besides, it can function to make good sow milk and to alleviate fatty livers in fowl. The effects described above are attributed to elevating brain 5-HT level, which can be artificially induced by administrating 5-hydroxy-tryptophan (5-HTP), a direct precursor of 5-HT (Mashiko et al. 1982) and feeding a diet containing high levels of carbohydrate (Fernstrom 1985) in mammals. Also, in fish, the brain 5-HT level of chum salmon fry can be controlled by tryptophan or 5-HTP levels or by adding p-chlorophenylalanine, an inhibitor of 5-HT synthesis, etc. (Akiyama et al. 1986b, 1989). Akiyama et al. (unpublished data) have observed that 0 year blue-fin tuna fed a diet with 0.03% tryptophan on a dry dietary basis shows a considerably higher survival rate and feed efficiency with slightly lower growth and feeding rate. Their swimming behavior was relatively slow in action, compared to the control fish. Thus, the physiological effects of tryptophan have been gradually proved even in fishes, and there is a recent trend toward practical use of tryptophan in some portions of the fish farming industry of Japan.

III. Conclusion

Amino acid requirements of most fishes may be speculated from the amino acid composition of whole body protein in each fish species. Dietary requirements for unknown species can be determined in this way, as Wilson (1989) has suggested. However, we should not ignore the gaps in information that exist for amino acid requirements in salmonids and catfish. Further studies on arginine requirements and metabolism are needed, including the biosynthetic ability of arginine and species-specific necessities of the me-

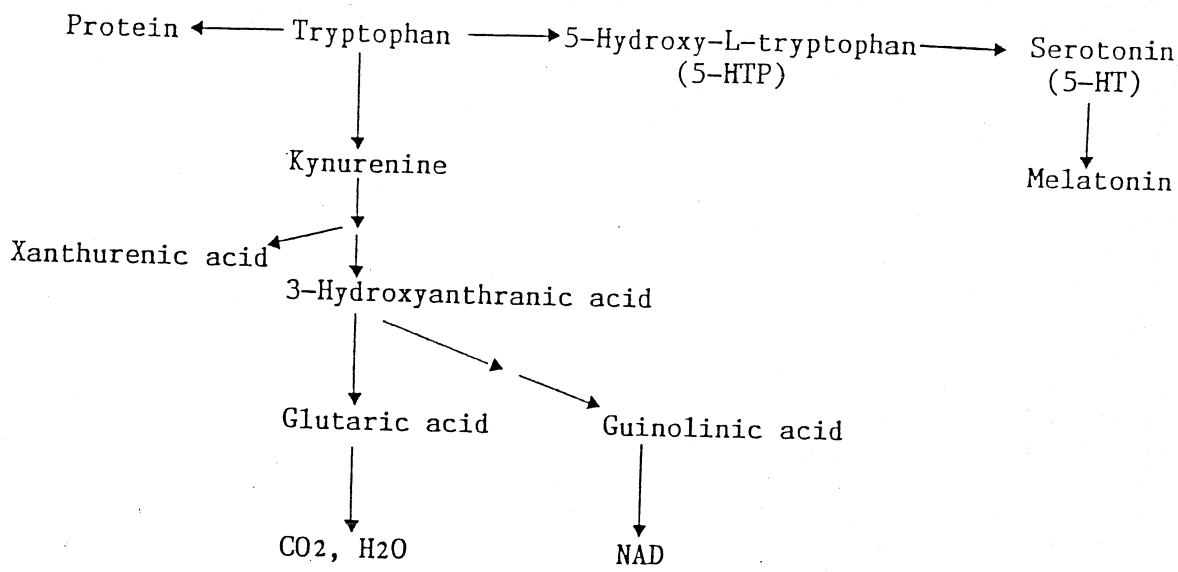

Figure 3. Map of tryptophan metabolism

tabolites for salmonids. In addition to determining the actual levels needed, not only fulfilling of any particular amino acid, the balance between each amino acid is very important. Therefore, A/E ratio of requirement is likely to be a particularly useful parameter for the evaluation of amino acid nutritional value in diet.

The addition of a little excess of certain amino acids may bring about positive physiological effects on fish and facilitate their culture. Tryptophan, producing many functional metabolites including 5-HT, is the most promising amino acid.

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AMINO ACID NUTRITION OF FISH: A NEW METHOD OF ESTIMATING REQUIREMENT VALUES

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ABSTRACT

Certain aspects of amino acid nutrition and metabolism in fish appear to differ from those observed in other animals. Fish are normally fed diets containing two to four times as much protein as other vertebrates. This observation has led certain investigators to suggest that the efficiency of protein utilization is lower in fish than other animals. However, when one compares several parameters relating protein intake to growth rate of fish and other vertebrates, the results are very similar. A similar comparison indicates that the lysine requirement of channel catfish, chickens , and swine is also very similar.

Recent developments in amino acid nutrition of swine have led to the use of the concept of an ideal protein as a means of expressing amino acid requirements of swine. The ideal protein concept is based on the idea that there should be a direct correlation between the whole body amino acid pattern of the animal and the dietary amino acid requirements of the animal. A comparison of the amino acid requirement values previously determined in my laboratory for channel catfish by conventional means and those estimated based on the ideal protein concept show excellent agreement. We are currently evaluating the use of this ideal protein concept as a means of estimating the amino acid requirements of fish.

Certain aspects of amino acid nutrition and metabolism in fish appear to differ from those observed in other vertebrates. Fish are normally fed diets containing two to four times as much protein as other vertebrates. This is because the optimal dietary protein level required for maximum growth of various cultured fish ranges from about 30 to 55% crude protein (see Bowen, 1987; Tacon and Cowey, 1985; Wilson, 1989). This observation has led certain investigators, including me, to suggest that the efficiency of protein utilization is lower in fish than in other animals.

Tacon and Cowey (1985) first noted that the dietary protein requirements of fish are not that dissimilar from those of other vertebrates when expressed relative to feed intake (grams of protein per kg body weight per day) and live weight gain (grams of protein per kg live weight gain). Bowen (1987) has compared several parameters relating protein intake to growth of fish and other vertebrates and found very little difference in protein utilization among the species compared (Table 1). The data used to make this comparison included median values for 18 studies for fish and 8 studies of other vertebrates, i.e., calf, chicken, lamb, swine, and white rat. The only parameters that differed significantly were the level of protein in the diet required for maximum growth and the food conversion efficiency. When the protein requirement data were recalculated to correct for differences in relative protein intake and growth rates, as suggested by Tacon and Cowey (1985), the resulting data were very similar for fish and other vertebrates. This indicates that the efficiency of protein utilization is very similar among the species compared.

The complete quantitative amino acid requirements have been established for only five species; namely, the chinook salmon, common carp, Japanese eel, Nile tilapia, and channel catfish. A limited number of requirement values have also been reported for chum salmon, coho salmon, sockeye salmon, rainbow trout, gilthead bream, European sea bass,

Table 1. Comparison of Various Parameters Relating Protein Intake to Growth of Fish and Other Vertebrates

Parameter	Fishes	Other Vertebrates
Specific growth rate	2.765	2.445
Protein in diet (%)	40.3	20.0
Protein intake at maximum growth (mg protein ingested/g body wt/day	16.5	12.0
Protein retention efficiency (100 x g protein retained/ g protein ingested)	31.0	29.0
Protein growth efficiency (g growth/g protein ingested)	1.945	1.965
Food conversion efficiency (g growth/g diet ingested)	0.78	0.26

Data from Bowen (1987)

and *Tilapia mossambica*. These requirement values have been summarized elsewhere (Wilson, 1989).

Most investigators have used the method developed by Halver and coworkers (1989) to determine the quantitative amino acid requirements of fish. This procedure involves feeding graded levels of one amino acid at a time in a test diet containing either all crystalline amino acids or a mixture of casein, gelatin, and amino acids, formulated so that the amino acid profile is identical to whole chicken egg protein except for the amino acid being tested. This procedure has been used successfully with chinook, coho, chum and sockeye salmon, Japanese eel, Nile tilapia, and rainbow trout. However, the amino acid test diets must be neutralized with sodium hydroxide for utilization by carp (Nose, 1979) and channel catfish (Wilson et al., 1977).

Even though most investigators have obtained a typical dose-response curve by feeding graded levels of each indispensable amino acid, the test diets do not normally support maximum growth. Several techniques have been utilized to access amino acid adequacy and to explain the lower growth rate observed. These studies have been summarized elsewhere (Wilson, 1979).

Some unique amino acid deficiency signs have been described for certain species of fish that have not been observed in other animals. These are summarized in Table 2. It is of interest that bilateral cataracts induced by methionine deficiency have only been observed in rainbow trout (Poston et al., 1977; Walton et al., 1982; Rumsey et al., 1983). The scoliosis and lordosis that have been observed in tryptophan deficient sockeye salmon (Halver and Shanks, 1960), coho salmon (Halver, 1965), rainbow trout (Shanks et al., 1962; Kloppel and Post, 1975; Poston and Rumsey, 1983; Walton et al., 1984) and chum salmon (Akiyama et al., 1985) appear to be related to a depletion of 5-hydroxytryptophan in the body or brain (Akiyama et al., 1986).

Amino acid requirement values for fish are normally expressed as a percentage of diet and as a percentage of dietary protein. Care should be taken when comparing requirement values among species because of the different manner in which the values are reported. For example, the requirements of channel catfish and rainbow trout for certain amino acids appear quite different when expressed as a percentage of diet; however, they are quite similar when expressed as daily intake to support maximum weight

Table 2. Unique Amino Acid Deficiency Signs in Fish

Amino Acid	Species	Deficiency Signs	Proposed Mechanism
Methionine	Rainbow trout	Bilateral cataracts	Unknown
Tryptophan	Rainbow trout Sockeye salmon Coho salmon Chum salmon	Scoliosis	Depletion of 5-hydroxy-L-tryptophan in the brain or body of the fish

Table 3. A Comparison of Selected Amino Acid Requirements of Rainbow Trout and Channel Catfish

Amino Acid	Percentage of Diet		mg/100g body wt/day	
	Trout	Catfish	Trout	Catfish
Arginine	1.80	1.03	28	31
Lysine	2.18	1.50	43	45
Methionine	1.00	0.56	20	20
Tryptophan	0.24	0.12	4.7	3.6

Data from Cho et al. (1985).

gain (Table 3). These data indicate that dietary amino acid utilization is very similar between these two species.

A comparison of the estimated lysine utilization of fish and other animals is presented in Table 4. These calculations were based on studies in our laboratory with channel catfish and data summarized in NRC tables for poultry (National Research Council, 1977) and swine (National Research Council, 1988). It seems quite interesting that when one estimates lysine utilization (g growth/ g lysine ingested) for these three species, with a wide range of relative growth rate and dietary lysine intake, the results are very similar. These data add additional support to the findings of Bowen (1987), indicating that protein utilization is very similar among fish and other vertebrates.

Some variation exists among the amino acid requirement values reported for the various species studied (Wilson, 1989) even when the values are expressed as percentage of dietary protein. It is unclear at this time if these differences represent real differences in metabolic needs among species or differences in the methodology used between laboratories in their assessment. A high degree of correlation has been demonstrated between the indispensable amino acid requirement pattern and the whole body indispensable amino acid pattern in channel catfish (Wilson and Poe, 1985). These researchers also found that the amino acid composition of the whole body of the fish did not change with the size of the fish. The whole body indispensable amino acid composition of five different fish species has been compared and found to be very similar (Table 5).

Table 4. A Comparison of the Estimated Lysine Utilization of Fish and Other Animals

Parameter	Channel Catfish	Broiler Chicken[a]	Swine[b]
Size	10 - 40 g	360 - 725 g	1 - 5 kg
Growth rate (g/day)	0.5	26	200
Lysine requirement (% of dietary protein)	5.0	5.7	5.8
Lysine utilization (g growth/g lysine ingested)	60.6	48.3	57.1

[a]Calculated from data in NRC (1977).
[b]Calculated from data in NRC (1988).

Table 5. Indispensable Amino Acid Composition of Whole Body Tissue of Various Fishes Expressed as g/100 g Amino Acids

Amino Acid	Rainbow Trout	Atlantic Salmon	Coho Salmon	Cherry Salmon	Channel Catfish
Arg	6.41	6.61	5.99	6.23	6.67
His	2.96	3.02	2.99	2.39	2.17
Ile	4.34	4.41	3.70	3.96	4.29
Leu	7.59	7.72	7.49	7.54	7.40
Lys	8.49	9.28	8.64	8.81	8.51
Met + Cys	3.68	2.78	4.76	4.48	3.78
Phe + Tyr	7.76	7.86	7.58	8.21	7.42
Thr	4.76	4.95	5.11	4.63	4.41
Trp	0.93	0.93	1.40	0.83	0.78
Val	5.09	5.09	4.32	4.85	5.15

Data from Wilson and Cowey (1985).

Table 6. A Comparison of the Amino Acid Requirement Values as Determined by Conventional Means and as Estimated Based on the Ideal Protein Concept for Channel Catfish

Amino Acid	Amino Acid Ratio	Determined Requirement	Estimated Requirement
Lysine	100	5.1	---
Arginine	78	4.3	4.0
Histidine	25	1.5	1.3
Isoleucine	50	2.6	2.6
Leucine	87	4.4	3.5
Met + Cys	44	2.3	2.2
Phe + Tyr	87	5.0	4.4
Threonine	52	2.0	2.7
Tryptophan	9	0.5	0.5
Valine	61	3.0	3.1

Cowey and Tacon (1983) have also suggested that there is a relationship between the amino acid composition of fish muscle and the dietary amino acid requirements of fish.

Recent developments in amino acid nutrition of swine have led to the use of the concept of an ideal protein as a means of expressing amino acid requirements of swine. The Agriculture Research Council (1981) has advocated that the concept of an ideal protein containing an ideal balance of amino acids be used for expressing amino acid requirements of swine. The ideal protein concept is based on the idea that there should be a direct correlation between the whole body amino acid pattern of the animal and the dietary amino acid requirements of the animal. In addition, since lysine is normally the first limiting amino acid in most feedstuffs, the requirements for the other indispensable amino acids are expressed relative to the lysine requirement (Agricultural Research Council, 1981; Wang and Fuller, 1989; Fuller, et al., 1989). Thus, if one knows the dietary lysine requirement and the whole body amino acid composition of an animal, then one should be able to estimate the dietary requirement for the remaining indispensable amino acid requirements of the animal relative to the lysine requirement. A comparison of the amino acid requirement values, as determined by conventional means in our laboratory and as estimated based on the ideal protein concept for channel catfish, is presented in Table 6. These data show excellent agreement.

We are currently evaluating the use of the ideal protein concept as a means of estimating the amino acid requirements of fish. If this procedure proves satisfactory, then all one will need to do to formulate diets based on the amino acid needs of a certain fish species is to determine the whole body amino acid composition and the lysine requirement of that specific species. This new procedure should be much less time consuming and less costly than determining amino acid requirements of the fish by conventional means.

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PROPERTIES OF FISH DIGESTION: PARTICULARLY IN THE LARVAL AND JUVENILE STAGE

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ABSTRACT

To develop microcapusulated diets for larvae and juveniles of marine fish such as flounder and red seabream, it is essential to understand their digestive systems during these stages. We are investigating the functional developmental pattern of the pancreas, stomach, and intestine to determine when these organs start to synthesize and secrete proteases. In this paper, we summarize the profile of proteases identified from teleosts and the developmental pattern of the pancreas and stomach in the flounder.

Introduction

The total production of marine fish culture using nets and ponds in Japan was approximately 255.5×10^3 tons in 1990. The major production came from: the yellowtail, *Seriola quinqueradiata*; red seabream, *Pagrus major*; coho salmon, *Oncorhynchus kisutch*; and flounder, *Paralichthys olivaceus* culture, which produced approximately 161.1×10^3, 51.6×10^3, 23.6×10^3, and 6.0×10^3 tons, respectively. Except for the yellowtails for which seeding production is under trial, the juveniles are supplied to fish farmers from public or commercial hatcheries. For example, more than 7×10^7 red seabream juveniles were produced by the private hatcheries in 1990. At present, juveniles of these fish are also released into the open sea to enhance the natural fish stock. Thus, the demands of marine fish juveniles seem to further increase.

The larvae of marine fish other than salmons are usually fed with zooplanktons such as rotifers, *Brachionus plicatilis*, and brine shrimp, *Artemia* sp. To maintain large volumes of zooplankton requires a great amount of time, space, and expense. Therefore, many research groups have been trying to develop microcapusulated diets for the larvae. However, a complete formula has not yet been defined. This is partially due to the lack of understanding of the digestive systems during the larval stage. As a result, an adequate protein source has not been determined. Elucidating the functional development process of larval digestive organs may help to reform the larval feed. We are now carrying out the biochemical and immunohistochemical studies on digestive proteases to determine when the stomach and pancreas start to secrete the digestive enzymes. In this paper, we summarize the features of the digestive system of fish, focusing on the larval and juvenile stages.

Digestive Organs and Proteases

The stomach, pancreas, and intestine are the organs responsible for digesting protein in most fish as is also the case in mammals, except for the stomachless fish such as carp. As for the digestive enzymes, stomach and pancreas proteases have been well characterized biochemically. Pepsinogen, like hog pepsinogen C, has been identified from the bonito, *Kathonus pelamis* (Kubota and Ohnuma, 1970). Among teleosts, most of which have diffused exocrine pancreatic cells into the liver or pyrrolic caecum, eel and catfish are unique in that each has a compact pancreas that runs along the intestine. For these two fish, pure pancreatic tissue can be collected. Various proteases have been biochemically characterized by Yoshinaka et al. (1978, 1982, 1983, 1984a-d, 1985a-f). According to these authors, when the autoactivated pancreatic extract of catfish, *Parasilurus asotus*, was applied to the DEAE- and CM-cellulose columns, approximately 15 protein peaks are eluted in total by the chromatographs. Seven of these peaks have been identified as trypsin, elastase, carboxypeptidase A or B (Fig. 1). In addition, chymotrypsin and collagenase

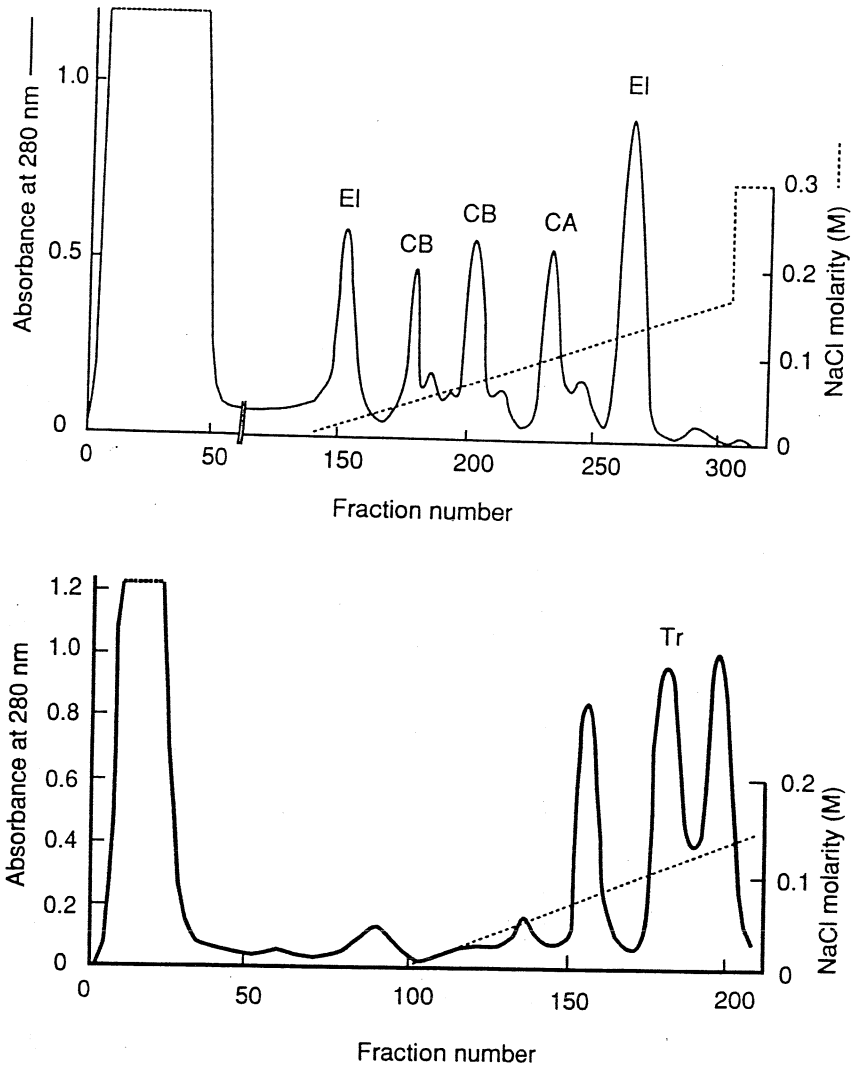

Figure 1. Elution pattern of autoactivated pancreatic extract of catfish, Parasilus asotus, on ion exchange chromatography using DEAE- (A) and CM-cellulose (B) columns. CA, carboxypeptidase A; CB, carboxypeptidase; El, elastase; Tr, trypsin.

activities are detectable from the extract, but their elution pattern has not been examined. Elastase and collagenase solubilize intact the structural protein elastin and collagen molecules. Possession of these enzymes may be related to the carnivorous habit of the fish and make it possible to utilize the proteins as amino acid source. Even though dipeptidase and tripeptidase activities have not yet been examined, it is possible to hypothesize that feed proteins are solubilized to peptones by the hydrolytic activity of pepsin under the acidic condition of stomach. Peptones are then hydrolyzed into amino acids by the pancreatic and intestinal enzymes, which are absorbed by the intestine.

Development of Digestive Organs

During the prelarval stage, the alimentary canal is still a closed duct without mouth or anus openings and where digestive organs have not yet differentiated. Most digestive organs, including the esophagus, intestine, rectum, and pancreas, differentiate during the succeeding prelarval stage. Only the stomach later differentiates when fish metamorphose prior

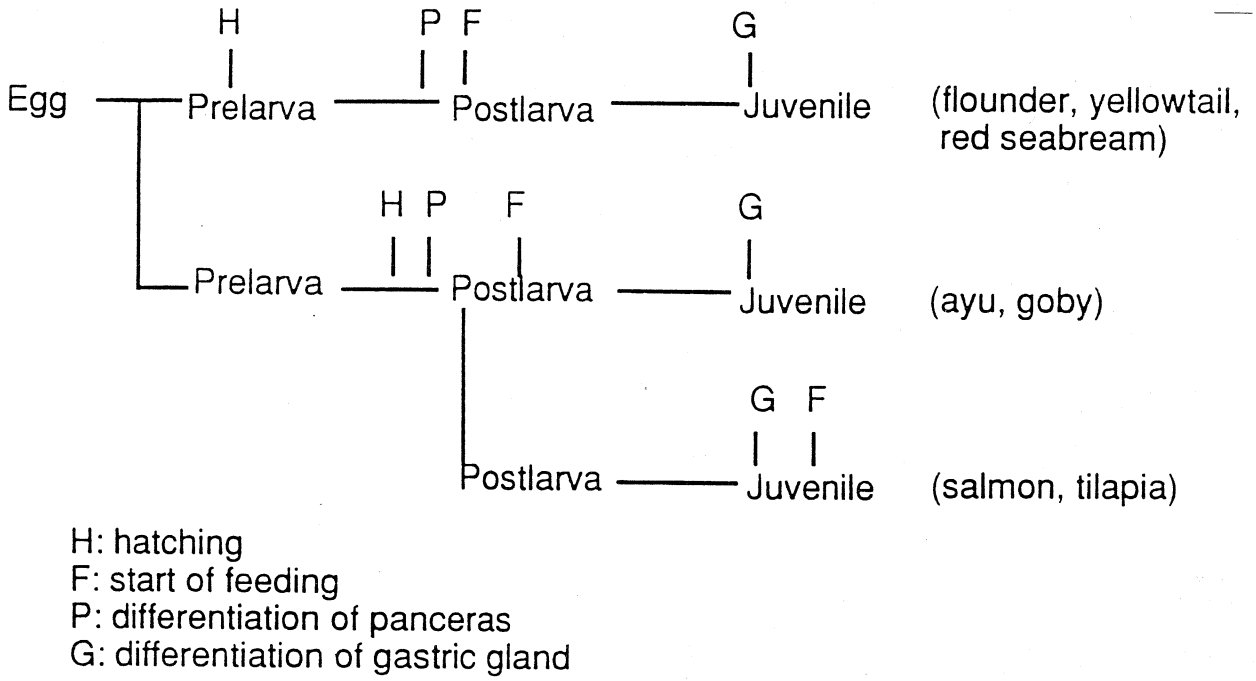

Figure 2. A possible classification of fish based on the times of hatching and the start of feeding.

to the juvenile stage. At this stage, fish become equipped with the digestive organs of adults. Such developmental patterns of digestive organs are likely to be common among teleosts.

On the other hand, the times of both hatching and starting to feed differ among teleosts. It is possible to schematize these patterns into three groups (Fig. 2). In the first group, fish hatch and start feeding before and after the prelarval stage, respectively. The flounder, red seabream, and yellowtail, which are commercially important species in Japan, are included in this group. In the second group, fish hatch in the late stage of the prelarval stage and start feeding during the postlarval stage. Ayu and goby belong to this group. In the third group which includes the salmons and tilapia, larvae continue to develop using yolk mass as a nutrient source and start feeding at the beginning of juvenile stage.

Salmon juveniles have developed adult-type digestive systems at the start of feeding, so that scrambled pellets are fully successful for them from this stage. In contrast, the larvae of both first and second groups have not developed stomachs at the start of feeding and have to digest nutrients without the help of stomachs during the postlarval stage. This process of digestion differs greatly from that of adults. Protein molecules are thought to be pinocytosed by the epithelial cells of the rectum at this stage (Watanabe, 1982). Since tryptic activity is detectable from the postlarvae of ayu, *Plecoglossus altivelis* (Tanaka et al., 1972), the proteins are probably pinocytosed not as intact molecules but as a partially hydrolyzed form by the pancreatic proteases. However, it is not clear to what degree the pancreatic enzymes take part in the digestive process. To develop the microcapusulated diets for larvae, more research needs to be done on the digestive systems of fish.

Appearance of Proteases

Recently, we prepared rabbit antiserum against eel trypsinogen (anti-Trg) and found that it

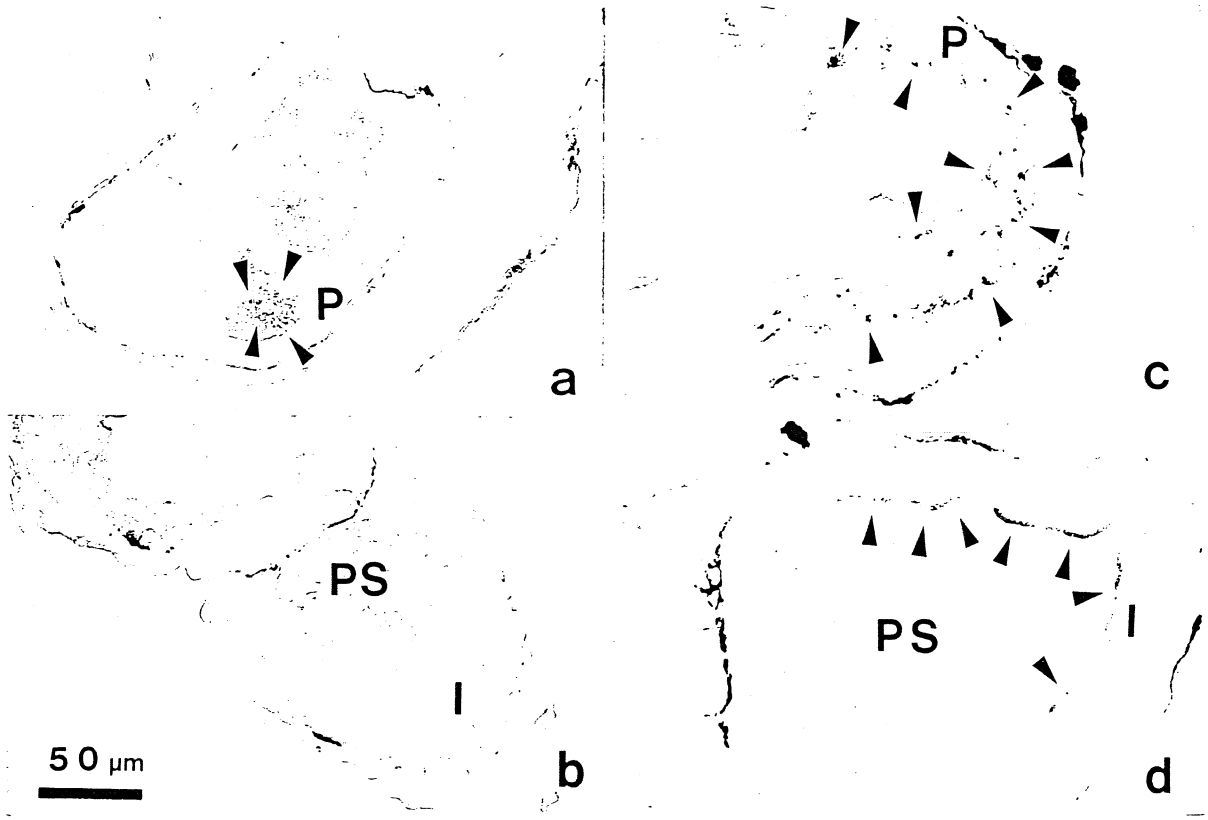

Figure 3. Immunolocalization of trypsinogen and trypsin in pancreas and intestine at the early stages in flounder, Paralichthys olivaceus; a and b: The proteins were immunohistochemically visualized using anti-Trg., the fourth day after hatching. c and d: fifteenth day after hatching. Trypsin secreted from pancreas can be seen along the surface of intestinal epithelium. Arrowheads indicate immunoreactions against anti-Trg. P, pancreas; I, intestine; PS, primordial stomach.

immunoreacts with most teleost trypsinogens and trypsins. Now we are trying to localize trypsinogen and trypsin in the larval alimentary canal of various teleosts using immunohistochemical techniques to determine when the pancreas starts to produce proteases and to secrete it into the intestinal lumen. In this section, we summarize the results obtained on the development of the pancreas as well as the stomach in the flounder larvae.

In immunohistochemistry using the enzyme-labelled antibody method, immunoreaction to anti-Trg does not appear from the alimentary canal of larvae during the lecithotropic stage (1 to 3 days after hatching), indicating that the pancreas has not yet started to synthesize trypsinogen. The trypsinogen appears at the pancreas for the first time on the fourth day after hatching, when the larvae start to feed (Fig. 3a). However, since any immunoreaction to anti-Trg cannot be detected from the intestinal lumen (Fig. 3b), it seems likely that trypsinogen has not yet been secreted from the pancreas. Immunoreaction to anti-Trg is detected from the luminal surface of intestine for the first time at the fifteenth day after hatching (postlarval stage), indicating that the pancreas has just started to secrete trypsinogen into the intestinal lumen (Fig. 3d).

As for the development of the exocrine cells of the stomach, Miwa et al. (1992) reported that pepsinogen appears at the stomach wall at the metamorphosis stage under the control of thyroid hormone.

Considering all of the above results, the developmental process of the digestive system of the flounder can be summarized (see Fig. 4). The pancreatic cells start to synthesize trypsinogen on the fourth day after hatching and secrete it beginning on the fifteenth day. Therefore, until the fifteenth day after the start of feeding, proteins are probably pinocytosed as intact molecules at the rectum. At the fifteenth day, the extracellular digestion starts at the intestinal lumen, where proteins are partially hydrolyzed by the pancreatic enzymes before being pinocytosed at the rectum. Such digestive processes combined with partial

Enzyme	Organ	Age (Days after hatching)							
		1	3	4	7	15	20	30	40
Trypsin	Pancreas	-	-	+	+	+	+	+	+
	Intestine	-	-	-	-	+	+	+	+
Pepsin	Stomach	-	-	-	-	-	-	-	+

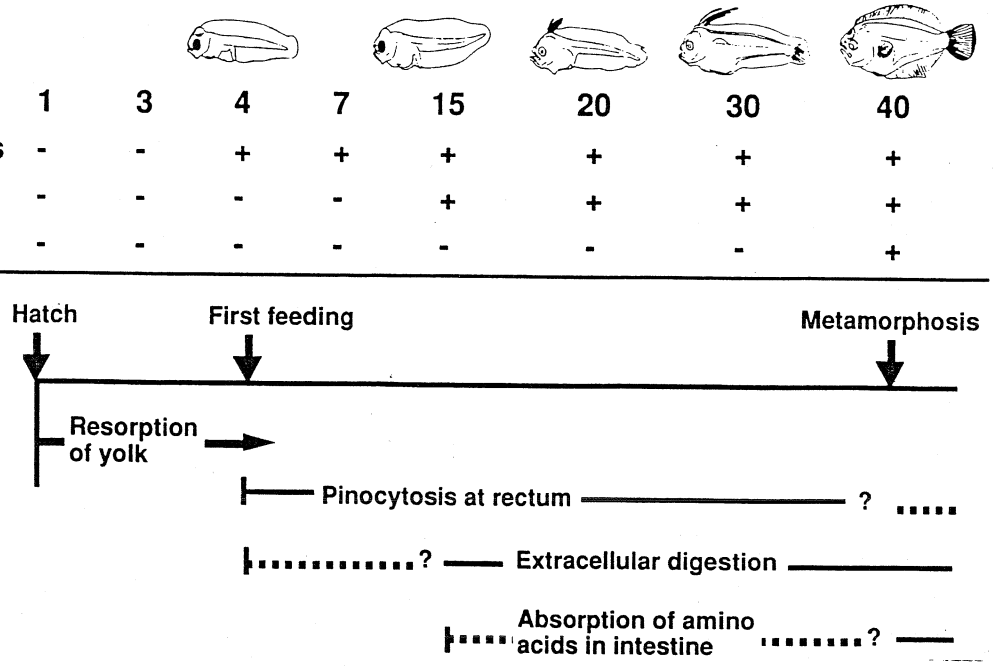

Figure 4. Development of digestive organs and possible systems of protein digestionduring the early stage of the flounder.

hydrolysis and pinocytosis possibly continue to the metamorphosis stage when the secretion of pepsinogen starts. After metamorphosis, it is probable that the proteins are completely hydrolyzed to amino acids. However, the exact time when absorption of protein is switched from pinocytosis to amino acid absorption is not currently understood.

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FEEDING PRACTICES AS RELATED TO AQUACULTURE NUTRITION STUDIES

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ABSTRACT

Experiments designed to help investigators determine the nutritional requirements of aquatic organisms are often difficult to interpret because a large number of variables exist, many of which may not have been considered during the experimental design phase. Results of similar experiments may be quite disparate because of the influence of one or more of those variables. One example is the range in the apparent optimum protein requirement for penaeid shrimp. Reasons for the variability include differences in the dietary ingredients used, method of diet preparation, and manner in which the animals were housed and handled before and during the experiments that are compared. Other, less obvious factors such as ingredient leaching and the differential attractant qualities of dietary ingredients may also affect results. Standardization of nutritional study methods with respect to aquatic animals may be difficult even for a given species, and lacking such standardization, it is critical that the methodology, ingredient analyses, and all other factors that could impact results are carefully recorded and reported.

Introduction

Completed studies aimed at determining the nutritional requirements of aquacultured species number in the thousands. From the literature on a given species or group of closely related species, we can learn such things as that the optimum protein requirement may be between 20 and 70%, an all animal protein diet is efficient, animal protein must be present in the diet, the level of a certain vitamin is 50 mg/kg or 800 mg/kg, and a great many other confusing bits of information. In many instances, the differences in results are undoubtedly due to variable quality of feedstuffs, not only from one nutrient source to another (e.g., casein, casein-gelatin, source of fish meal) but also between batches of the same feed ingredient. In some cases the reasons for variability in results may be related to the manner in which the fish were fed (ad libitum to apparent satiation or based on percentage of body weight daily), feeding frequency (once, twice, several times a day; five, six, or seven days a week), amount and frequency of handling, and the frequency at which feeding rate was adjusted if the fish were not fed ad libitum. In addition, nutrition studies vary with respect to such things as:

1. Duration - fixed time period of weeks (which varies considerably from one study to another) or until the fish have increased in size by some percentage over their initial size (also variable);

2. Initial animal size - research has been conducted on first feeding animals and larger fish ranging from small fingerlings to adults;

3. Rearing history - the animals have been held under quite different rearing conditions from one study to another, and they may have been acclimated to the experimental conditions for variable periods of time before the feeding trial was initiated;

Table 1. Percent Composition of Diets Used to Determine the Growth Response of Channel Catfish Fed Dietary Lipids Ranging From 6 to 14% of the Diet (From Gatlin and Stickney, 1982)

Ingredient	Fish Oil Percentage in Diet				
	6%	8%	10%	12%	14%
Casein	33.7	33.7	33.7	33.7	33.7
Fish oil	6.0	8.0	10.0	12.0	14.0
Corn starch	46.3	41.2	36.0	30.9	25.7
Cellulose	8.1	11.2	14.4	17.5	20.7
Vitamins & mineral	5.8	5.8	5.8	5.8	5.8

4. Acclimation to feed - animals may have been provided a commercial feed up until the initiation of an experiment, or they may have been offered a conditioning diet similar to the experimental diets for some period of time prior to the onset of the feeding trial;

5. Natural food availability - some studies have been conducted in environments where nutrients other than those provided in the experimental diets were available; and

6. Source of ingredients - some ingredients, such as fish meal, vary considerably in proximate composition and nutritional value depending on the source of the ingredient (e.g., species of fish in the case of fish meal) and method of manufacture; thus the source should be specified.

There are a number of other variables among feeding trials that could easily have an effect on the results and, therefore, the interpretation of those results. One variable that cannot be avoided, even when everything else is carefully controlled, involves the water in which the animals are reared. Water quality varies to some extent from one laboratory to another and often varies temporally within a given laboratory. While laboratories used for nutritional studies on mammals can be made virtually identical to one another in terms of the environment to which the animals are exposed, it is not realistic to provide an identical environment in every fish nutrition laboratory. Some standardization may, however, be appropriate.

The list of confounding factors surrounding aquaculture nutrition experiments seems to be almost endless. In the above compilation, nothing was mentioned about palatability; yet some ingredients in experimental diets may impart flavors to the feed that either attract the species being evaluated or cause the animals to avoid one or more of the diets. Feed particle size, texture, color, and the way the feed particles behave in the water may also influence the results of a feeding trial, with different experimental diets having different characteristics. In those cases, the results may be a measure of something other than nutrient composition.

Whenever one attempts to evaluate the requirement for a protein, carbohydrate, or lipid, the array of experimental diets that are manufactured not only varies in the quantity of one ingredient, but, if the diets are to be isocaloric and/or isonitrogenous, must also vary with respect to at least one of the other energy-bearing components. For example, in the diets shown in Table 1 that were designed to evaluate the growth response of channel catfish to graded levels of dietary lipid, one could easily make the case that the experiment actually evaluated the response of catfish to different levels of carbohydrate.

Aquaculture nutritionists have commonly been guilty of employing 'control' diets that were not defined. While not as common today as it was a decade or so ago, a large number of studies have used closed formula commercial feeds as the ration to which the experimental diets were compared, rather than using an available open formula feed or an experimental formulation of proven reliability. In reality, to be even more comparable, the open formula feed should be prepared in the same laboratory that prepares the experimental diets so that the same manufacturing techniques are employed. All of the ingredients should not only be clearly specified (e.g., 'steam-dried menhaden meal' instead of 'fish meal')

but the same batch of each ingredient should be used in each of the experimental diets that incorporate that ingredient.

It should be obvious from the above that it will be virtually impossible to develop a protocol for nutritional studies that will obviate all of the concerns, even with respect to a given species, let alone all animals of interest to aquaculturists. However, it is incumbent on aquaculture nutritionists to be aware of the problems and to design their research to avoid as many of them as possible.

Problems associated with the design and conduct of nutritional studies are not the sole province of the aquaculture community. Researchers working with terrestrial animals have experienced the same difficulties and frustrations. With respect to the establishment of dietary nutrient requirements, Baker (1986) stated the following:

> A nutrient requirement must be defined in terms of a specific criterion of response for animals of a given age, weight, sex and body composition. The experimental diet employed must be carefully defined in terms of protein source and level, energy source and level and a multitude of biological availability factors, both negative (e.g., phytate and fiber) and positive (e.g., anabolic bioactivity).

The same author also made the point that existing levels of nutrients in body stores can profoundly affect estimated nutritional requirements. Note that our compilation of potential problems did not mention that importance of age, weight, sex, and body composition, all of which can make a difference in the results obtained with aquatic animals.

Many aquaculture nutritional studies take into account the concerns expressed by Baker (1986). In dealing with aquatic species, we must add some things to the list of cautions. For example, leaching of dietary nutrients is not typically a problem for nutritionists working with terrestrial animals. The following sections of this paper look at the difficulty involved in determining the nutritional requirements of certain species and provide some suggestions for dealing with at least a few of the problems mentioned above.

Shrimp Protein Requirements

Shrimp are a wonderful model for demonstration of the problems associated with the nutritional requirements of aquatic animals. The interest in shrimp culture over the past few decades has given rise to a large number of nutritional studies. Reviews by New (1976, 1980) provide an excellent overview of the first few hundred studies that were conducted.

Compounding the other problems associated with studying the nutritional requirements of aquatic animals are the facts that shrimp eat very small particles (tens to hundreds of microns in size) and they eat slowly. Shrimp diets need to be highly water stable since the animals may gnaw on a single pellet over a period of hours. Nutrient leaching can certainly affect experimental results.

For purposes of this discussion, we have limited our evaluation to studies that evaluated the protein requirements of shrimp. Other examples could also be used. Since so much work has been done to determine optimum protein level and source, the literature is ample as well as confusing. Just a few examples from the early studies on shrimp protein requirements will illustrate that point.

Increases in dietary protein from either casein or de-fatted fish meal from 18.3 to 45.8% led to growth increases in *Penaeus monodon* (Lee, 1971). Andrews et al. (1972) felt that the optimum protein level of *P. setiferus* was about 30%, while Shewbart et al. (1973), who used menhaden meal based diets, reported an optimum protein level between 28 and 32% for *P. setiferus*. *P. aztecus* growth was reduced when the dietary protein level was above 40% in a study conducted by Venkataramiah et al. (1975). Zein-Eldin and Corliss (1976) found that a 51.5% protein diet produced the best growth (the experimental diets ranged from 24 to 63% protein) in the same species.

Xu and Li (1988) placed the dietary protein optimum at 40% for *P. orientalis*. *P. indicus* was reported to grow best on a diet containing 43% crude protein (Colvin, 1976), but Sambasivam et al. (1982) indicated that food conversion ratio and growth were high in the same species fed diets containing 60% protein. Survival was higher in shrimp fed 50% protein diets. The optimum protein level reported for *P. merguiensis* appeared to lie between 34 and 42% (Sedgwick 1979).

Optimum protein for *P. japonicus* was also placed at 40% of the diet or possibly higher by Balazs et al. (1973). Other studies have placed the optimum for the same species between 40 and 75% (Deshimaru and Shigeno, 1972; Shigeno, 1975; Deshimaru and Yone, 1978).

Diets ranging in protein content from 25 to 60% were fed to *P. monodon* by Alava and Lim (1983). They found that the 40% protein diet produced the best growth, but that comparable growth was ob-

tained from diets containing 30, 35, and 45% protein. Colvin and Brand (1977) indicated that postlarval *P. californiensis* and *P. stylirostris* required a 44% dietary protein level.

Apparent protein requirement can be influenced by other nutrients in test diets. For example, Teshima and Kanazawa (1984) found that the effects of dietary protein level on the growth and survival of *P. japonicus* varied with the level of dietary carbohydrate but not with lipid.

New (1976) pointed out a number of possible reasons for the variability in protein requirement that had been reported. He concluded that the optimum protein level for penaeid shrimp may be from 27 to 35%, but that a lower optimum may be achievable if the appropriate amino acid balance can be established and maintained in the diet. He indicated that diets with 14 to 15% protein had sometimes been satisfactory.

The source of protein in the diet of shrimp has been the subject of a certain amount of research. Smith et al. (1985) felt that growth of small *P. vannamei* was influenced more by protein level, while protein source (animal or vegetable) was more important in larger shrimp. Soybean meal seemed to be an excellent source of protein for penaeid shrimp in studies by Kanazawa et al. (1970) and Sick and Andrews (1973). The same plant protein led to poor performance in a study conducted by Forster and Beard (1973). Lim and Dominy (1990) found that feed conversion, protein efficiency ratio, and apparent protein utilization did not change when *P. vannamei* were fed diets containing from 0 to 56% soybean meal. A diet containing 70% soybean meal was poorly utilized in that study. Soybean cake was felt to be an acceptable protein source for protozoal *P. japonicus* in a study by Hirata et al. (1975).

Successful commercial shrimp feeds sometimes have crude protein levels below 25%. Such feeds are typically used in ponds where natural food is available. The presence of natural food in the form of fungi and bacteria may also be responsible for some of the variability observed in laboratory studies. When feed pellets sit for periods of hours they can be expected to develop a covering of microflora that may have the ability to convert even the most nutrient-poor prepared feed into living tissue containing a better balance of nutrients for shrimp. Similar microfloral communities often become established on the walls and floor of culture containers, where they obtain their nutrients from those dissolved in the water and from waste feed and fecal pellets. When consumed by shrimp, these micro-organisms, which may not even be apparent to the researcher who religiously cleans the tanks on a routine basis, could provide a source of nutrients that are not present at the proper levels in the experimental feeds. The micro-organisms may be able to manufacture nutrients from precursors in the feed via biochemical pathways that are not available to the shrimp. The interpretation of results may be impacted to a greater or lesser extent. How much a feed pellet serves as a substrate for micro-organisms as opposed to supplying shrimp with nutrients directly could be a very important consideration.

Determination of the nutritional requirements of shrimp is difficult in a controlled indoor environment; while in ponds, where natural food is diverse and substrates for the development of fungi and bacteria are plentiful, the task may become hopeless. Supplementation of pelleted rations with a source of detritus such as rice straw may be a good management strategy for shrimp culture in some situations (Miltner et al., 1983), and even pellets manufactured from bagasse (the remains of sugarcane stalks after crushing) will support shrimp growth in ponds (Wood, 1989). Some of the positive response may be attributable to the consumption of micro-organisms growing in association with what might otherwise have been food sources of nutrition for shrimp.

It is possible to obtain what seems to be reliable nutritional information for shrimp if sufficient care is exercised, and we assume that reproducibility in results is a determining criterion for success. Taechanuruk and Stickney (1982) examined the apparent protein digestibility in diets fed to *Macrobrachium rosenbergii* in a recirculating water system consisting of a 1200-l reservoir, 27 aquaria of 20-l capacity each, and 8 individual 80-l biofiltration tanks. Only one shrimp was stocked per aquarium, so the density in the system was very low. The system was extremely clean, and the freshwater shrimp were fed up to 3 times a day for a maximum of 45 minutes at each feeding. While some contribution to the results from indigenous microflora cannot be ruled out, the study was conducted under the most stringent conditions possible, short of total asepsis, to avoid that influence on the results.

Other Considerations

Our literature review of material published over the past decade provided a surprising paucity of information on such topics as attractants in feeds, palatability, texture, color, frequency of feeding, and so forth. Those topics seem to have had more lip service than actually being the focus of research projects. A few examples of studies that have been conducted, both on vertebrate and invertebrate

aquaculture species, are provided in the following subsections. The review is not exhaustive, nor does it cover all the possible areas and issues surrounding feeding practices. It does demonstrate that the general topic is one that deserves more thought and attention from aquaculture nutritionists.

Nutritional Status

Historically, aquaculture feeding trials were typically conducted by randomly stocking replicated culture chambers each with a given number of fairly uniformly sized animals and immediately placing them on the experimental diets. Little or no acclimation time to the culture system was allowed, and the switch from commercial feeds to experimental diets was precipitous and followed by immediate onset of the experiment. Over the past decade or so, more and more investigators have allowed a period of time, often 1 or 2 weeks, to pass between stocking the fish into the experimental units and the onset of the experiment. Alternatively, the fish to be used in an experiment are pooled for a period of time before being stocked in the experimental units. During the period prior to the onset of the experiment, a conditioning ration of similar composition to the experimental diets is offered so the fish become accustomed to the new formulation. For example, fish may have been fed a commercial feed but will be placed on a casein-gelatin conditioning diet before an experiment. The conditioning diet is typically fed for a period of at least a week or two so that the fish become accustomed to it. Thereafter, the same diet may be used as a control and will be fed to one of the experimental groups. The other experimental diets, which typically differ only in terms of a single nutrient, are offered to the remaining experimental groups. While this change has been widely accepted, there seems to be little literature surrounding the rationale and efficacy of the approach, and it has certainly not been standardized. Interestingly, the nutritional status of aquatic animals used in experiments is not only important in nutritional studies but has also been shown to affect their response to chemical toxicity (Lanno et al., 1989).

Since fish are used in various types of studies where nutritional status may have an influence on results, some type of standardization of pre-test feeding appears to be appropriate. It makes sense not only to provide experimental fish with a conditioning ration but also to use the same feeding regimen that will be used during the experiment. For example, if experimental fish are to be fed twice daily at 3% of body weight and at 1000 and 1800 hrs, the same feeding rate and times should be used during the conditioning period.

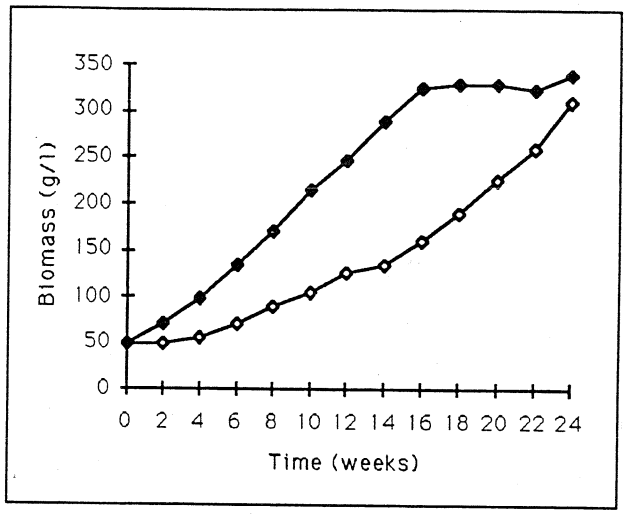

Figure 1. Hypothetical Growth Curves for an Experiment in Which One Group of Fish Received a Complete Ration (Closed Diamonds) and the Other Received a Deficient Ration (Open Diamonds).

Stocking Density

The density at which fish are stocked in experimental units can have a considerable effect on behavior, food intake, and overall performance. Agonistic behavior may be increased or eliminated as a function of stocking density. For example, growth rate of rainbow trout (*Oncorhynchus mykiss*) has been shown to be retarded as stocking density increases (Holm et al., 1990). The opposite response may also occur. Fry Arctic charr (*Salvelinus alpinus*) exhibited less agonistic behavior as stocking density was increased (Wallace et al., 1988).

Density can be presented as a function of numbers or weight of fish per unit water volume. If experimental fish are retained long enough and achieve sufficiently high biomass within a limited capacity experimental chamber, it is quite possible that density limitation will confound the influence of a nutritional variable. That concept is presented graphically in Figure 1. The deficient diet depicted in the figure quickly produced reduced growth in the hypothetical fish. Yet, toward the end of the experimental period, growth becomes asymptotic in the fish fed the complete diet, and the fish receiving the deficient diet almost reach the same biomass by the end of the 24 week study. Analysis of the data could show that the final weights are not significantly different, yet the

fish receiving the complete diet could well have become limited because of excessive density per unit volume of water and because the carrying capacity of the culture system had been reached.

Duration of Feeding Trials

There appears to be a paucity of research aimed at determining how long a feeding trial should be conducted. By convention, and apparently adopted from standard practices established by nutritionists working with terrestrial animals, aquaculture nutritionists have often kept feeding trials going until the fish or invertebrates increased in weight by some specified percentage. In many cases, investigators have set minimums of a few hundred percent (e.g. 500%) increase in body weight of the fastest growing group as the point at which the trial will be terminated. That amount of increase is often utilized in conjunction with fingerling fish and juvenile invertebrates. Typically, the animals will realize the requisite weight increase in from 10 to 14 weeks. Postlarval and fry animals are often fed for a designated period of time that is typically 8 to 14 weeks during which weight gains of up to several thousand percent may be realized. The amount of time required to turn over the body store of a particular nutrient is usually unknown. Assuming that turnover times for different nutrients vary, and that variability may also be related to the size and previous nutritional history of the animal, those rates should be determined for each nutrient. The timing of studies should be adjusted accordingly. We are not aware of instances with aquatic species in which that type of determination has been made. As previously discussed in conjunction with stocking density, the carrying capacity of the culture system should always be kept in mind. It could become a confounding factor as shown in the long duration hypothetical study depicted in Figure 1.

Feeding Frequency, Rate, and Method

The response of aquaculture species to feeding frequency has been investigated to some extent. For many species, frequent feeding is recommended for fry and early fingerlings. In the case of juvenile fish and invertebrates stocked in growout ponds, most experts agree that once or twice a day feedings are sufficient for optimum growth. While that strategy may be generally acceptable, there is some evidence that feeding frequency is positively correlated with density of the culture species (Holm et al., 1990), and for at least one species, milkfish (*Chanos chanos*), increasing feeding frequency from four to eight times daily led to significantly better growth and improved food conversion ratio (Chiu et al., 1987). That result held over two feeding levels (5 and 9% of body weight daily). In another study with milkfish, Teshima et al. (1984) fed a 35% casein:15% gelatin diet at 10, 30, or 50% of body weight a day in one or two feedings to fingerlings and reported that the twice a day feeding rate at 30 or 50% of body weight daily led to best performance.

Red tilapia (*Tilapia mossambica* x *T. nilotica*) fry growth was not significantly different when the fish were fed once, twice, or three times a day for 5 weeks at 10% of body weight daily. Growth of fish in those groups was significantly better than for fish fed at the same rate every other day and a group that was fed ad libitum (Siraj et al., 1988). Silva et al. (1986) had earlier found that optimum feeding frequency and amount varied with fish size in their study with *T. nilotica*. They indicated that performance with size can be maintained in fish of various sizes if the proper combination of feeding level and frequency is used.

In another experiment with *T. nilotica*, Teshima et al. (1986) examined the effects of feeding rate, feeding frequency, stocking density, dietary vitamin level, and a binder on growth using diets with purified protein sources. Weight gain was only significantly correlated with increased feeding frequency (fish fed at 7% of body weight daily performed better than those fed 3.5%). Food conversion ratio was improved in the fish fed at the lower rate. Weight gain, food conversion ratio, and protein efficiency ratio were unaffected by the other experimental variables.

Carlos (1988) fed bighead carp (*Aristichthys nobilis*) at three different feeding rates and one, three, or five times daily and determined that growth was correlated with feeding rate but not frequency. Charles et al. (1984) worked with common carp (*Cyprinus carpio*) and found that feeding frequency was an important factor in the amount of food the fish consumed daily, with three times per day being optimum. (The experiment included feeding frequencies of one, two, three, and six times a day.) Feeding frequency has also been shown to be important in reducing the agonistic effects associated with fish hierarchy in Arctic charr (Jobling, 1983), although as we have already seen, that problem is also related to fish density (Wallace et al., 1988). Thus, standardization of either feeding rate or frequency will be difficult among aquaculture species. Any standards that are imposed in the future will have to be species-specific.

Various methods are used to feed fish. They include hand feeding at a percentage of body weight daily, hand feeding to satiation, automatic feeding of a certain amount of feed daily, and demand feeding. Feeding method for nutritional studies has not been

standardized. Some methods can lead to overfeeding or underfeeding, and others can lead to faulty conclusions based on human error. Distribution of the feed to ensure that all of the population has equal access is another potential problem area, particularly when large populations are being fed. Agonistic behavior is also an important consideration that may be enhanced or mitigated against depending on the feeding method that is employed. Hand feeding may represent the best technique, if the individual responsible for feeding the fish can feed objectively to apparent satiation or feeds at a given body weight percentage daily. Feeding in one of those manners can lead to less waste and the calculation of feed conversion ratio can be made with confidence.

Palatability

Some feedstuffs, and in particular, some purified ingredients, may be poorly accepted by aquatic animals. If there is a difference in palatability, growth differences between diets may be related to that factor rather than to the nutrient composition differences in the diets. Careful observation of the animals to determine that all diets are being consumed similarly by the experimental animals should be practiced at a minimum, although the determination of palatability and implementation of paired feeding trials is recommended.

The two-stage process that is required to rule out palatability as an influence in a feeding trial aimed at determination of the nutritive value of an ingredient should begin with a palatability trial. Treatment groups of aquatic animals should be fed to apparent satiation on each of the experimental diets. The rate at which the least palatable diet is consumed should then be used as the feeding rate for all treatments in the subsequent nutritive value trial.

This strategy may not work well for all aquatic species. Shrimp, for example, break pellets into small particles as previously mentioned. There is at least anecdotal evidence that shrimp may also discriminate between particles of feed they find palatable and particles that are rejected. Since the particles are very small, they may not be accounted for in a palatability trial, and the diets may all appear to be consumed equally based on weighing the pellets that remain after the animals appear to be satiated. To further complicate matters, shrimp may eat over a period of hours and determination of satiation is difficult.

Another factor that has been shown to affect palatability is the presence of certain chemicals in the feed. One example is the use of Romet-30 as an antibiotic in channel catfish feed. Robinson et al. (1990) recommended increasing the fish meal content of medicated catfish feed to 16% (catfish feeds may contain as little as 4% fish meal or other animal proteins) to overcome the acceptability problem associated with the presence of the antibiotic. A similar problem with palatability has been reported in chinook salmon (*Oncorhynchus tshawytscha*) fed diets containing the antibiotic erythromycin (Schreck and Moffitt, 1987).

Color of Feed Particles

Color vision has been demonstrated in fishes (Lagler et al., 1962), and it stands to reason that, at least for some species, the color of the feed might be important to pellet acceptability. Relatively little research information on the relationship with pellet color and feeding behavior was uncovered during our literature search. Jakobsen et al. (1987) fed Atlantic salmon (*Salmo salar*) parr brown pellets, yellow pellets, and a mixture of the two and found that growth was significantly higher on the mixed color ration.

Pellet Shape, Texture, and the Use of Attractants

Training some species of aquaculture animals to accept pelleted feed has been difficult. Not only are species such as largemouth bass (*Micropterus salmoides*) reluctant to accept prepared feeds, there may be differences in training success from one strain of fish to another (Williamson 1983). Even species which will generally accept hard pellets may perform better if shape and texture are optimized. For example, Stradmeyer et al. (1988) fed Atlantic salmon parr pellets of different shapes (round, long and thin, long and fat) and textures (hard and soft) and determined that the fish preferred long pellets over round ones and soft pellets over hard ones. The long-term significance of that type of preference, particularly in instances where the fish are not given a choice, remains to be determined.

Attractants of various types have been added to feed as a means of stimulating feeding activity in finicky species. Training of largemouth bass has been a problem for fish culturists for many years. Lovshin and Rushing (1989) found that training of that species to moist diets was easier than getting them to accept dry diets. Coating dry feed with a commercial product used to attract fish to lures increased trainability to the dry feeds, but there was no change in acceptability with respect to the moist diets when the attractant was added.

Extracts from natural foods and a mixture of synthetic amino acids served as attractants to the glass eel phase of first feeding European eels (*Anguilla anguilla*) in a study conducted by Kamstra and Heinsbroek (1991). Those authors also showed that

feeding level was an important factor. At relatively high feeding levels, the attractants played no significant role in increasing feeding behavior. A sulfonium compound, dimethyl-beta-propiothetin, was shown to promote striking behavior in certain species of freshwater fishes in a study conducted by Nakajima et al. (1989). In a follow-up study, Nakajima et al. (1990) found that certain dietary levels of the same compound would increase the growth rate of red sea bream and yellowtail. A number of peptides have also been shown to attract abalone and yellowtail (Harada, 1989). Abalone can also be attracted to a variety of extracts from brown algae (Harada and Akishima, 1985).

Evaluating extracts from the natural foods of aquaculture species to determine whether attractants exist has not been limited to abalone. Gilthead sea bream (*Sparus aurata*) have also been attracted to extracts of certain invertebrates (Tandler et al. 1982). For species that do not aggressively attack pelleted feeds, such attractants may be required for maximum growth.

Replication and the Culture Environment

While it does not have a direct bearing on the performance of animals in a feeding trial, the number of replicates used in nutritional experiments should be sufficient to allow for proper statistical analysis of the data collected. In many instances, ANOVA is used to determine if growth, food conversion ratio, and survival are significantly different from one treatment group to another. The only proper way to determine mean values for use in the ANOVA test is to use the average value for a group of fish or invertebrates reared within an experimental unit; e.g., an aquarium, tank, raceway, or pond. To have sufficient degrees of freedom, a minimum of three replicates should be provided for each treatment. Thus, to evaluate 7 experimental diets, a total of at least 21 identical culture chambers are required.

Culture chambers of different sizes and shapes should not be used within an experiment, and all of the culture chambers should receive identical flow rates from the same water supply and be otherwise treated identically. There should be no variation in temperature, amount of ambient light, or other physical differences from one culture chamber to another. Handling strategy should not vary from one culture chamber to another nor should exposure to human activity.

Other Factors

A host of environmental considerations in addition to those discussed under various subsections above can influence how fish respond in an experiment. One of those considerations is the shape of the experimental chambers in which the animals are grown. Degani and Levanon (1984) found that elvers of the European eel maintained in round indoor tanks grew more rapidly but had lower survival than elvers maintained in rectangular indoor tanks. Cannibalism was more frequently observed in round than in rectangular tanks. In the same study, mortality was higher in outdoor tanks as compared with indoor tanks, so location relative to cover is another consideration.

In addition to tank shape, background color can have a major role in the performance of certain culture species. Other factors that have been mentioned include food particle size and the method used to dry pellets in conjunction with shrimp diets (Teshima and Kanazawa, 1983), feed particle size and growth of Arctic charr (Tabachek, 1988) and Atlantic salmon (Gaignon et al., 1982), and fish sex with respect to various species including tilapia (Bondari, 1982) and catfish (Lee, 1991). Substances used as pellet binders have been shown to affect digestibility in such species as rainbow trout (Storebakken and Austreng, 1987), although in many cases binders are routinely added without consideration to such an effect.

Various studies on the efficacy of different binders have been conducted in recent years, but little attention has been given to the possible effects of those binders on growth and survival of the animals under study. Most of the studies aimed at finding suitable binders have been conducted in conjunction with shrimp because they feed so slowly (Boonyaratpalin, 1981; El-Dakour and George, 1982; Bautista et al., 1989).

For some species, notably marine aquaculture animals, successful first feeding has only been accomplished to date by providing live food. At some point in the culture process, many species can be weaned from live to prepared feeds, and that conversion is considered to be a critical step in aquaculture by many researchers. As only one example from among many that could be provided, Tuncer et al. (1990) found that hybrid striped bass fry could survive on a prepared feed only if they were first fed brine shrimp (*Artemia*) nauplii and then converted to the dry ration.

Leaching or volatilization of dietary ingredients from pellets that are allowed to stand in water for even several minutes has long been considered a factor in nutritional studies with aquatic animals. Similarly, some nutrients can be affected and even destroyed during feed manufacture. One nutrient that has received considerable attention is vitamin C. Ascorbic

acid is known to be heat labile, and most feed formulations that undergo extrusion pelleting have been overfortified by 400% or more of the known requirement when vitamin C was supplied in the form of ascorbic acid. Differences in processing temperatures and other factors associated with manufacture still led to significant variations in the amount of vitamin C in the finished product.

Some relief from this problem came when protected forms of vitamin C became available. Grant et al. (1989) demonstrated that L-ascorbyl-2-polyphosphate is much more stable than ascorbic acid under the same thermal conditions. Ascorbate-2-sulfate is another form of vitamin C and is much more heat stable than ascorbic acid (Schuep et al. 1989). Ascorbate-2-sulfate is more stable than glyceride-coated ascorbic acid or the sodium salt of the acid (Soliman et al. 1987). Many aquaculture nutritionists maintain experimental diets under frozen conditions throughout each experiment to protect the ingredients from degradation.

Pelleting conditions should be the same for all diets used in an experiment, and, in fact, standardization through interlaboratory calibration might be a good idea. Viola et al. (1985) warned that pellets containing the same ingredients that have been exposed to different temperatures during manufacture can produce different growth results in fish.

Extruded pellets are often preferred by fish farmers and researchers because they are water stable and float, assuring the culturist that the fish are actively feeding and making ad libitum feeding easier. The manufacturing technique can have an influence on consumption rate, however. Hilton et al. (1981) found that when rainbow trout were fed extruded pellets, gastric emptying time was prolonged in comparison with fish fed pressure pellets made in the presence of steam. Those authors also indicated that extrusion processing may increase the bioavailability of dietary carbohydrate, which can lead to impaired liver function in trout but might be beneficial for certain other species.

A Need for Standardization

The various factors discussed above do not represent a complete list but are merely representative of variables that can affect the results of aquacultural nutrition experiments. Also, the references cited represent only a sampling of the number of studies that could have been used as examples.

From what we have seen, it would not seem possible to develop a set of standards that can be applied to all aquatic species being studied by aquaculture nutritionists. Some problems are clearly more critical with respect to one species or species group than to another. Water stability of pellets is certainly more important for shrimp than it is for catfish or trout, for example. Yet, the nutritionist should be aware of how ingredients, manufacturing techniques, storage of feed, method of feed presentation, frequency of feeding, age of the culture animals, and so many other factors can affect results.

Examination of the literature does not provide sufficient information for the formulation of hard and fast recommendations for most of the variables that are discussed above. At this point we can only reiterate that the various factors associated with feeding practices that can affect animal performance always need to be considered as studies are designed and implemented. Critical for proper interpretation of results is the need to carefully record all experimental conditions; details of feed formulation and manufacture; age, size, nutritional plane, and source of stock of the animals used; and any other type of information that might affect the experiment.

At some time in the future it may be possible to standardize nutritional experiments, at least by species, for aquatic animals. At this time it does not appear possible to do so. The number of variables present in any experiment with aquatic animals is prodigious. While we cannot control all of them, it is incumbent upon us to report the conditions that existed with respect to our experiment in sufficient detail that another investigator can repeat the study with a very high probability that the same trends in results will be obtained. At the present time, repetition of an experiment often leads to confusion rather than confirmation.

If standardization of experimental protocols is to be accomplished, aquaculture nutritionists should consider holding an international workshop on the topic during which a series of recommendations could be established. Aquaculture nutritionists associated with the UJNR could take the lead in developing such an international workshop.

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METABOLISM OF DIETARY CRYSTALLINE AMINO ACIDS IN TILAPIA

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ABSTRACT

Metabolism of dietary amino acids in relation to growth performance was investigated in the tilapia, *Oreochromis niloticus,* fed two types of diets with the same amino acid profile as casein, a protein-amino acid diet containing a mixture of casein-amino acid (1 : 1) (CA-diet) and an amino acid test diet (AA-diet) as a nitrogen source. Four test diets, CA-diets and AA-diets with either agar or carboxymethylcellulose (CMC) as a binder, were prepared. AA-diets gave a poorer growth rate than CA-diets for the tilapia, regardless of the kind of binder, agar or CMC.

Tracer experiments were conducted to examine the loss of amino acids into water during feeding and incorporation of the absorbed amino acids into body protein. The loss of dietary amino acids was less in both CA- and AA-diets with agar than it was in those with CMC. Especially, more than 85% of the dietary amino acids was lost into the water in the fish receiving the AA-diet with CMC. There was no difference in the loss (%) of total radioactive amino acids into the water between AA- and CA-diets with agar, but the former showed much more loss of leucine, valine, threonine, and glycine into water than the latter did. In the fish receiving the diets with agar, the incorporation of the ingested [^{14}C] amino acids into body proteins 24 hours after feeding was also lower in the AA-diet than in the CA-diet. A similar tendency on the protein synthesis was also observed in the fish receiving AA- and CA-diets with CMC.

The results of the present study indicate that the low nutritive value of AA-diet with CMC for the tilapia are due not only to the leaching of dietary amino acids but also to the low incorporation of ingested amino acids into body protein. The AA-diet with agar was conceived to give the poorer growth performance because of the low utilization of ingested crystalline amino acids for body protein synthesis.

Introduction

In contrast to the salmonids (Klein and Halver, 1970), the fish and crustaceans such as the carp (Aoe et al., 1970), catfish (Dupree and Halver, 1970), tilapias (Mazid et al., 1978), and prawns (Cowey and Forster, 1971; Deshimaru and Kuroki, 1975) grow poorly on an amino acid test diet that contains crystalline amino acid mixture with the same amino acid profile as that of a protein such as casein. Various reasons for the inability of the amino acid test diet to support the growth of these animals have been suggested: (1) low pH value of the diet (Nose et al., 1974; Murai et al., 1983) and inadequate level of dietary electrolytes (Murai et al., 1983); (2) the short intestinal retention or rapid absorption of amino acids (Tanaka et al., 1977a; Yamada et al., 1982); (3) amino acid imbalance in the tissues due to the difference in rate of intestinal absorption among free amino acids (Tanaka et al., 1977b; Murai et al., 1981); (4) stimulated amino acid catabolism; (5) increased excretion of dietary amino acids (Murai et al., 1984); and (6) the loss or leaching of dietary amino acids during mastication (Yamada and Yone, 1986). However, the utilization and metabolism of free amino acids in these animals have not been well established.

Table 1. Composition of Test Diets (g/100g diet)

Ingredient	Diet			
	AA-Agar	CA-Agar	AA-CMC	CA-CMC
Casein (vit.-free)	-	20	-	20
Amino acid mixture	40	20	40	20
Dextrin	30	30	30	30
Pollack liver oil	6	6	6	6
Vitamins[a]	3	3	3	3
Minerals[b]	4	4	4	4
α-Cellulose	11	11	11	11
Agar	6	6	-	-
CMC[c]	-	-	6	6

[a]Halver's recipe (Halver, 1957).
[b]USP XII. salt mixture No. 2 with trace elements (Halver, 1957).
[c]Carboxymethylcellulose (Serogen; Daiichi Kogyo Seiyaku Co., Japan).

In the present study, we intended to clarify the reason why the amino acid test diet shows the low dietary value for the tilapia, *Oreochromis niloticus*. To approach this, tracer experiments using a mixture of [^{14}C]-amino acids along with feeding trials were conducted to compare the leaching of dietary amino acids to body protein synthesis between two types of diets with the same amino acid composition as casein, a protein-amino acid (1 : 1) diet (CA-diet), and an amino acid test diet (AA-diet).

Materials and Methods

Feeding Experiments of Tilapia

Oreochromis niloticus juveniles were provided by a tilapia farm in Kagoshima, transported to this laboratory, and maintained on a commercial carp feed until ready to use. Four test diets (Table 1) with the same amino acid profile as casein were prepared as described by Yamada and Yone (1986). The diets contained equal ingredients except the protein sources and binders: diet AA-Agar, 40% casein + 6% agar; diet CA-Agar, 40% casein-amino acid mixture (1 : 1) + 6% agar; diet AA-CMC, 40% casein + 6% CMC; and diet CA-CMC, 40% casein-amino acid mixture (1 : 1) + 6% CMC. The tilapias were acclimated to the rearing conditions for 1 week and then fed test diets. Feeding trials were conducted under the conditions given in Table 2. The duplicate tanks were assigned for all of the test diets. The weight gain and feed efficiency (FCE) data were analyzed by analysis of variance (Steel and Torrie, 1960).

Radioactive Measurements and Radio-HPLC

Radioactivities were measured with a liquid scintillation counter, United Technology Packard TRI-CAB 460C, using a toluene solution containing 0.6% PPO as a scintillator. Solid samples such as diets, proteins, etc., were solubilized by Soluene-350 (Packard Instrument Co., Ltd., U.S.A.) for 24 hours, added to the scintillator, and then measured with the liquid scintillation counter. Amino acid composition was analyzed by high performance liquid chromatography (HPLC) as previously described (Teshima et al., 1986). The radioactivity distribution of different amino acids was determined by Radio-HPLC using a Shimadzu HPLC LC-3A (Shimadzu, Kyoto, Japan) with a radioactive monitoring system, an Aloka Model CRC-2201 radio-gas analyzer (Aloka, Tokyo, Japan). The instrument was constructed such that the column effluent went to the mass detector and then entered into the system for radioactive measurements. The mass and radioactivity were recorded on the data processor, a Shimadzu Chromatopac C-R4A.

Table 2. Rearing Conditions of the Tilapia

Item	Condition
Body weight of fish	About 4 g
Stock density	10 fish/tank (12L)
Feeding period	30 days
Water temperature	27 ± 1°C
Water supply	120% exchange/day
Diet size	1-2 mm diameters
Feeding rate	Ad libitum feeding
Feeding frequency	Once/day

The retardation of radioactive peaks from mass peaks due to instrument origin was corrected in the radioactive monitor tracing. In most cases, the specific activity of material chromatographed was high enough to give a radioactive peak.

Preparation of Diets Containing Crystalline [^{14}C] Amino Acids

A mixture of [^{14}C] amino acids was obtained from New England Nuclear, U.S.A., and contained the following amino acids (kBq/MBq mixture): L-alanine, 80; L-arginine, 70; L-aspartic acid, 80; L-glutamic acid, 125; L-glycine, 40; L-histidine, 15; L-isoleucine, 50; L-leucine, 140; L-lysine, 60; L-phenylalanine, 80; L-proline, 50; L-serine, 40; L-threonine, 50; L-tyrosine, 40; and L-valine, 80. Radiochemical purities of these radioactive compounds were checked by Radio-HPLC before use. Four test diets containing [^{14}C] amino acid mixtures and the binder, either agar or CMC, were prepared as follows: To about 200 mg of wet diet, the [^{14}C] amino acid mixture was added to give the radioactivity of about 8.5 kdpm/mg diet (Table 3).

Feeding of Diets Containing [^{14}C] Amino Acids

Tracer experiment-1 was conducted to measure feed intake and loss of dietary amino acids during feeding of diets. Prior to the initiation of the experiment, the test animals underwent a 1 week conditioning period during which they adjusted to the test diets and rearing conditions in the radioisotope laboratory. A diurnal light:dark cycle was regulated at 12:12 hours. Each test diet containing [^{14}C] amino acid mixture was given to 3 fish at 1-2% of their body weight and held for 15 minutes in a 500ml-beaker.

Tracer experiment-2 was conducted to clarify the fate of ingested crystalline amino acids when the tilapia were fed diets containing free amino acid mixture alone or casein-amino acids (1 : 1) as protein sources. The fish were fed AA-Agar and CA-Agar diets containing [^{14}C] amino acids for 15 minutes in a similar manner to that of tracer experiment-1, transferred to other aquaria, and then kept for 24 hours at 24-26°C. No food was given to them during the holding period.

Radioisotopic Analysis of the Whole Body and Rearing Water

Radioactivity of diets (kdpm/mg diet) was determined with a liquid scintillation counter after dissolving aliquots of diets with Soluene-350. Diets given (kdpm/3 animals) were gravimetrically quantified by an electric micro-balance. Quantities of diets ingested actually were obtained by subtracting the loss of diets into rearing water from quantities of diets given. Loss of diets into rearing water was determined as follows: All of the rearing water was taken out of the tanks 15 minutes after giving diets and then separated into the filtrate and residue by filtration through glass wool. The sum of the radioactivities of filtrate and residue was regarded as the loss of [^{14}C] amino acids from diets during the feeding.

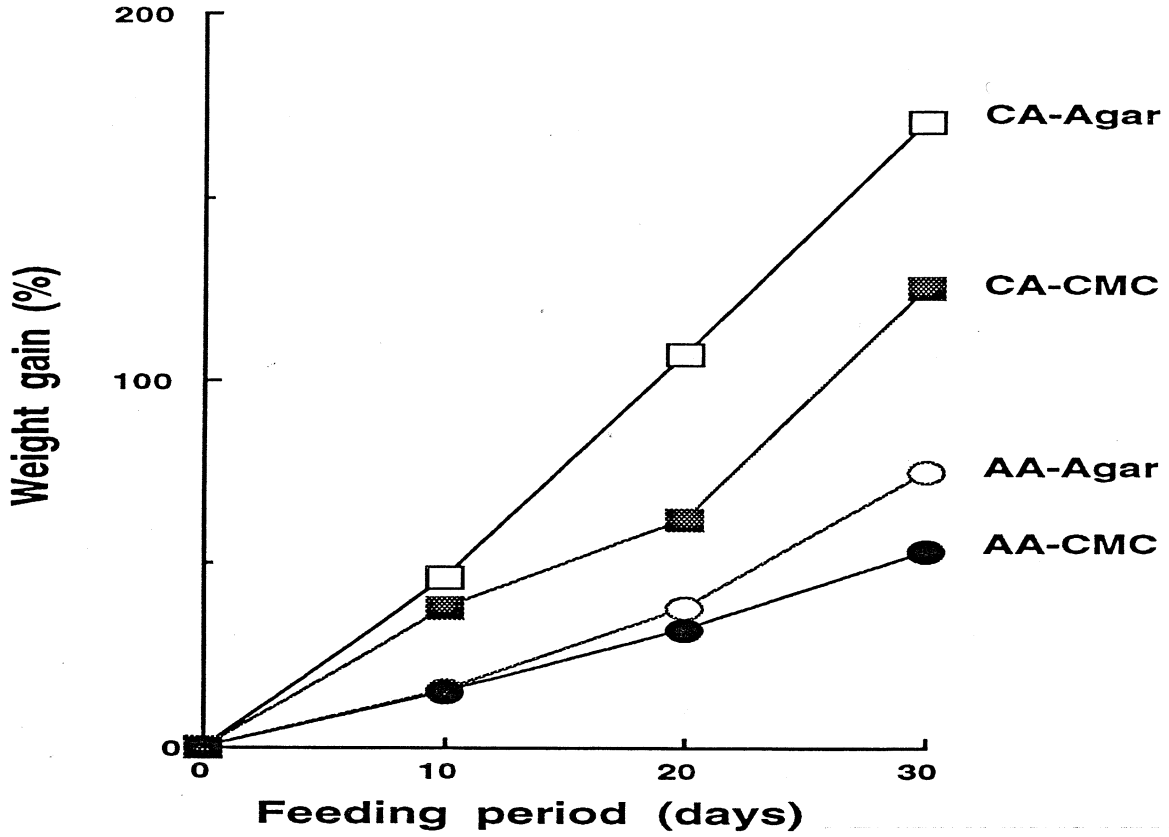

Figure 1. Growth of the Tilapia fed 4 test diets

Table 3. Growth and Feed Conversion Efficiency of the TilapiaFed Amino Acid Casein-Amino Acid (1 : 1) Diets

Diet	Body weight (g) Initial	Final	Weight gain (%)	FCe (%)[a]	Survival (%)
AA-Agar	4.1±0.1	7.2±0.2	75	30	100
CA-Agar	4.1±0.1	11.1±0.5	170	85	100
AA-CMC	4.1±0.1	6.3±0.4	53	15	100
CA-CMC	4.1±0.1	9.2±0.3	125	60	100

[a]Feed conversion efficiency = weight gain (g) x 100 / feed given (g).

Results

The results of the feeding trial are shown in Figure 1 and Table 3. Regardless of the kind of binders, agar or CMC, AA-diets showed lower weight gain and feed conversion efficiency (FCE) than CA-diets ($P < 0.01$). Percentage weight gains from AA-diets with agar or CMC were less than half of those from the corresponding CA-diets. The kind of binders affected the performance of tilapia in both AA- and

Table 4. Ingestion and Fate of Dietary [^{14}C] Crystalline Amino Acids in the Tilapia Fed Amino Acid and Casein-Amino Acid (1 : 1) Diets with Either Agar or CMC as a Binder

Feeding and incorporation of radioactivity	Radioactivity (dpm x 10^3/3 fish/tank)			
	AA-Agar	CA-Agar	AA-CMC	CA-CMC
Diet (kdpm/mg)	(8.39)	(8.61)	(8.57)	(8.68)
Feeding of diets[a]				
Diet given	59.5	60.0	58.9	61.0
Loss of [^{14}C] amino acids	19.1	16.4	51.1	26.6
(% loss)	(32.1%)	(27.3%)	(86.8%)	(43.6%)
Diet ingested				
Whole body (after 24 hr)	4.06	4.52	0.63	2.71
Protein fr.	0.81	3.00	0.36	2.20
Non-protein fr.	3.25	1.52	0.27	0.51
Body retention (%)[b]				
Whole body	10.0	10.4	8.0	7.9
Protein fr.	2.0	6.9	4.6	6.4
Non-protein fr.	7.9	3.5	3.4	1.5

[a]Test diets were given to 3 fish in the beaker (500L) at 23°C for 15 minutes, and the radioactive measurements were conducted to determine the quantities of feeds ingested actually and lost into the water during the period of feeding.

[b]Retention (%) of the ingested radioactivity in the whole body 24 hours after feeding of test diets.

CA-diets, with agar giving the better weight gain and FCE than CMC.

The results of tracer experiments are given in Tables 4 and 5. Among the groups of fish fed 4 test diets, a marked difference was found in the loss (%) of total radioactive amino acids into water during the 15-minute feeding (Table 4).

In the groups fed the diets with agar, about one-third of the administrated radioactivity was recovered from the rearing water, but there was no marked difference in the loss (%) of total radioactive amino acids between AA-Agar and CA-Agar diets. Table 5 shows the composition 1(%) of radioactive amino acids in the rearing water 15 minutes after feeding when the diets with agar were given to the fish. The results showed the loss of essential amino acids (EAA) such as aspartic acid, glutamic acid, proline, alanine, etc., but hardly any leaching of lysine, arginine, histidine, and isoleucine into the water. There was no remarkable difference in the loss of individual amino acids into the water between the two groups of tilapias fed the AA-Agar and CA-Agar diets, except for a slightly higher loss of leucine, valine, threonine, and glycine in the fish receiving the AA-diet.

In the case of the diets with CMC, 43.6% and 86.8% of administrated radioactivity were lost into the water in diets AA-CMC and CA-CMC, respectively (Table 4). Thus, when CMC was used as the binder, particularly in AA-diet, a large amount of dietary crystalline amino acids was lost into the water possibly due to the leaching and/or spilling during the 15-minute feeding. The fish receiving diets with CMC showed the lower incorporation of radioactivity into the whole body 24 hours after feeding than those receiving diets with agar. This is probably a reflection of the considerable loss of radioactive dietary amino acids into the water, especially in diet AA-CMC.

Body retention rates(%) of the ingested total radioactivity 24 hours after feeding were similar

Table 5. Composition (%) of Radioactive Amino Acids in Rearing Water 15 Minutes after Feeding of Amino Acid and Casein-Amino Acid (1 : 1) Diets Containing [^{14}C] Amino Acid Mixture and Agar as a Binder

Amino acid	Distribution of radioactivity (%)[a]		
	Amino acid mixture	Rearing water (after 15 min.) AA-Agar diet	CA-Agar diet
EEA[b]			
Thr	5	9	5
Val	8	11	7
Ile	5	0	4
Leu	14	17	12
Phe	8	7	7
Lys	6	0	0
His	2	0	0
Arg	7	1	1
Non-EEA			
Asp	8	10	27
Ser	4	2	3
Glu	12	15	17
Pro	5	9	7
Gly	4	6	0
Ala	8	8	7
Tyr	4	5	4

[a] Percentage of total radioactive amino acids.
[b] EEA = Essential amino acids.

among the four different fish diets, ranging from 7.9 to 10.4%, but the incorporation of radioactivity into body protein was markedly different among the dietary groups. The two groups of fish fed the diets with agar, AA-Agar and CA-Agar diets, showed a similar incorporation of radioactivity into the whole body 24 hours after feeding, but more than 60% of the radioactivities were associated with the non-protein fraction in the former and with the protein fraction in the later, respectively. These results indicate that dietary amino acids from AA-diets with agar were less utilized by *O. niloticus* for protein synthesis than those from CA-diet with agar. In the diets with CMC, the ratio of radioactive protein/non-protein in the whole body was also higher in CA-diet than in AA-diet, as observed in the diet with agar.

Discussion

The results of the present study on *O. niloticus* juveniles confirmed the earlier observation on other tilapia species that AA-diets sustained poor growth of *Tilapia zillii* (Mazid et al., 1978). A similar finding was obtained not only in the fish such as the carp and catfish but also in the crustaceans such as *Palaemon serratus* (Cowey and Forster, 1971) and *P. japonicus* (Deshimaru and Kuroki, 1974). The carp has been known for a long time not to sustain normal growth on the amino acid test diet containing CMC as the binder (Aoe et al., 1974; Nose, 1979; Plakas et al., 1980; Plakas and Katayama, 1981). Yamada et al. (1981) revealed that increased feeding times per day of the amino acid test diet improved markedly the growth

and FCE of the carp. Plakas et al. (1980) suggested that stimulated amino acid catabolism is responsible for the poor utilization of crystalline amino acids by carp. Whereas, Murai et al. (1984) indicated that poor utilization of crystalline amino acids by carp resulted from excretion of amino acids rather than stimulated catabolism of amino acid absorbed. Also, there are reports showing that the low nutritive values of test diets containing amino acid mixture or casein-hydrolysate as nitrogen sources were mainly due to the leaching of these nutrients into water during mastication (Yamada and Yone, 1986; Nakazoe et al., 1987). All of these suggestions were drawn from the experiments using cold amino acid test diets followed by the amino acid analysis of diets, rearing water, plasma, etc.

The present study demonstrated that by using tracer techniques in diets with CMC the AA-diet probably would give the lower nutritive value for the tilapia, *O. niloticus,* than the CA-diet due to much more leaching of amino acids into the water as well as lower incorporation of ingested amino acids into the body protein. In the case of the diets with agar, however, the AA-diet was conceived to give the lower nutritive value than the CA-diet mainly due to the low utilization of ingested amino acids for body protein synthesis rather than to the leaching of dietary amino acids into the rearing water. Yamada and Yone (1986) showed that about 70% of all dietary amino acids, except cystine and tyrosine, were lost into the water during the mastication within 15 minutes after the feeding when the carp was fed the AA-diet with agar as a binder. In the case of the tilapia receiving the CA-Agar diet, the loss (%) of total amino acids into the water was lower than the case observed in the carp receiving the AA-Agar diet (Yamada and Yone, 1986).

Yamada et al. (1982) have reported that the inferiority of the amino acid diet compared to the casein diet in sustaining growth of the tilapia may be related to the more rapid absorption of dietary free amino acids from the intestines. They suggested that variations in the timing of intestinal absorption of individual essential amino acids may cause an imbalance of amino acids in the body tissue. This may be related to the low body protein synthesis in the tilapia receiving the AA-Agar diet (Table 4). On the other hand, the addition of a small amount of casein (7.7%) to an amino acid diet improved growth and feed efficiency in the carp (Murai et al., 1981, 1982a, 1982b). Yamada and Yone (1986) suggested that such a growth-enhancing effect of casein supplemented to the amino acid diet was because using casein as a binder prevented the loss of dietary soluble nutrients into water. This is also likely in the tilapia fed the CA-diet with CMC, but not in those fed the CA-diet with agar as shown in Table 4.

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EFFECT OF DIETARY pH ON THE UTILIZATION OF CRYSTALLINE AMINO ACID BY SHRIMP (*PENAEUS VANNAMEI*)

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ABSTRACT

A study was conducted to determine the effect of dietary pH on amino acid utilization by juvenile *Penaeus vannamei*. A basal diet (diet 1), five diets (diets 2-6) supplemented with crystalline amino acids (AA) to simulate the AA pattern of shrimp protein and in which the dietary pH was adjusted from pH 4.8 to 5.0, 6.0, 7.0 and 8.0 ± 0.2, and a 28% shrimp protein control diet (diet 7) were fed to shrimp to satiation six times per day for 10 weeks. Growth and feed consumption of shrimp were improved ($P<0.05$) by supplementing AA and increasing the levels of dietary pH. Maximum weight gain and feed intake were obtained when the AA diet was adjusted to pH 8.0 (diet 6). However, the weight gain of this group was only about 85% that of shrimp fed the control diet (diet 7). Shrimp fed diet 7 also had statistically the best feed conversion ($P<0.05$), which was about two-fold better than those obtained with other diets. There were no significant differences among the survival rates of shrimp fed the various diets.

The pellet water stability at 1 and 3 hours was similar for diets 2-6 and diets 1 and 7. However, the dry matter loss of diets 2-6 was approximately twice that of diets 1 and 7. The crude protein losses were negligible for diet 1, 7.0% for diet 7, and 21.2 to 22.3% for diets 2-6. The total essential amino acids (EAA) including cystine, at 1 hour soaking, slightly increased for diets 1 and 7 but decreased by 26.5% for diet 6.

Supplementing AA and increasing dietary pH had no appreciable effect on moisture and crude protein contents of whole shrimp. Body fat increased ($P<0.05$) and ash decreased with increasing levels of dietary pH. Shrimp fed the control diet had the highest contents of crude protein and fat and the lowest levels of moisture and ash.

This study indicates that growth and feed conversion of *P. vannamei* fed the AA supplemented diets were inferior to that of shrimp fed the control diet. This may be attributed to the lower water stability of the AA supplemental diets and the apparent loss of AA from these diets. However, improved growth and feed consumption were obtained with increasing pH values of the AA supplemented diets.

Introduction

Tracer experiments using labeled acetate or glucose have shown that marine shrimp such as *Penaeus japonicus* (Kanazawa and Teshima, 1981), *P. aztecus* (Shewbart et al., 1972; 1973), *P. monodon* (Coloso and Cruz, 1981), and *Palaemon serratus* (Cowey and Forster, 1971) require the same 10 amino acids as fish, rats, and pigs. However, the quantitative requirements of the essential amino acids for shrimp are still unknown.

The amino acid test diets consisting entirely of crystalline amino acids or a mixture of intact protein and amino acid are important tools for the study of

Table 1. Percentage Composition of Experimental Diets

Ingredient	Basal diet (Diet 1)	pH adjusted diets (Diets 2-6)	Control diet (Diet 7)
Squid meal	6.00	6.00	——
Peanut meal	20.00	20.00	——
Amino acid mix[1]	——	15.71	——
Shrimp protein	——	——	32.00
Dextrin	36.30	21.30	28.10
Cod liver oil	6.10	6.10	6.50
Cholesterol	0.50	0.50	0.50
Soybean lecithin	1.00	1.00	1.00
Glucosamine-HCl	1.00	1.00	1.00
Binder[2]	3.50	3.50	3.50
Vitamin mix[3]	1.00	1.00	1.00
Mineral mix[4]	7.50	7.50	7.50
Celufil	16.10	15.39	17.90

[1] See Table 2.
[2] Mixture of 2.5% Kelvis and 1.0% sodium hexametaphosphate.
[3] Supplied the following in mg/kg of diet: vitamin A, 80000 IU; vitamin D_3, 2000 IU; vitamin E, 500; vitamin K, 20; niacin, 300; riboflavin, 60; pyridoxine, 57; thiamin, 60; pantothenic acid, 147; biotin, 2; folic acid, 20; vitamin B_{12}, 0.1; choline chloride, 3000; inositol, 200; and ascorbic acid, 500.
[4] Supplied the following in g/kg of diet: modified BT_m salt mixture, 9.771; $CaHPO_4.2H_2O$, 35.1; K_2HPO_4, 14.5; NaCl, 10.0; $MgSO_4.2H_2O$, 4.0; $CaCO_3$, 1.4; $ZnSO_4.7H_2O$, 0.22; KI, 0.007; Na_2SeO_3, 0.001; $CoCl_2$, 0.001.

protein nutrition. However, it has been reported that juvenile *P. japonicus* (Deshimaru and Kuroki, 1974; 1975; Deshimaru, 1982) and *P. orientalis* (Mai et al., 1988) utilized crystalline amino acids very poorly. Studies at our laboratory to determine lysine and arginine requirements of *P. vannamei*, using amino acid test diets containing a combination of intact proteins and crystalline amino acids to simulate the amino acid profile of 28% shrimp protein diet resulted in poor growth and no differences in weight gains between the lysine or arginine levels in the diets. Likewise, it has been demonstrated that common carp (Aoe et al., 1970; Nose et al., 1974) and channel catfish (Dupree and Halver, 1970; Wilson et al., 1977) showed little growth when fed amino acid test diets. However, Nose et al. (1974) reported improved growth response of common carp by adjusting the pH of amino acid diets to 5 or greater. In channel catfish, Wilson et al. (1977) obtained a significant growth improvement by increasing the pH of the amino acid test diets from 4.35 to 6 or 7.

The objective of this study was to determine the effect of dietary pH on the utilization of crystalline amino acids by juvenile *Penaeus vannamei*. Pellet water stability and losses of dietary nutrients after immersion in seawater were also evaluated.

Materials and Methods

Seven isocaloric diets (Table 1) were formulated to contain 13% and 28% crude protein for diets 1 and 2-7, respectively. Squid meal and peanut meal served as natural protein sources in diets 1-6, and freeze-dried *P. vannamei* tail muscle were used in the control diet (diet 7). Diets 2-6 were supplemented with a mixture of crystalline L-amino acids (Table 2) to provide an amino acid pattern found in the 28% shrimp protein diet. The diets were maintained isoca-

Table 2.Composition of Amino Acid Mixture

L-amino acid	g/100 g diet
Alanine	0.940
Arginine-HCl	2.053
Aspartic acid	1.570
Cystine	0.177
Glutamic acid	1.970
Glycine	1.164
Histidine	0.255
Isoleucine	0.673
Leucine	0.981
Lysine-HCl	1.762
Methionine	0.549
Phenylalanine	0.454
Proline	0.855
Serine	0.383
Threonine	0.679
Tryptophan	0.134
Tyrosine	0.397
Valine	0.714

loric by adjusting the levels of dextrin and cod liver oil. Cholesterol, lecithin, glucosamine hydrochloride, and vitamin and mineral mixes were constant in all diets. Kelvis[1] (Kelco International, San Diego, California) and sodium hexametaphosphate were used as a binder and celufil (U.S. Biochemical Corp., Cleveland, Ohio) as a filler.

The diets were prepared and stored as described by Lim and Dominy (1990) with some modifications. After all dietary ingredients were thoroughly mixed, 6 N NaOH was added to amino acid supplemental diets 3-6 to obtain pH levels of 5.0, 6.0, 7.0, and 8.0 ± 0.2 according to the procedures given by Nose et al. (1974) and Wilson et al. (1977). No pH adjustment was done for diets 1, 2, and 7. The pellets were broken into small pieces and dried in a forced-air despatch oven at 50°C for 2 1/2 h.

Juvenile *P. vannamei* were obtained from the Oceanic Institute, Waimanalo, Hawaii and acclimated for 2 weeks. During this period, they were fed a commercial shrimp feed twice daily. After acclimatization, shrimp of 0.82 to 1.23 g size (average 0.98 ± 0.03 g) were selected, individually weighed, and stocked at a density of 15 shrimp to each of 28 flow-through (0.85 l/min) 55-l glass aquaria. Each aquarium, filled with 52 l of sand-gravel filtered seawater, was provided with a tight-fitting lid, plastic netting shelter, and continuous aeration through an air stone. Any shrimp that died within 72 h after stocking was replaced by a shrimp of similar size.

Each test diet was fed to shrimp in four replicate aquaria six times daily to satiation for 10 weeks. Feeds were offered three times in the morning between 0800 and 1000 h and three times in the afternoon between 1200 and 1400 h. The quantity of feed consumed per aquarium was determined daily.

All aquaria were cleaned daily by siphoning off accumulated waste materials and exuviae. Water flow rates were checked and adjusted daily to insure proper water exchange rate. Water temperature, salinity, dissolved oxygen, and pH were measured in four randomly selected aquaria twice a week. Water temperature ranged from 25.0 to 28.1°C with an average of 26.5 ± 0.7°C, salinity varied from 30.0 to 32.0 ppt with an average of 30.8 ± 0.5 ppt, dissolved oxygen ranged from 5.6 to 8.6 mg/l with an average

[1]Mention of firms or trade products does not imply endorsement by the U.S. Department of Agriculture.

Table 3. Averages of Weight Gain, Feed Intake, Feed Conversion, and Survival of Shrimp Fed Different Diets[1]

Diet	pH (±0.2)	Wt. gain (g)	DM feed intake (g/shrimp)	FCR (DM feed fed)	Survival (%)
1 (Basal)	6.7	1.73[a]	6.02[a]	3.47[a]	96.67
2 (+AA)	4.8	2.47[b]	7.93[b]	3.21[a]	91.67
3 (+AA)	5.0	2.53[b]	7.89[b]	3.12[a]	88.34
4 (+AA)	6.0	3.33[c]	10.46[c]	3.14[a]	90.00
5 (+AA)	7.0	4.05[c]	12.77[d]	3.18[a]	93.33
6 (+AA)	8.0	4.79[d]	15.98[e]	3.34[a]	96.67
7 (Control)	7.0	5.62[e]	8.89[bc]	1.60[b]	83.34

[1] Means in the same column having the same superscript are not significantly different at P>0.05.

Table 4. Water Stability of Experimental Diets[1]

Diet	pH (±0.2)	Pellet water stability(%) at:	
		1 hour	3 hours
1 (Basal)	6.7	91.45[a]	84.74[a]
2 (+AA)	4.8	79.79[b]	70.84[b]
3 (+AA)	5.0	79.43[b]	71.15[b]
4 (+AA)	6.0	78.35[b]	71.39[b]
5 (+AA)	7.0	80.13[b]	70.81[b]
6 (+AA)	8.0	78.81[b]	71.16[b]
7 (Control)	7.0	92.10[a]	85.59[a]

[1] Means in the same column having the same superscript are not significantly different at P>0.05.

of 6.4 ± 0.7 mg/l, and the pH values ranged from 7.2 to 7.8 with án average of 7.5 ± 0.2.

Every 14 days, the shrimp in each aquarium were counted and weighed. When the shrimp were removed for weighing, the aquaria were cleaned thoroughly and drained. On sampling day, shrimp were fed once in the afternoon with 60% of the amount of feed consumed the previous day to minimize cannibalism.

Water stability of pellets was determined at 1 and 3 h, following the method of Dominy and Lim (1991). Moisture, crude protein, and crude fat of diets before soaking (0 h) and after 1 and 3 h soaking in seawater at room temperature were determined using Association of Official Analytical Chemists methods (AOAC, 1980). Amino acid contents were determined by a private laboratory for diets 1, 6, and 7 before soaking and after 1 h soaking. All analyses were performed in triplicate.

At the conclusion of the experiment, shrimp were collected and stored frozen at -30°C for subsequent determination of whole body composition.

Table 5. Percentages of Crude Protein and Fat (Dry Matter Basis) of Experimental Diets Before (0 hour) and After (1 hour) Immersion in Seawater[1]

Diet	pH (±0.2)	Crude protein		Crude fat	
		0 hour	1 hour	0 hour	1 hour
1 (Basal)	6.7	16.88	16.77 (-0.7)	7.78	8.89 (+14.3)
2 (+AA)	4.8	32.09	25.18 (-21.5)	8.17	10.52 (+28.8)
3 (+AA)	5.0	31.92	25.48 (-20.2)	8.23	10.55 (+28.2)
4 (+AA)	6.2	31.79	24.77 (-22.1)	8.33	10.66 (+28.0)
5 (+AA)	7.0	31.83	25.11 (-21.1)	8.39	10.37 (+23.6)
6 (+AA)	8.0	31.54	24.52 (-22.3)	8.65	10.22 (+18.5)
7 (Control)	7.0	32.03	29.79 (-7.0)	8.03	8.93 (+11.2)

[1] Numbers in parentheses represent percentages of nutrient gain or loss.

Chemical analyses were done in triplicate following the same methods previously described.

All data, except for the diet proximate and amino acid composition, were subjected to analyses of variance and Dunnett's test to determine the differences between the treatment means (Steele and Torrie, 1960). Results were considered statistically significant at the 0.05 probability level.

Results

The average final weight gain, dry matter feed intake, feed conversion, and survival rate of shrimp fed different diets are presented in Table 3. Shrimp fed the basal diet (diet 1) had the lowest mean weight gain which was significantly lower ($P<0.05$) than those of shrimp in all other treatments. Crystalline amino acid supplementation to the basal diet significantly improved the growth of shrimp. There was a trend of increased growth with increasing dietary pH, with shrimp fed the diet having a pH of 8 being heavier ($P<0.05$) than shrimp in all other diets with lower pH (diets 2-5). Shrimp fed the control diet (diet 7) had the highest weight gain ($P<0.05$).

Total feed intakes (dry matter basis) reflected the weight gains for diets 1-6. Because of this, the feed conversion values (g dry feed fed/g wet weight gain) for the groups fed diets 1-6 were not different ($P<0.05$). On the other hand, because feed consumption of shrimp fed the control diet was less and the weight more, the resulting feed conversion was approximately half that of all other groups. Survival rates were generally high and ranged from 83.3% for diet 7 to 96.7% for diets 1 and 6. No significant differences were found among the survival rates of shrimp receiving various dietary treatments.

Pellet water stability of the diets measured at 1 and 3 h are given in Table 4. The percentages of dry matter remaining after 1 or 3 h of submersion in aerated seawater at room temperature were nearly the same for diets 1 and 7. The amino acid supplemented diets with pH adjustment (diets 2-6) had similar water stability that was significantly ($P<0.05$) lower than those of diets 1 and 7.

Dry matter percentages of crude protein and crude fat of test diets before (0 h) and after 1 h submersion in seawater are given in Table 5. The percentage of crude protein in all diets decreased after 1 h submersion in seawater. The percentage of protein loss was only 0.7% for the basal diet (diet 1) and 7.0% for the control diet (diet 7). Diets 2-6 lost considerably higher amounts of crude protein (20.2 to 22.3%). The apparent level of crude fat after 1 h of soaking were inversely related to the pellet water stability. Diets 2-6, which had the poorest water stability, gained substantially more fat than diets 1 and 7.

Dry matter percentages of essential amino acids (including cystine) of diets 1, 6, and 7 before (0 h) and after 1 h soaking in seawater are presented in Table 6. There was no difference in total essential amino acid content after 1 h submersion for diets 1 and 7, but amino acids were considerably decreased for diet 6 (26.5%) ranging from 7.1% for cystine to 53.2% for methionine.

Table 6. Essential Amino Acid Content (Percent Dry Matter) of Experimental Diets Before (0 hour) and After (1 hour) Immersion in Seawater[1]

Amino acid	Diet 1 (basal)		Diet 6 (+AA)		Diet 7 (Control)	
	0 hour	1 hour	0 hour	1 hour	0 hour	1 hour
Arginine	1.32	1.34	2.74	2.19 (-20.1)	2.39	2.21 (- 11.3)
Cystine	0.13	0.14	0.28	0.26 (- 7.1)	0.23	0.27 (+17.4)
Histidine	0.39	0.42	0.62	0.59 (- 4.8)	0.62	0.68 (+ 9.7)
Isoleucine	0.58	0.56	1.15	1.00 (-13.0)	1.16	1.23 (+ 6.0)
Leucine	1.08	1.06	1.86	1.51 (-18.8)	2.07	2.13 (+ 3.0)
Lysine	0.66	0.65	1.82	1.32 (-27.5)	2.05	2.25 (+ 9.8)
Methionine	0.20	0.22	0.79	0.37 (-53.2)	0.83	0.77 (- 7.2)
Phenylalanine	0.68	0.68	1.11	0.97 (-12.6)	1.16	1.16
Threonine	0.52	0.50	1.21	0.87 (-28.1)	1.10	1.21 (+10.0)
Tryptophan	0.19	0.20	0.32	0.28 (-12.5)	0.34	0.37 (+ 8.8)
Valine	0.64	0.65	1.28	0.95 (-25.8)	1.21	1.23 (+ 1.6)
Total	6.39	6.42	13.18	10.35 (-26.5)	13.16	13.42 (+ 2.0)

[1]Numbers in parentheses represent percentage gain or loss.

Whole body composition of shrimp expressed in percent dry matter is given in Table 7. Body moisture content was highest for shrimp fed the basal diet (diet 1) and tended to decrease with increasing dietary pH, becoming significantly ($P<0.05$) lower than diet 1 at pH 7 and 8. On the other hand, body fat tended to increase with increasing pH, becoming significantly higher at pH 5 and above. Carcass protein values did not appear to be related to dietary pH. Ash followed a similar trend to that of body moisture, decreasing with increasing pH, becoming statistically significant ($P<0.05$) at pH 6 and above.

Discussion

Supplementation of crystalline amino acids to the basal diet and/or increasing dietary pH levels did not affect the survival rate but significantly improved the growth performance of shrimp. Highest weight gain, which was approximately 85% of that fed the shrimp protein control diet, was obtained with the amino acid test diet adjusted to pH 8. However, this growth improvement was due to increased feed consumption rather than improvement of nutritional value of the diets. Feed efficiency values were nearly the same for the basal and amino acid test diets and were only about 50% of that obtained with the control diet. Nose et al. (1974) reported a growth improvement of common carp by adjusting the pH of the amino acid test diets with sodium hydroxide to 5 or higher. However, the growth was only about 60%, and feed efficiency did not exceed 20% of those on the casein control diet. Wilson et al. (1977) found that it was necessary to adjust the pH of the amino acid diets to 6 to 7 for channel catfish to improve growth and feed conversion, although these parameters remained inferior to those of the whole egg protein control diet.

Akiyama (1991) reported that mixtures of synthetic amino acids are effective attractants for shrimp diets. Moreover, crystalline amino acids are readily absorbed by shrimp since they require no further digestion. Retention time of an amino acid diet in the intestine of common carp has been found to be less than half that of a casein diet (Tanaka et al., 1977). Thus, increased feed consumption of the amino acid supplemented diets observed in this study may be due to improvement in diet palatability and faster rate of absorption. Likewise, Murai et al. (1981) observed that fingerling common carp and channel catfish fed diets with crystalline amino acids as a major nitrogen source consumed more feed than those with casein

Table 7. Whole Body Percentage Composition (Dry Matter) of Shrimp Fed Various Experimental Diets[1]

Diet	pH (±0.2)	Moisture	Crude protein	Crude fat	Ash
1 (Basal)	6.7	78.01[a]	73.53[a]	5.76[a]	15.76[a]
2 (+AA)	4.8	76.39[abcd]	75.07[ab]	5.56[a]	15.31[a]
3 (+AA)	5.0	77.76[ac]	74.53[a]	6.11[b]	14.99[a]
4 (+AA)	6.0	77.17[abc]	74.96[ab]	6.30[bc]	13.85[b]
5 (+AA)	7.0	75.57[bd]	72.47[a]	6.53[cd]	13.64[b]
6 (+AA)	8.0	76.06[cd]	73.57[a]	6.38[bcd]	13.44[b]
7 (control)	7.0	74.58[d]	77.37[b]	6.58[d]	13.39[b]

[1] Means in the same column having the same superscript are not significantly different at P>0.05.

and/or gelatin. They indicated that short retention time of the amino acids may have stimulated feed consumption.

The higher feed consumption rate among shrimp fed diets with higher pH may be due to faster rates of digestion and absorption of amino acids. Previous studies on the proteolytic enzymes in the digestive tracts of penaeid shrimp revealed the absence of peptic activity (Gates and Travis, 1969; Maugle et al., 1982; Lee and Lawrence, 1982; Lee et al., 1984) but the presence of a broad range of alkaline proteases with optimum pH values of 7.0 to 9.5 (Gates and Travis, 1969; Maugle et al., 1982; Galgani et al., 1984; Tsai et al., 1986; Honjo et al., 1990). Higher levels of sodium as a result of increasing dietary pH may also be a possible cause for the increased rate of amino acid metabolism. Chiu et al. (1984, 1987) suggested that dietary electrolytes balance (Na + K - Cl) influenced the growth, feed efficiency, and amino acid metabolism of rainbow trout fed amino acid test diets. However, Wilson et al. (1985) observed that changes in dietary sodium and chloride levels had no effect on growth and feed conversion and metabolism of the amino acid of rainbow trout fed the intact protein diets. Murai et al. (1983) concluded that dietary pH and level of dietary electrolytes or a combination of both factors play a certain role in amino acid metabolism of common carp but suggested that further studies are needed to clarify the role of electrolytes on amino acid metabolism.

The poor feed conversion obtained with the amino acid test diets as compared to that of the control diet may be due to loss of amino acids during ingestion process, since shrimp are very slow feeders. The loss of total essential amino acids was 26.5% for the amino acid test diet whereas that of the control diet increased by 2.0%. The imbalance of essential amino acids resulting from the loss of individual amino acids, such as 53.2% for methionine and 4.8% for histidine, may also have contributed to the poor utilization of the amino acid test diets. Another possible contributing factor is the inability of shrimp to effectively utilize crystalline amino acids as have been reported for *P. japonicus* (Deshimaru and Kuroki, 1974, 1975; Deshimaru, 1982) and *P. orientalis* (Mai et al., 1988). Deshimaru (1982) demonstrated that the rate of assimilation of dietary free arginine into the muscle protein by *P. japonicus* was extremely low (0.6%) compared to that of protein-bound arginine (90.5%). He suggested that the inability of free amino acid to substitute for intact proteins in supporting normal growth of shrimp is probably due to differences in the rate of amino acid absorption. Likewise, Mai et al. (1988) showed that juvenile *P. orientalis* could not absorb supplemental methionine and lysine synchronously with protein-bound amino acids.

Increasing the dietary pH levels had no significant effect on body moisture and crude protein content of shrimp. The shrimp fed the intact protein control diet had the lowest moisture and highest protein content. Percentage of body fat seemed to be directly related to the growth rate, whereas ash content was inversely related to the size of shrimp.

Results of this study clearly indicate that the growth response and feed consumption of shrimp can be greatly enhanced by increasing the dietary level of

pH to 8. This improvement in feed consumption was probably because of improved diet palatability and faster rate of digestion and absorption of amino acids. However, more studies are needed to elucidate the roles of dietary pH and electrolytes on the amino acid metabolism in shrimp.

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APPLICATION OF MICROENCAPSULATION TECHNIQUES FOR THE DELIVERY OF NUTRIENTS TO MARINE SUSPENSION-FEEDERS

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ABSTRACT

Culture of the larval stages of many species of fish and crustaceans, as well as the larval and adult stages of bivalve molluscs, is dependent on the use of live foods, such as algae, rotifers, and *Artemia*. Rapid leaching of water-soluble nutrients from microparticulate diets, physical instability of suspended particles in seawater, and high microbial population development associated with microparticle breakdown are some of the reasons why it is difficult to develop satisfactory artificial diets for many of these suspension-feeding organisms.

Microencapsulation techniques offer an approach that can help overcome some of the problems associated with the delivery of microparticulate diets to suspension-feeders. Microencapsulation can reduce leakage of dietary nutrients from food particles and, therefore, reduce the occurrence of high microbial populations often associated with artificial diets.

Microparticulate Diets for Larval Crustacea and Fish

Microbound Diets

In the last decade, there has been an explosive growth of aquaculture in many countries, with production of some commodities, such as shrimp, increasing by more than an order of magnitude. Paralleling this growth of aquaculture, there have been many exciting advances in the development of microparticulate diets for marine suspension-feeders. Some of these developments were reviewed by Langdon, Levine, and Jones (1985).

Among the most exciting advances is the development of satisfactory artificial diets for larvae of the Kuruma shrimp (*Penaeus japonicus*) by Kanazawa, Teshima, and coworkers, based on the use of carrageenan-microbound diets (MBD; Kanazawa et al., 1982; Teshima, Kanazawa, and Sakamoto, 1982). In addition, Kanazawa, Teshima, and co-workers have developed satisfactory microbound diets for larvae of Milkfish (*Chanos chanos*), Starry Founder (*Paralichthys olivaceus*), and the Ayu (*Plecoglossus altivelis*), although culture of larval Red Sea Bream (*Chrysophrys major*) is still partially dependent on rotifer supplements (Kanazawa and Teshima, 1988). Most commercial artificial diets for shrimp and fish larvae are presently based on microbound diets and involve entrapping nutrients in hydrocolloid particles, such as carrageenan, calcium alginate, or agar.

Microencapsulated Diets

Microcapsules differ from microbound diets in that encapsulated material is separated from the external environment by the capsule wall. Ideally, the walls of capsules designed to deliver artificial diets to suspension-feeders prevent leakage of nutrients and yet can be broken down by feeding or digestive processes of the consumer.

The use of microencapsulation techniques for delivering nutrients to shrimp larvae was pioneered by Jones and coworkers (Jones, Mumford, and Gabbott, 1974). The microcapsule-type used by these researchers was initially a nylon-protein-walled capsule, first described by Chang, MacIntosh, and Mason (1966). In subsequent modifications of the method, nylon and biologically toxic detergents were eliminated from the capsule preparation method (Jones, Kurmaly, and Arshad, 1987). In the last decade, this technique has been used in commercial products (e.g., Frippak Feeds). Commercially produced en-

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capsulated diets have improved to the point where it is apparently possible to completely replace live foods with microencapsulated diets for shrimp larvae, with no significant reduction in larval survival and growth, compared to that of larvae fed on live foods (Kurmaly et al., 1989).

Artificial Diets for Bivalve Molluscs

Mixed-Particle Diets

Research on artificial diets for bivalve molluscs has lagged behind that for shrimp and fish larvae, mainly because bivalve molluscs require particles that are about an order of magnitude smaller than those required for shrimp and fish larvae. With decreasing food particle size, problems associated with nutrient leaching become more severe.

Langdon and Seigfried (1984) and Langdon and Bolton (1984) described an artificial diet for juvenile American oysters (*Crassostrea virginica*) that was made up of three particle types: (1) alginate-bound microparticles containing high-molecular weight, water-soluble nutrients (proteins, complex carbohydrates, and nucleic acids); (2) lipid-walled capsules containing low-molecular weight, water-soluble nutrients, with potentially required lipid nutrients added to the wall material; and (3) kaolin particles.

Growth of oysters fed on the mixed-particle diet was not predictable between experiments, varying from 19 to 89% of algal-fed oysters (Langdon and Bolton, 1984). When non-toxic antibiotics were added to cultures of oysters fed on artificial diets, variation in oyster growth between experiments was reduced to between 41 and 64% of algal-fed oysters. The results with antibiotics strongly suggested that the microbial flora was important in determining growth of oysters fed on the artificial diet. The problem perceived by Langdon and others was that these microbial effects were neither defined nor predictable, limiting the usefulness of the diets for research and for commercial aquaculture.

Subsequent research on the development of artificial diets for bivalve molluscs in my laboratory has centered on three areas: (1) isolation of bacterial strains beneficial to oyster growth and survival; (2) development of improved microencapsulation techniques for the delivery of high-molecular-weight, water-soluble nutrients to bivalves; and (3) development of microencapsulation techniques for the delivery of low-molecular-weight, water-soluble nutrients to bivalves.

Isolation of Bacterial Strains Beneficial to Oyster Growth and Survival

Crosby, Newell, and Langdon (1990) demonstrated that isolated cellulolytic bacteria incubated with finely ground cellulose increased the nitrogen content and carbon availability of dietary cellulose for adult American oysters (*Crassostrea virginica*). This research demonstrated that bacteria associated with particulate foods can benefit the nutrition of bivalves, both by improving the digestibility of the diet and by enriching the diet with nutrients associated with bacterial cellular products.

Beneficial effects of bacteria on the growth and survival of oyster (*Crassostrea gigas*) larvae were found when selected bacterial isolates were added to axenic cultures of oyster (*Crassostrea gigas*) larvae fed on axenic algal diets (Douillet, 1991). Many of the bacterial isolates tested were either harmful or had neutral effects on larvae; however, one strain (CA2) consistently enhanced larval survival (21-22%) and growth (16-21%), compared with that of axenic larvae fed on algae alone. Subsequent experiments demonstrated that the beneficial effects of strain CA2 also occurred when it was added at 10^5 cells ml^{-1} (final concentration) to non-axenic larval cultures. Similar results showing beneficial effects of isolated bacterial stains on cultures of scallop (*Pecten maximus*) larvae have been reported by Samain et al. (1992). Douillet (1991) showed that oyster larvae directly filtered and ingested CA2 bacteria and estimated that bacterial carbon contributed up to 146% of larval carbon requirements.

In summary, some of the mechanisms underlying the observed beneficial effects of bacteria associated with bivalve cultures have been elucidated. Further developments in this research area may lead to improved control of the microbial flora of aquaculture facilities and, consequently, improved the predictability and performance of bivalves fed on artificial diets.

Development of Improved Microencapsulation Techniques for the Delivery of High-Molecular-Weight, Water-Soluble Nutrients to Bivalves

The alginate-bound microparticle type used by Langdon and Siegfried (1984) and Langdon and Bolton (1984) was not considered to be an ideal particle type for feeding to bivalve molluscs because microscopic examination of prepared particle suspensions often revealed free particles of denatured

dietary protein (egg albumen). In an attempt to improve the delivery of dietary protein containing ^{14}C-labelled protein, cross-linked-protein-walled capsules were prepared and evaluated (Langdon, 1989; Langdon and DeBevoise, 1990). The use of a trifunctional cross-linking agent (1,3,5-benzenetricarbonyl trichloride; BHT) instead of a bifunctional cross-linking agent (sebacoyl chloride; SB) in wall formation significantly reduced leakage of ^{14}C-protein from capsules suspended in seawater over a 24 h period. For example, Langdon (1989) reported that about 16% encapsulated ^{14}C-protein was lost from SB-cross-linked capsules compared to only 4% loss from BTC-cross-linked capsules, when 100 ppm of the bactericidal agent Thimerosal was added to capsule suspensions. Losses of ^{14}C-protein from BTC-cross-linked capsules increased from 4% to 24% when capsules were incubated in seawater without bactericides, indicating significant microbial breakdown of capsule walls with the consequent release of ^{14}C-protein. However, recent studies by Kreeger (1992) indicated that protein loss from BTC-cross-linked capsules prepared with purified (TCA-precipitated) crab protein was less than 5%, even in the absence of bactericides, suggesting that the resistance of cross-linked protein capsules to bacterial breakdown may be partly determined by the type of protein used in wall formation.

Juvenile oysters (*Crassostrea gigas*) fed on cross-linked-protein capsules containing ^{14}C-labelled protein, assimilated ^{14}C-protein with an efficiency of 39 to 40% (Langdon, 1989; Langdon and DeBevoise, 1990). Although addition of nylon to the capsule wall, together with a lipid coating, reduced leakage of ^{14}C-protein, assimilation efficiencies of oysters fed on lipid-coated, cross-linked, nylon-protein capsules was significantly lower (29%) than that of oysters fed on cross-linked-protein capsules (39%). Recent experiments indicated that the mussel *Mytilus edulis trossulus* could also utilize dietary ^{14}C-protein encapsulated within BTC-cross-linked capsules. Interestingly, assimilation efficiencies varied from 5 to 31%, depending on seasonal cycles in the mussel's digestive physiology (Kreeger, 1992).

Development of Microencapsulation Techniques for the Delivery of Low-Molecular-Weight, Water-Soluble Nutrients to Bivalves

Lipid-walled microcapsules (LWM's) designed for the delivery of low-molecular weight, water-soluble nutrients to suspension-feeders were first described by Langdon (1983a). Subsequent research has involved manipulation of the wall composition and nature of the internal, aqueous capsule core to maximize encapsulation efficiencies (proportion of the capsule made up of core material) and retention efficiencies (proportion of core material retained after suspending capsules in seawater for 24 h). Encapsulation efficiencies of 5 to 15% and retention efficiencies greater than 70% are obtained with many capsule formulations; for example, Villamar and Langdon (1992) report that after 24 h in suspension, LWM's retained up to 86% L-ascorbyl-2-polyphosphate, 84% ^{14}C-thiamine and 53% riboflavin. Low encapsulation efficiencies of lipid-walled capsules mean that they cannot be used to deliver bulk nutrients to suspension-feeders, but they may be useful in supplying micronutrients, such as water-soluble vitamins.

A "complex" microparticle (CXM) consisting of LWM's embedded in alginate beads has been recently described by Villamar and Langdon (1992). This particle type was designed to provide shrimp larvae with all potentially essential dietary nutrients with minimum nutrient losses due to leakage. Retention efficiencies for ^{14}C-glucose ranged from 69 to 92% when ^{14}C-glucose was encapsulated in CXM-embedded LWM's and the CXM's were suspended in seawater for 24 h. Shrimp larvae fed on these ^{14}C-glucose-containing CXM's were able to break down the particles and incorporate ^{14}C into their body tissues. Studies also indicated that larval *Penaeus vannamei* could utilize CXM diets for growth.

Conclusions

Development of microparticulate diets for marine suspension-feeders has progressed to the stage where there are now satisfactory, microparticulate, diets available for the commercial culture of some species of marine fish and crustacea. Improvements are required in diet delivery techniques to minimize leakage of nutrients from food particles and reduce the consequent development of high microbial populations, especially in the case of diets for marine bivalves that require food particles less than 20 μm in size. Furthermore, a better understanding of the role of microbes in the nutrition of cultured suspension-feeders is required to obtain predictable and satisfactory growth of animals fed on artificial diets. Axenic culture techniques (Langdon 1983b) will facilitate isolation of beneficial bacteria that may be used to modify the microbial flora of culture systems.

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A CHALLENGE IN AQUACULTURAL NUTRITION OF SALMON: BALANCING THE MICRONUTRIENT SELENIUM

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ABSTRACT

Selenium as a micronutrient and antioxidant is reviewed briefly in fish and other animal research. In fish, wild coho salmon have been shown to have significantly higher concentrations of hepatic and whole body selenium than their hatchery counterparts. The same is true for Atlantic salmon. Most fish diets contain from 0.7 to 1.2 ppm derived mainly from fish by-products in the diet. In animal diets, as in human diets, the minimum daily requirement for selenium is yet to be published. A cursory examination was made of the effects of a chemical stress on fish by measuring excretory loss of selenium. Atlantic salmon maintained on an elevated dietary level of selenium were subjected to stressful light test with survival related to hepatic and body burdens of selenium. The relationship of selenium to better fish health is discussed.

Background

Although selenium (Se) is widely distributed in the environment, its availability throughout the world varies considerably for animals as well as man. In some areas of the world, as in the United States, there is an abundance of Se in the soils and the food chain. Conversely, there are areas with deficiencies in each. Hence, in animal husbandry and fish rearing, one cannot safely assume sufficiency or therapeutic needs without close analysis of the food source and the animal in question.

With the discovery by Schwarz and Foltz in 1957, selenium was recognized as a nutritionally important trace element. It was later demonstrated as having an active role in the enzyme glutathione peroxidase (Ratruck et al., 1973). Glutathione peroxidase (GP-X) has now been identified as an extremely important enzyme in the oxygen detoxifying system. GP-X has been found in all respiring cells.

Selenium is known to play a very important role in health maintenance of farm animals and commercially important animals (Combs and Combs, 1984). Its importance in fish has been indicated by Hicks et al. (1984), yet its therapeutic need has not been documented.

Selenium deficiency has a demonstrable effect on the important GP-X activity (Lieber, 1980; Baker and Autor, 1983; Casaril et al., 1985). The role of GP-X in the immune response mechanism has been gaining much attention in recent years. Spiers et al. (1985) conclude that the major physiological consequence of GP-X deficiency is a selective loss of hexose monophosphate shunt (HMS) activity. The HMS pathway serves two major functions in the respiratory burst of phagocytic cells: (1) It provides the substrate for the superoxide ion generating nicotinamide adenine dinucleotide phosphate, reduced form (NADPH), oxidase; and (2) it regenerates NADPH oxidized by the H_2O_2 detoxifying glutathione cycle, of which GP-X is an essential component.

Leukocyte GP-X is important for survival of the phagocytic cells' protection from reactive O_2 species diffusing from the phagalyosome, and GP-X is also involved in the phagocytic activity (Diploc and Chaudhry, 1988). GP-X deficiency was found to be associated with defective microbicidal activity in phagocytic cells of Se-deficient animals. As well as being toxic to the ingested microorganisms, the superoxide ion, H_2O_2, and its metabolites may also attack the neutrophil in which they are produced, especially in cases where there is insufficient Se for the needed GP-X activity. It is clearly indicated that Se deficiency or insufficiency could have a telling effect on the "well functioning" of an immune response system.

Since 1957, when Schwarz-Foltz first elucidated the importance of selenium in the diet, nutritionists have pondered over the nutritional requirements for selenium in animal and human diets. A narrow threshold seems to exist between deficiency and toxicity. However, sufficiency may well depend on factors yet to be determined by nutritional research.

Selenium Deficiency

Considerable research has been conducted on the beneficial and adverse effects of dietary selenium in mammals and birds. Koller and Exon's (1986) review of selenium deficiency/toxicity in man and animals demonstrated a narrow threshold between minimum and maximum tolerance as far as daily requirements and biological activities are concerned. Evidence suggests that, in humans, 90 µg/day is the minimum daily requirement for optimum biological performance. This value lies between 30 µg/day, which is the amount necessary to prevent selenium syndrome (Keshan disease), and 1,000-1,500 µg/day, which is the maximum tolerance level.

Selenium deficiency causes muscular dystrophy in birds and mammals. Milder deficiency conditions may be characterized by muscular weakness in newborns, reduced weight gain, diarrhea, stillbirths, abortions, and diminished fertility (Underwood, 1977; Herrick, 1979; Reynolds and Oldfield, 1976; Lannek and Lindberg, 1976).

Selenium Effects on Immune Response

Selenium deficiency in dogs and mice has resulted in impaired mitogenic stimulation of lymphocytes (Sheffy and Schultz, 1979; Purnham et al., 1983). Rats receiving selenium-supplemented diets exhibit suppressed humoral and cell mediated immune responses, but enhanced natural killer cell cytotoxicity. In swine, Kelley and Easter (1987) indicated that vitamins A and E and selenium are the nutritional factors that most influence immune responses. Animals deficient in selenium and vitamin E develop lower antibody titers in response to vaccination than do selenium-sufficient animals (Sheffy and Schultz, 1979). Excess selenium seems to potentiate the effect of a killed vaccine (Desowitz and Barnwell, 1980). It is reported that selenium enhances antibody synthesis, thereby leading to an amplification of the immune system in response to antigenic stimulation (Desowitz and Barnwell, 1980; Koller et al., 1979; Spallholz et al., 1973). Evidence also suggests that selenium deficiency can lead to decreased superoxide dismutase (SOD) activity (Koller and Exon, 1986). Reffett et al. (1988) found that selenium deficiency may depress the immune response of calves challenged with a foreign pathogen. The evidence seems to favor the notion that adequate and therapeutic dietary selenium is needed to enhance the immune response system. In a recent review of antioxidants and disease, Diploc (1991) states that selenium is emerging as a dietary factor that may prove to be of major significance as a prophylactic agent against cancer.

Effects of Selenium on Stress Response

Marginal selenium deficiencies may not be noted until an animal is subjected to physical or possibly chemical stressors. For example, severe myopathy can be precipitated in yearling cattle by the stress of exercise, transportation, etc. Low temperature stress in marginally deficient cattle can produce weakness in newborn calves (Koller and Exon, 1986). Peters et al. (1988) described leucocytosis in fish resulting from social interactions in hatchery situations. Other investigators have noted similar increases in the leucocrit in fish that are stressed (Tomasso et al., 1983; Wedemeyer et al., 1983). Elsaesser and Clem (1987) compared the blood chemistry of normal and acutely stressed channel catfish. Among 25 parameters measured, only three (glucose, lactic dehydrogenase, and creatine phosphokinase) showed significant differences. These parameters may have been responding to energy expenditure rather than to stress. Since selenium deficiency can alter immunocompetence and an animal's ability to withstand stress, it is quite likely that selenium-deficient animals are more susceptible to infectious diseases than are selenium-sufficient animals.

Selenium and Fish Health

Very little is known about the relationship of selenium to fish health. King (1975) showed that selenium deficiency causes muscular dystrophy and steatitis in juvenile rainbow trout. Hilton et al. (1980) studied the nutritional requirements and toxicity levels in rainbow trout and found that 13 µg/g dry feed produced chronic toxicity and that 0.15-0.38 µg/g provided maximal glutathione peroxidase activity. Hilton and Hodson (1983) demonstrated an effect of increased dietary carbohydrate on selenium metabolism and toxicity. Selenium deficiency in Atlantic salmon (Poston et al., 1976) and rainbow trout (Hilton and Hodson, 1983) has been shown to cause a reduc-

Table 1. Experiment 1: Urinary Selenium Measured via Catheters and Corresponding Liver Glutathione Peroxidase Activities Resulting From Alcohol Exposure (NC = Not Collected)

	Daily average of urine vol. (ml)	Blood alcohol level(gm%)	Total Se excreted (µg)	GPX act. NADPH oxidized (µMs/min/mg prot)	Percent of actv loss exp/cont.
Fish 2A	35.07	0.178	10.42	3.2	22.5
Fish 3A	NC	0.210	NC	2.5	39.0
Fish 4A	20.13	0.173	22.00	2.3	42.0
Fish 9A	NC	0.185	NC	2.8	31.0
Fish 10A	NC	0.184	NC	2.7	35.0
Fish 11A	NC	0.196	NC	3.2	23.0
Fish 7B	45.71	0.103	14.57	3.4	18.0
				Mean = 2.87 ± 0.41	
Control					
Fish 5A	NC	0.010	NC	4.1	100
Fish 6A	26.75	0.010	5.30	4.3	100
Fish 7A	42.25	0.020	4.80	4.0	100
Fish 8A	NC	0.020	NC	4.1	100
				Mean = 4.13 ± 0.13	

tion in plasma GPX activity. Gatlin and Wilson (1984) established the dietary requirement for fingerling channel catfish.

Bell et al. (1986) investigated selenium in rainbow trout and Atlantic salmon. They found GPX activity in rainbow trout to be significantly reduced with selenium deficiency. They also found hepatic glutathione-s-transferase and pyruvate kinase activity to be increased in selenium-deficient Atlantic salmon parr (Bell et al., 1987). In addition, they showed that pyruvate kinase increased and that liver GPX activity decreased in Atlantic salmon with pancreatic disease (Bell et al., 1987).

Poppe et al. (1985) compared liver selenium levels in wild and farm-reared adult Atlantic salmon and found approximately a seven-fold increase in wild fish. They also found significant differences between wild fish, farm-reared fish, and farm-reared fish afflicted with a hemorrhagic syndrome known as Hitra Disease (Poppe et al., 1986). They found muscular dystrophy in apparently healthy fish from farms with hemorrhagic syndrome, thus supporting their hypothesis that Hitra Disease (HD) is a nutritional or metabolic disorder. They noted that bacteria are seldom detected or isolated from such fish, although they are easily demonstrated in later stages of this disease. They postulated that the slow-growing, cold-water *Vibrio salmonicida* that is isolated from salmon with Hitra Disease may be a secondary invader in a host already weakened and stressed by nutritional or metabolic disorders in addition to other environmental conditions.

This laboratory has demonstrated differences of selenium concentrations in coho salmon outmigrant smolts and returning adults when comparing wild and hatchery-reared fish (Felton et al., 1990). In both stages, the wild fish concentration was almost double that of the hatchery counterpart. This is contrasted by an almost seven-fold difference in adult Atlantic salmon found by Poppe et al. (1985).

Table 2. Experiment 2: Excretory Selenium Measured in Aquarium Water Resulting From Alcohol Exposure

SampleSP	Daily vol. of tank water assayed	Average alcohol conc. in tank in gm% µg/L	Highest conc. of Se for peak day in µg/L
Tank 1	1000	0.332	0.953
Tank 2	1000	0.375	54.075
Tank 3	1000	0.261	22.133
Tank 4	1000	0.254	23.765
Control			
Tank 6	1000	0.000	0.508

Current Research

An interest in selenium and its role in disease resistance was first stimulated by an observation of Dworkin et al. (1984), who found that alcoholics have blood selenium levels that are only 50% of those found in non-alcoholics. This fact, coupled with the knowledge that chronic alcoholics have a very low resistance to diseases, set the stage for experiments described below.

The first two experiments were to test whether fish would lose selenium, as humans apparently do, in the presence of waterborne alcohol. Tables 1 and 2 summarize the results of two experiments conducted utilizing two modes of collecting urinary selenium. In both experiments, fish were exposed to waterborne alcohol in 40 liter tanks at static conditions. The water alcohol concentration in both experiments was maintained at 0.254% and 0.375%. The tanks were aerated with filtered laboratory air.

Experiment 1 was performed on trout bearing urinary catheters held in chambers. It proved extremely difficult to maintain urinary catheters in place once the trout became intoxicated and lost their equilibrium. In spite of the technical difficulties, it was possible to collect urine from three experimental and two control fish for a period of 7 days. The GP-X, a sensitive indicator of selenium change, decreased with concomitant loss of selenium in the fish. The levels of GPX were lower in the experimental fish than in the control fish (Exp.=2.87±0.41, Cont.=4.13 ±0.13).

Experiment 2 was performed on free-swimming, non-catheterized trout. Selenium changes due to excretion were measured in the tank water. A loss of selenium was noted on the first day in both the control and the experimental fish, presumably due to handling stress. However, levels of excreted selenium in the control tank did not continue to rise as they did in the experimental tank. In contrast, the three experimental tanks displayed a continued increase in selenium concentration peaking on the fifth day and decreasing slightly on the remaining 2 days (Felton, et al., 1989). The decrease in the experimental tanks observed in the last 2 days could be attributed to a reabsorption of waterborne selenium through the gills.

Experiment 3 was designed to test survival of salmonids reared on elevated dietary levels of selenium and to test any protective effects these enhanced dietary levels might offer when fish were subjected to temperature and photo stress. Atlantic salmon fingerlings were placed on four different dietary levels of selenium, made by adding sodium selenite in the amounts 0.0 µg/g, 0.5 µg/g, 2.5 µg/g, and 5.0 µg/g to a commercial OMP diet. The basic OMP diet contained 1.10 µg/g of selenium. Duplicate groups of fish (300 fish per group) were maintained on the diets for six months. At the end of that time, one set of duplicates was transferred by truck from the Big Beef Creek Research Station (where well water temperatures averaged 12°C) to the University of Washington School of Fisheries (where surface water tempera-

Table 3A. Selenium Concentration and Survival of Stressed Atlantic Group, Held at Water Temperature of 20°C (Concentration of Se Added to OMP Diet; ND = Not Determined)

Exp. group	Number of fish	Analysis of diet conc. Se µg/g	Body burden Se µg/g	Liver conc. Se µg/g	Percent survival light exp.
Control					
1-5					
1-1	177	0.68	0.3	ND	23
1-2					
Experimental					
1-2 (0.5µg/g)	90	0.93	1.1	1.1	14
1-3 (2.5µg/g)	76	1.62	1.24	0.5	38
1-4 (5.0µg/g)	73	4.80	3.4	1.3	34

Table 3B. Selenium Concentration and Survival of Unstressed Atlantic Salmon Held at Water Temperature of 12-14°C (Concentration of Se Added to OMP Diet; ND = Not Determined)

	Number of fish	Selenium measured in diet µg/g	Body burden of selenium µg/g	Liver Conc. selenium µg/g	Percent survival
Control					
11-5	60	0.68	1.4	ND	90
Experimental					
11-2 (0.5µg/g)	60	0.93	0.84	ND	100
11-3 (2.5µg/g)	60	1.62	1.97	ND	91
11-4 (5.0µg/g)	60	4.80	2.1	ND	97

tures averaged 20°C). Fish at the School of Fisheries were subjected to light guiding stress, a very rigorous treatment, and then maintained in the laboratory for 30 days on the same four dietary regimens of selenium. Tables 3A and 3B compare the survival of stressed fish held in 20°C versus non-stressed fish in the lower temperature water 12°C at Big Beef Creek Laboratory. It may be seen in Table 3A that survival was better in stressed fish given the highest dietary levels of selenium than it was in the control. In fish held at the colder temperatures of Big Beef Creek Research Station and unstressed, the selenium effect was less remarkable (Felton et al., 1989).

Experiment 4 was stimulated by the findings of Poppe et al. (1985) where they compared liver selenium levels in wild vs. farm-reared Atlantic salmon. Our study differed from Poppe's in that it was performed on coho salmon—(1) wild adults vs. hatchery-reared adults, and (2) wild smolts vs. hatchery-reared smolts. The levels were assayed on a wet-

Table 4. Liver Selenium Concentrations of Returning Coho Adult Salmon Compared With Farmed Coho Adults (Felton et al., 1990)

Sample	Number of fish	Mean total liver weights (g)	Mean of Se conc. on dry wt. basis (µg/g)	Mean of Se conc. on wet wt. basis (µg/g)
Ocean caught	10	76.79 ±18.98	8.76 SD±1.9	1.71 SD±0.40
Estuary caught	10	59.44 ±14.57	8.12 ±2.39	1.64 ±0.47
Spawning ground caught	10	28.80 ±19.11	7.56 ±2.86	1.58 ±0.60
Farmed fish	10	39.93 ±10.50	4.82 ±2.50	1.00 ±0.50

Table 5. Selenium Concentrations (Mean and Standard Deviation) in Whole Body Samples of Hatchery-Reared and Naturally Reared Coho Salmon Smolts (Felton et al., 1990). Statistical Significance of Selenium Difference, $p < 0.0025$

	n	Tissue wt. (g)	Selenium concentration (µg/g, dry wt)	Selenium concentration (µg/g, wet wt)
Hatchery-reared	20	X 0.88 SD 0.05	1.974 1.160	0.407 0.239
Naturally reared	20	X 0.86 SD 0.03	3.630 2.050	0.749 0.424

weight basis, in order to compensate for differences due to diets. A summary of findings is seen in Tables 4 and 5 (Felton et al., 1990).

Although overall selenium levels differ in coho salmon from those found in Atlantic salmon, similar trends were noted. The livers from hatchery-reared adult cohos contained considerably lower selenium levels than did the livers of wild adults. A similar difference was noted between hatchery-reared smolts and wild smolts.

Discussion

In the title, a selenium nutritional challenge was mentioned for coho salmon. What exactly is the meaning of this statement, and what is meant by "sufficiency"? Sufficiency may well depend on factors yet to be determined by nutritional research.

In answer to the first question, the challenge for diets may hinge on establishing the upper limits of selenium for the various species of salmonids without toxic manifestations, a level that can compensate for losses resulting from hatchery stresses of crowding, food competition, water chemistries, and myriad other conditions in a given hatchery situation. In order to ascertain the dietary level of selenium needed for a given species in a given hatchery, selenium measurements should be made on the hatchery fish and their wild counterparts. This measurement admittedly would be hard to do in a hatchery. However, there is

a very credible laboratory at Oregon State University's Department of Agricultural Chemistry that can easily accommodate this need.

In our current research, we are using coho as our test fish. To date we have established the dietary level of selenium needed to bring concentrations in the hatchery fish in line with concentrations found in their wild counterparts. We have conducted some stress experiments in order to elicit loss of selenium and possibly other micronutrients. We are investigating the effects of stress on other micronutrients such as copper, zinc, vitamin E, and vitamin C.

Stress in fish, as in any animal, is a complex study involving many responses of animal life—physiology, adaptation, metabolic energy diversion, long-term survival impairment, and biochemical changes resulting from losses of micronutrient and concomitant immune response impairment. The last response has occasioned a new approach to the study of stress effects upon fish. In a recent publication titled "Biological Indicators of Stress in Fish" (Adams, 1990), there is little or no mention of this type of response. Hence, we hope that our present research will provide some of the information lacking on this response. When this laboratory study has produced sufficient information, we anticipate a 3- to 5-year study at an existing salmon hatchery that has repeatedly poor returns.

Conclusion

The difficulty in establishing the optimum balance of selenium in fish, therefore, is complicated by stress factors such as hatchery location, size, handling procedures, and water quality, to name a few. These factors as well as the species differences will influence the correct balance of selenium and other dietary supplements needed to produce fish with competent immune and energy systems capable of meeting challenges of the wild. Current work is addressing these issues and the indications to date are promising.

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GROWTH AND PIGMENTATION OF RAINBOW TROUT REARED ON A DIET CONTAINING SPRAY DRIED RED YEAST (*PHAFFIA RHODOZYMA*)

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ABSTRACT

Pigmentation and growth was determined for juvenile rainbow trout (*Oncorhynchus mykiss*) reared on a reference diet supplemented with different amounts of spray dried, red yeast (*Phaffia rhodozyma*). Diets were prepared with 0, 5, 10, or 15% intact red yeast (IY) and 5, 10, or 15% mechanically broken red yeast (MY) and fed to the trout for 9 weeks. Skin pigmentation of fish fed diets with red yeast ranged from 33.7 to 43.5 μg astaxanthin/g tissue and was not influenced by milling or yeast concentration ($p<0.05$). Muscle pigmentation of animals fed diets containing red yeast ranged from 12.1 to 26.5 μg/g tissue and was greater for animals fed diets with 10 or 15% yeast ($p<0.05$) than with diets containing 5% red yeast. Average weight gain of trout on the seven diets ranged from 6.35 ± 0.19 to 5.33 ± 0.57 g / week and ranked in the following order: 15% MY = Control = 5% IY >15% IY = 10% IY = 5% MY = 10% MY ($p<0.05$).

Introduction

Astaxanthin is a carotenoid pigment responsible for the red color in the flesh, skin, and eggs of salmonids (March et al., 1990) and the epithelial tissues of other aquaculture species including goldfish (Hata and Hata, 1972), sea bream (Katayama, Kanji, and Chichester, 1973), red carps (Katayama, Tsuchiya, and Chichester, 1971) and shrimp (Yamada et al., 1990). Although animals have some capability for metabolic inter-conversion (Ando et al., 1990; Tanaka et al., 1976) and assimilation of dietary carotenoids they cannot synthesize carotenoids *de novo*. Therefore, animals that accumulate carotenoids require either a dietary source of the pigment or appropriate biosynthetic intermediates. Astaxanthin is the key xanthophyll responsible for red coloration of farmed fish and shellfish (Simpson, Chichester, and Katayama, 1981). The current need for astaxanthin as a dietary component for the salmon farming industry alone is close to 20,000 kg/year (Torrissen, Hardy, and Shearer, 1989).

Relatively little is known about the biological function(s) of xanthophylls in live fish. It has been suggested that xanthophylls function as fertilization hormones, reduce mortality rate during embryonic development, increase ability to tolerate harsh environmental conditions, and enhance growth, maturation rate, and fecundity (Tacon, 1981). Recently, a mechanism for astaxanthin in protecting biological membranes from oxidative injury has been proposed (Kurashige et al., 1990). Nutritional deficiency with respect to astaxanthin may be the cause of blue disease in farmed Tiger prawns (Howell and Mathews, 1991).

The red yeast *Phaffia rhodozyma* produces and stores an isomer of astaxanthin (3-R,3'-R-dihydroxy-

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4,4'-diketo-b-carotene, Andrewes, Phaff, and Starr, 1976). Wild type strains of this yeast normally accumulate astaxanthin at less than 1000 µg/ g cell dry weight (Johnson and Lewis, 1978; Haard, 1988). Recently, mutant strains of red yeast that accumulate astaxanthin up to 10,000 µg/g cell dry weight have been isolated (An, Schuman, and Johnson, 1989; Lewis et al. 1990; Evans, Adams, and Wisdom, 1991; Johnson et al., 1990, 1991; Miura, Yoshima, and Minato, 1990).

Feeding mechanically ruptured cells of red yeast achieves astaxanthin deposition in juvenile rainbow trout and lobster (Johnson, Conklin, and Lewis, 1977; Johnson, 1978; Johnson and Lewis, 1978; Gentles and Haard, 1990, 1991). Astaxanthin in the intact yeast was not biologically available to juvenile trout when the cells had been freeze dried prior to incorporation into the feed (Johnson, Conklin and Lewis, 1977; Johnson and Lewis, 1978; Johnson, Villa and Lewis, 1980). Removing the capsule or cell wall of red yeast by co-fermentation with *Bacillus circulans* (Okagbue and Lewis, 1983) or enzymic digestion with a specific preparation of β-1,3-glucanase (Gentles and Haard, 1990) greatly improved the bioavailability of astaxanthin in juvenile trout. Although digestion of the wall by such treatments appears to be impractical for industrial use at this time, feeding intact red yeast that had been spray dried to sexually mature trout resulted in about 88% flesh pigmentation compared to mechanically disrupted yeast (Gentles and Haard, 1990, 1991).

Previously, workers have noted faster weight gain of rainbow trout fed diet supplemented with red yeast than with trout fed the same diet without red yeast supplement. Johnson (1978) fed a base diet of commercial trout ration containing either 15% *Phaffia rhodozyma* cells (broken, UCD strain 210) or 15% Bakers yeast to juvenile trout at an estimated 1.5% of body weight and observed growth rates of 2.07 and 1.30 g/day, respectively. However, definite conclusions could not be made since the diets were not adjusted to the same energy and nitrogen content. Red yeast contains more energy than Brewers yeast because of differences in lipid content, 17.0 and 4.0 % w/w total lipid, respectively. Gentles and Haard (1990, 1991) fed a reference diet supplemented with either 15% *Phaffia rhodozyma* (broken, UF stain 24) or 15% Bakers yeast to sexually mature trout at 1.5% of body weight and observed growth rates of 6.9 and 4.2 g/week, respectively. However, because of the large variation in weight within treatments and small number of fish employed in the study, the results were not significantly different ($p<0.05$). Other studies have also led investigators to suggest that dietary xanthophylls promote weight gain. Deufel (1965) reported enhanced growth, maturation rate, and fecundity in rainbow trout fed canthaxanthin. Torrissen (1984) reported that Atlantic salmon fry fed commercial diets supplemented with astaxanthin or canthaxanthin grew significantly faster ($p=0.02$) than did the control groups after 35 days of feeding. Growth weight was mainly promoted during the early start-feeding period in the third week. Long and Haard (1988) showed that feeding juvenile rainbow trout a reference diet supplemented with *Artemia* carotenoprotein, but not *Artemia* carotenoids, synthetic canthaxanthin, or synthetic astaxanthin, resulted in significantly ($p<0.05$) greater growth than a pigment free diet.

Materials and Methods

Culture System

A flow-through system consisting of 21 tanks, each with a capacity of 57 L, was maintained at 15°C and a flow rate of 50 L/h. All tanks were painted black inside to facilitate calmness and feeding efficiency. Rainbow trout (Mount Lassen strain) were purchased from Mount Lassen Trout Farm (Lassen, CA) and maintained on commercial feed (Silvercup, Murry Elevators, Murry UT) for 2 weeks prior to presentation of experimental diets.

Each experimental diet was fed in triplicate at 1.3% of tank biomass, based on the collective weight of the fish in each tank at the start of each week. The replicate tanks were randomly distributed among the 21 tanks. Automatic feeders were set to feed ration for each day at 1-h intervals 12 times during daylight hours. At the start of feeding experimental diets, each tank contained 15 fish having an average weight of about 60 g.

Feed Preparation

A salmonid reference diet (Hardy, Cassilas, and Matsumato, 1987) containing 40% casein, 15% marine oil, and other required nutrients for trout, was modified to contain red yeast (*Phaffia rhodozyma*, strain UFC 24) as described previously (Gentles and Haard, 1991). A total of seven diets were prepared, i.e., supplemented with 5, 10, and 15% milled, spray dried red yeast or with 5, 10 and 15% intact, spray dried yeast as well as a control diet without added yeast. The diets were made isocaloric and comparable in composition of major nutrients by adjusting the composition of casein, oil, starch, and cellulose based on the proximate composition of the milled or intact yeast (table 1). Menhaden oil was from Zapata Haynie

Table 1. Composition of Experimental Diets[a]

	Diet						
	1	2	3	4	5	6	7
Ingredient	Content (%)						
Casein	40.00	37.95	35.91	33.86	37.56	35.12	32.68
Gelatin	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Corn starch	14.70	14.00	13.20	11.20	14.00	13.24	12.46
Dextrin	9.20	9.20	9.20	9.20	9.20	9.20	9.20
Glucose	4.70	4.70	4.70	4.70	4.70	4.70	4.70
Cellulose	3.00	1.58	0.16	0.00	1.98	0.97	0.00
Amino Acids	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Bernhart Tom	4.00	4.00	4.00	4.00	4.00	4.00	4.00
Vitamins	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Choline Cl	0.30	0.30	0.30	0.30	0.30	0.30	0.30
Menhaden oil	15.00	14.22	13.44	12.66	14.19	13.37	12.56
Mineral Mix	0.10	0.10	0.10	0.10	0.10	0.10	0.10
Red Yeast (Milled)	0.00	5.00	10.00	15.00	0.00	0.00	0.00
Red Yeast (Intact)	0.00	0.00	0.00	0.00	5.00	10.00	15.00

[a] Base diet based on Hardy et al., 1987. Red yeast was *P. rhodozyma*, UF strain 24. Astaxanthin content of diets were approximately: diets 2 & 5 (37µg/g), 3 & 6 (74µg/g), 4 & 7 (112µg/g), 1 (0µg/g).

(Hammond, LA), and red yeast was provided by Universal Foods Corp. (Milwaukee, WI). Diets were formulated into 0.5 cm pellets, vacuum packaged in oxygen barrier bags, and stored at -20°C prior to the week of feeding. The amount of feed provided each day was adjusted for gain after each weekly weighing and individually adjusted in each tank after each mortality.

Growth

Each tank was taken as one experimental unit, i.e., tank biomass instead of individual biomass was measured. Fish were weighed by placing all 15 fish from a tank in a tared bucket of water. Any mortalities were individually weighed before disposal. Tank biomass was determined once a week for 9 weeks at which time all remaining fish were sacrificed for pigment analysis. Average weight gain for each tank was determined from the slope of an equation developed by linear regression analysis of each of ten weighings at 0, 1, 2, 3, 4, 5, 6, 7, 8, and 9 weeks on experimental diets.

Astaxanthin Analysis

The astaxanthin content of skin and muscle tissues was determined at the completion of the feeding trial. A sample of three fish was taken from each tank. The ordinary muscle and skin was removed and lyophilized prior to analysis. Total carotenoid was extracted, analyzed, and calculated as astaxanthin from pooled tissues of each tank by a method previously described (Gentles and Haard, 1990, 1991).

In Vitro Digestibility

The digestibility of red yeast by trout digestive enzymes was determined by a pH-stat method recently developed in our laboratory. An enzyme extract obtained from the pyloric ceca of trout is used to hydrolyze the protein to be assayed at pH 8.0 and 15°C. Protein hydrolysis is measured by calculating the hydrolysis equivalent (h) from the volume of standard alkali required to maintain the pH at 8.0.

$$h = (B \times 1/\alpha \times N_b) / (M \times (S\%/100))$$

Table 2. Pigment Content of Flesh and Skin of Trout Fed Diets Containing Different Amounts of the Red Yeast, *Phaffia rhodozyma*

Yeast	Added to Diet %	Flesh Astaxanthin mg/ g dry matter	Skin Astaxanthin mg/g dry matter
Spraydried whole yeast	5	12.10 ± 0.98	33.70 ± 1.45[a]
	10	22.23 ± 4.74[a]	41.09 ± 13.87[a]
	15	22.63 ± 2.23[a]	36.36 ± 8.66[a]
Spraydried, milled yeast	5	16.88 ± 1.32	35.53 ± 3.27[a]
	10	26.47 ± 3.02[a]	43.53 ± 9.30[a]
	15	22.53 ± 2.59[a]	37.09 ± 4.77[a]
Control	0	0.73 ± 0.44	2.47 ± 0.17

Data are average of three groups of fish per diet; ± indicates standard deviation. Values in the same column followed by the same letter are not significantly different, $p<0.05$.

where $\alpha = 10^{pH-pK}/1 + 10^{pH-pK}$; N_b = normality of titrant; M = mass (g) of reaction mixture; and S = protein concentration in reaction mixture. The degree of protein hydrolysis (DH%) is calculated from h as follows:

$$DH(\%) = (h/ h_{tot}) \times 100$$

where h_{tot} is the total number of peptide bonds in the protein, calculated from the mean average molecular weight of amino acid residues. Output from a titration burette, temperature thermister, and pH output is interfaced to a Mac IIcx computer with a Molytek datalogger and Parameter Manager Plus software (Genrad, Milipitas, CA). The DH(%) of the reaction is calculated from the following algorithm so that the progress of the reaction is monitored:

Volume (ml) = mv output of burette/ 50.00
pH = 7 + (mv output of pH meter/10)
pK = 8.275 - 0.0233 x T (°C) of reaction
$\alpha = 10^{pH-pK}/1 + 10^{pH-pK}$
$h = (B \times 1/\alpha \times N_b)/ (M \times (S\%/100))$
$DH(\%) = (h/ h_{tot}) \times 100$.

Enzyme for use in the *in vitro* assay was prepared by a method similar to that described by Grabner (1985). Pyloric ceca tissue was homogenized with cold (4°C) 0.05 N Tris-HCl buffer (pH 7.8) at a ratio of 1:3 (w/v). Chloroform was added to the homogenate (1:3 v/v), and the mixture was centrifuged at 3000 x g for 30 min at 4°C. The residue was treated twice with 50 ml buffer and again centrifuged at 3000 x g for 30 min. The supernatants were pooled and freeze dried. The resulting dry matter was dissolved in Tris-HCl buffer, 0.05 N, pH 9.0 and dialyzed against the same buffer for 48 h at 4°C and then centrifuged at 10,000 x g for 30 min. The resulting supernatant was adjusted to pH 8.0 and freeze dried, and the dried preparation was stable when stored at -15°C prior to use in the *in vitro* assay.

The *in vitro* assay system contains 2 mg of pyloric ceca enzyme protein, feed sample (e.g. yeast) containing 16 mg protein N in a 15.0 ml volume of distilled/deionized water. The initial reaction pH is adjusted to 8.0 after the sample has equilibrated for 15 min, and the reaction is started by addition of enzyme. The assay system is constantly mixed with a magnetic stirrer and purged with nitrogen gas to prevent in-

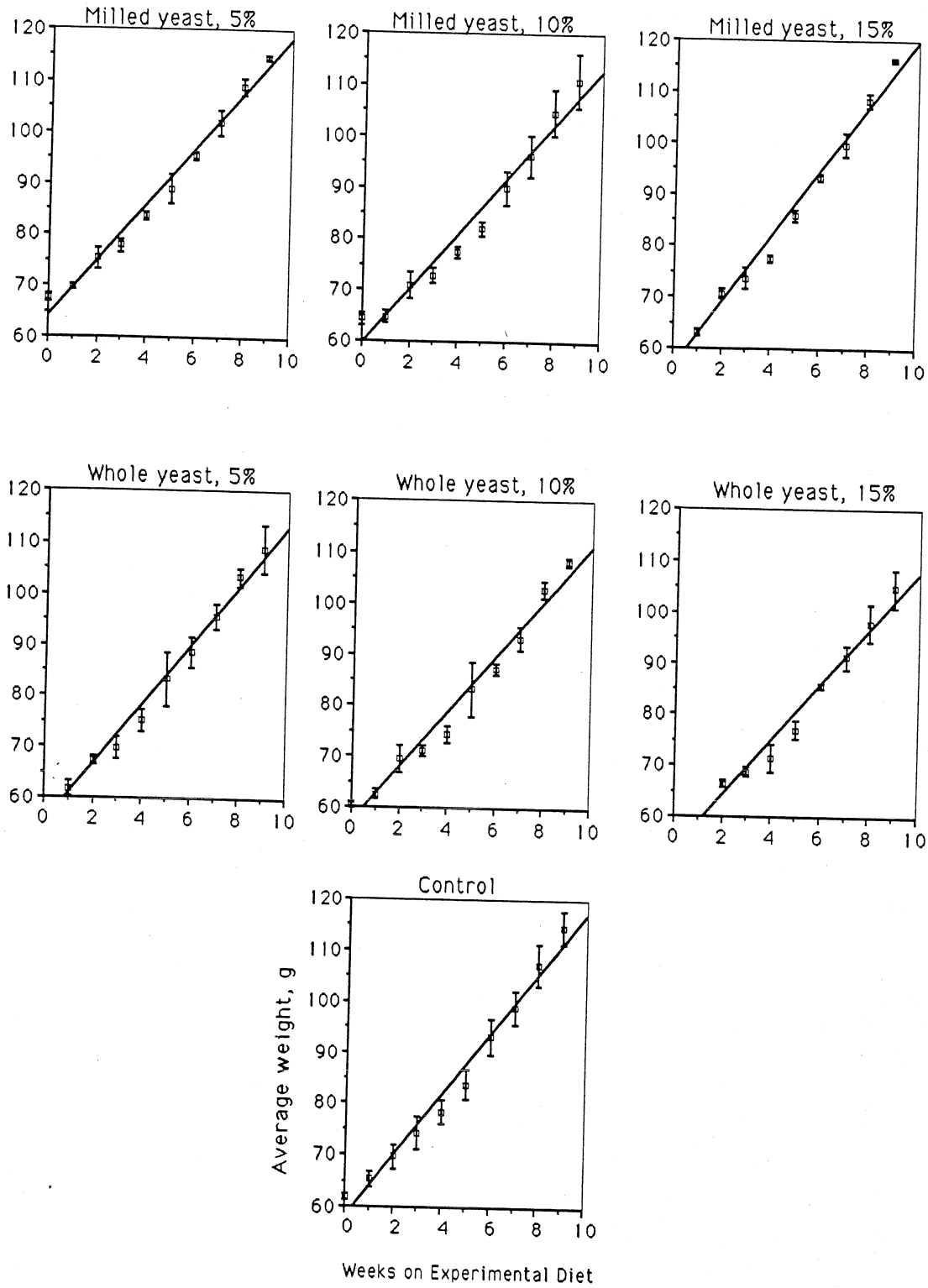

Figure 1. Weight of trout fed experimental diets containing milled and intact red yeast. Data are average for triplicate tanks, and bars are standard deviation.

Table 3. Growth of Trout Fed Diets Containing Different Amounts of the Red Yeast, *Phaffia rhodozyma*[a]

Yeast	Added to Diet %	Initial Wt. g/fish	Final Wt. g/fish	Av. Wt. Gain g/week-fish	R^2
Spray dried, whole yeast	5	57.63±0.19	108.96±4.62	5.81±0.35[ac]	0.99
	10	60.20±0.81	108.33±0.83	5.38±0.19[bc]	0.98
	15	55.98±0.62	104.75±3.68	5.39±0.40[bc]	0.98
Spray dried, milled yeast	5	67.83±1.23	114.66±0.43	5.38±0.20[bc]	0.99
	10	64.27±1.23	110.85±5.32	5.33±0.57[b]	0.97
	15	58.54±0.41	116.25±0.30	6.35±0.19[a]	0.99
Control	0	61.90±0.55	114.53±3.19	5.95±0.35[a]	0.98

[a]Data are average of values obtained from three groups of fish per treatment; ± indicates standard deviation. Average weight gain values followed by the same letter are not significantly different, $p<0.05$. R^2 is "goodness of fit" for linear regression analysis of weight vs. time of feeding experimental diet. Average weight gain was determined from slope of linear regression analysis.

corporation of atmospheric CO_2 into the reaction mixture. Hydrolysis is allowed to continue until reaction completion, normally less than 500 min at 15°C.

Statistical Methods

The statistical significance of differences in pigment content and weight gain was determined by the Student t-test. and linear regression analysis using Statview software.

Results

Pigmentation

Trout reared on diets containing red yeast for 9 weeks contained significantly more astaxanthin than fish fed control diet ($p<0.05$, table 2). There was no difference ($p<0.05$) in skin astaxanthin as a function of yeast concentration (5, 10, 15%) or mechanical disruption of yeast (milled vs. intact). The astaxanthin content in the flesh of fish fed a ration including 5% yeast (intact or milled) was less than that of animals fed diet with 10 or 15% yeast ($p<0.05$, table 2). The astaxanthin content in the muscle of trout fed a diet with 15% red yeast (intact or milled) supplement was about 23 µg/ g.

Growth

The weight of trout fed experimental diets for 9 weeks is shown in figure 1. There was good agreement in growth rate for replicates fed the same diet, as shown by the relatively low standard deviations. Fish reared on experimental diets containing 5% intact yeast or 15% milled yeast did not significantly differ ($p<0.05$) in average weight gain from fish fed the control diet (table 3). On the other hand, fish reared on diets containing 10% or 15% intact yeast, and 5% or 10% milled yeast grew at a slightly lower rate than those fed the control diet without added yeast ($p<0.05$) (table 3).

In vitro digestibility

Hydrolysis of protein evaluated by *in vitro* analysis is shown in figure 2. Red yeast was hydrolyzed at a slower initial rate than casein, and the degree of hydrolysis at the completion of the reaction was also lower for red yeast (table 4). Recent studies using the chromic oxide method for *in vivo* digestibility by post-juvenile rainbow trout showed that the apparent digestibility of casein, intact red yeast and milled red yeast are 97, 79, and 63 %, respectively (Hardy and Forster, 1991). Although caution should be used

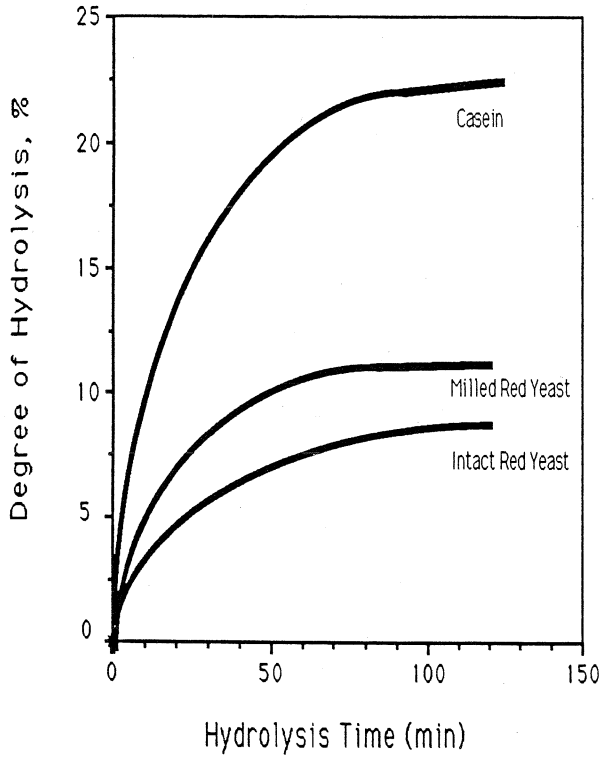

Figure 2. Degree of hydrolysis of protein in red yeast (strain UF 24) and casein using in vitro *system with digestive enzymes from the pyloric ceca of trout. Data are for one experiment and are representative of results obtained in at least three other experiments.*

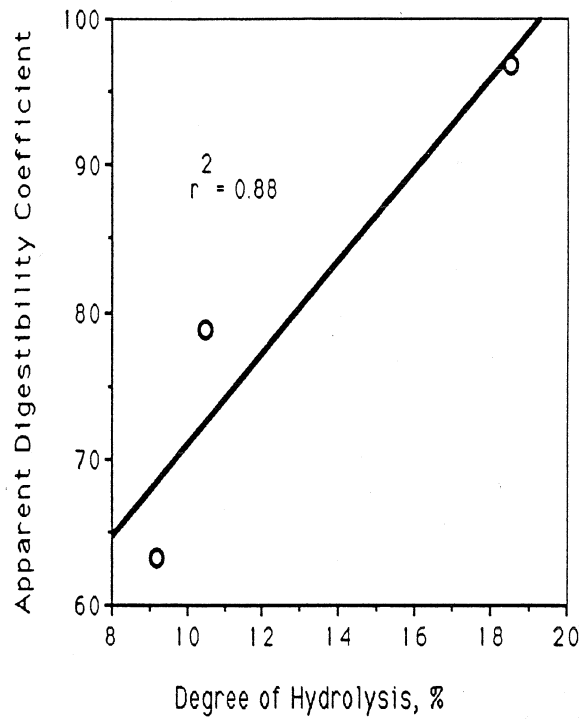

Figure 3. Relationship between degree of protein hydrolysis obtained by in vitro *method and the apparent digestibility coefficient of casein, milled red yeast, and intact red yeast. Data for apparent digestibility coefficient are from Hardy and Forster (1991) for a different strain of red yeast.*

in comparing these data, since a different strain of red yeast was used by these workers, a comparison of *in vitro* and *in vivo* protein digestibility suggests that the degree of protein hydrolysis is a good indication of apparent protein digestibility (fig. 3).

Discussion

Pigmentation

The results of this study show that the astaxanthin in spray dried, intact and mechanically broken red yeast has similar bioavailability for juvenile rainbow trout. Earlier we observed that the skin pigment content of mature rainbow trout fed a diet including 15% red yeast for 8 weeks at 1.5% of body weight was greater, i.e., 46-70 µg/g. The flesh pigment content for mature trout fed rations with 15% red yeast for 8 weeks was similar, i.e., 21-27 µg/g (Gentles and Haard, 1991). Torrissen (1986) suggested a greater affinity for pigment deposition in mature fish than in small fish since maturation increases skin pigmentation.

Our results differ from those reported by Johnson, Villa, and Lewis (1980) and Johnson and Lewis (1978) who did not observe pigmentation when juvenile trout were fed intact red yeast. Since the later studies were done with freeze dried yeast, we conclude that the spray drying process improves the bioavailability of astaxanthin in *Phaffia rhodozyma* cells. The comparable bioavailability of astaxanthin in intact spray dried and mechanically broken red yeast is supported by the similarity in degree of protein hydrolysis of intact and milled yeast cells used in this feeding study (table 4). Apparent protein digestibility using the chromic oxide method and degree of protein hydrolysis using the *in vitro* method are positively correlated (fig. 3). Moreover, the degree of hydrolysis of protein in freeze dried red yeast of the same strain used by Johnson, Conklin, and Lewis (1977) is considerably lower than the spray

Table 4. *In Vitro* Degree of Hydrolysis of Red Yeast Protein and Casein

Sample Analyzed	Pyloric Ceca Source	Degree of Hydrolysis
	Rainbow Trout (g)[a]	%
Spray Dried Red Yeast[b]	500.	9.4
	50.	9.2
Milled Red Yeast[b]	500.	11.3
	50.	10.5
Freeze Dried Red Yeast[c]	500.	4.9
Casein	500.	22.5
	50.	18.5

[a]Mount Lassen strain, approximate weight of fish. Trypsin activity of enzyme preparation from 50 g and 500 g fish was 33.4 and 54.5 BAPA units/mg protein respectively.

[b]*Phaffia rhodozyma*, strain UF-24

[c]*Phaffia rhodozyma*, strain UCD-210

dried cells used in this feeding trial (table 4). However, additional research is needed to determine the contributions of yeast strain and processing method to nutrient and pigment bioavailability in red yeast.

Growth

Weight gain of trout fed the experimental diets containing red yeast was not increased compared to fish reared on the control diet. Indeed, the growth of trout fed diet supplemented with 10% intact yeast, 15% intact yeast, and 5% or 10% milled yeast was slightly less than the growth of fish reared on the control diet ($p<0.05$). Curiously, the one diet (15% milled red yeast) that resulted in greater, although not statistically significant ($p<0.05$), growth rate than the control diet was the same as that used in previous feeding trials growth promotion as suggested (Johnson, Conklin, and Lewis, 1977; Gentles and Haard 1990). The weight gain of fish reared on a diet containing 15% red yeast was lower when the yeast was intact rather than milled (table 3). This may be due to the somewhat lower digestibility of intact yeast (fig. 2). Additional research is needed to determine the relationship between growth and protein digestibility since other nutritional factors may be involved.

Conclusions

Since both milling and freeze drying are expensive processing steps, and astaxanthin is less stable in milled yeast than intact yeast, the finding that astaxanthin in spray dried red yeast is bioavailable to juvenile as well as mature trout (Gentles and Haard, 1991) is of considerable practicable significance.

Although feeding rainbow trout a reference diet containing 5, 10, or 15% red yeast at 1.3% of body weight results in excellent pigmentation of skin and flesh, it does not consistently result in a promotion of weight gain compared to the reference diet without added yeast. We observed that fish fed diets containing red yeast reacted more vigorously to the feed than those fish fed the control diet. Therefore, it is possible that trout may grow faster on a diet containing red yeast when it is fed at a higher proportion of body weight or *ad libitum*. Further study will be required to fully understand the influence of red yeast on growth of rainbow trout.

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NUTRITIONAL RESPONSE OF JUVENILE *PENAEUS SETIFERUS* AND *PENAEUS VANNAMEI* TO DIFFERENT QUALITY FEEDS IN THE PRESENCE AND ABSENCE OF NATURAL PRODUCTIVITY

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ABSTRACT

The objectives of this study were to determine the growth and survival responses of juvenile *P. setiferus* and *P. vannamei* to animal versus plant protein in formulated feeds containing 25% protein in laboratory tanks and pens in earthen ponds and to evaluate the relative contribution of dried diets and natural productivity in earthen ponds to the nutritional requirements of shrimp.

The contribution of natural productivity in ponds to nutritional requirements of penaeid shrimp was evaluated for two species of shrimp, *Penaeus setiferus* and *P. vannamei*. This was done by determining growth and survival for juvenile shrimp placed into tanks in the laboratory in the absence of natural productivity and into bottomless pens in ponds in the presence of natural productivity and fed isocaloric and isonitrogenous feeds containing different percentages of animal and plant protein. One group of shrimp in pens in each experiment was not fed. Initial stocking densities for *P. seterferus* and *P. vannamei* were $15/m^2$ and $30/m^2$ in pens and $29/m^2$ in tanks and $15/m^2$ and $20/m^2$ in pens and $20/m^2$ in tanks, respectively. There was no significant difference in survival for any of the variables evaluated in each experiment conducted using tanks or pens.

Growth of *P. setiferus* but not *P. vannamei* in laboratory tanks fed feeds containing 1:1 animal to plant protein was significantly less than those fed feeds containing all animal protein (1:0) suggesting that *P. setiferus* requires a higher quality protein in the feed than *P. vannamei*. For both species, growth was significantly less in tanks for animals fed feeds containing no animal protein (0:1) than those fed feeds containing a 1:1 ratio of animal to plant protein. This suggests that the protein quality as represented by all plant protein at a 25% protein level is inadequate for both species.

The growth and survival of shrimp stocked outside versus inside the pens at the same density and fed the 25% protein feed containing all animal protein were not significantly different for both species. This indicates that there was no adverse effect of the pens upon shrimp survival and growth under these experiment conditions.

Significant growth occurred for the unfed groups for all four experiments conducted in pens in earthen ponds. Percent contribution of natural productivity versus presented feeds was estimated to be 69% to 76% for *P. setiferus* and 77% to 83% for *P. vannamei*. The lower the initial stocking density and harvest shrimp biomass the higher the estimated percent contribution of natural productivity for shrimp nutritional requirements.

The weight gain and harvested biomass were always significantly more for all the groups for both species of shrimp presented feed versus the respective groups of shrimp not presented feed in pens in earthen ponds. This indicates that the presented feed was contributing significantly either directly or indirectly to the nutritional requirements of shrimp in earthen ponds.

For all the experimental feeds fed, there was no significant difference in growth for *P. vannamei* stocked at either 15 or 20/m^2 with a harvest biomass of 71.9 gm/m^2 and 88.1 gm/m^2, respectively, and for *P. setiferus* initially stocked at 15/m^2 with a harvest biomass of 61.9 gm/m^2 in pens in ponds. These data indicate that the ingestion of natural productivity was adequate to compensate for the nutritional inadequacies of the feeds containing all plant protein. Growth of *P. setiferus* was significantly less for those groups initially stocked at 30/m^2 with a harvest biomass of 108 to 133 gm/m^2 fed feeds containing all plant protein versus those groups fed feed containing either some or all animal protein. This indicates that the presented feed containing all plant protein plus natural productivity were inadequate to maintain growth of *P. setiferus* for this stocking density and harvest shrimp biomass under these experimental conditions.

These data indicate that (1) natural productivity significantly contributes to the nutritional requirement for growth for shrimp cultured in earthen ponds, (2) *P. setiferus* requires higher quality protein in the feed than *P. vannamei*, (3) the greater the initial stocking density and higher the harvest biomass the less the percent contribution of natural productivity versus the presented feed to the nutritional requirements of shrimp, and (4) there are species differences for shrimp either in terms of protein requirement and/or utilization of natural productivity for growth.

These data indicate that a lower quality feed and/or lower feed rate can be used to culture shrimp in earthen ponds when natural productivity is being consumed by the shrimp. These data also indicate that only laboratory research concerning nutritional requirements for aquacultured species are inadequate for determining absolute nutritional requirements for a desirable commercial feed when natural productivity is contributing to the nutritional requirements of an aquatic species.

Introduction

Food is a major cost item for a commercially successful shrimp farm representing 30 to 55% of the operating cost depending on the pond management strategy and species (Griffin et al., 1985; Johns et al., 1983; Lawrence et al., 1983a; Pardy et al., 1983). It is recognized that the nutritional requirements for shrimp in earthen ponds comes from two sources, presented feed and natural productivity. Schroeder (1983) indicated that natural productivity associated with ponds affected the shrimp nutritional response to feeds. Maguire and Leedow (1983) concluded that food items from natural productivity of ponds contributed substantially to nutrition of the shrimp, *Metapenaeus macleayi*. Stomach analysis of *M. macleayi* suggested that natural productivity comprised approximately 42% of the shrimp diet (Maguire and Bell, 1981). Rubright et al. (1981) concluded that both natural productivity and presented feeds are important for semi-intensive pond culture of *P. stylirostris*. Using stable carbon isotope tracer techniques, Anderson et al. (1987) and Parker et al. (1989) obtained data indicating that the percent contribution for assimilation of organic carbon for *Penaeus vannamei* stocked into earthen ponds at 20/m^2 for 4 weeks was 53 to 77% and 44 to 86%, respectively. Using a higher initial stocking density of 40/m^2 and with a harvest biomass of 612 to 637 g/m^2 in pens in earthen ponds, Robertson et al. (1992c) estimated that the percent contribution of natural productivity to the increased *P. vannamei* biomass was 39 to 42%. Robertson et al. (1992a) estimated that the percent contribution of natural productivity to increased *P. setiferus* biomass in pens in earthen ponds initially stocked at 40/m^2 and with a harvest biomass of 400 to 429 g/m^2 was about 68%. These reports indicate that natural productivity is nutritionally important to shrimp cultured in earthen ponds, even at high stocking densities and a harvest biomass up to 637 g/m^2.

As a dietary constituent, protein is both a major growth nutrient and a major cost item. Protein requirements for marine shrimp change with age, environmental conditions, and species. For example: (1) the protein level in feeds for *P. vannamei* has been recommended to be 30% (Colvin and Brand, 1977; Smith et al., 1985); for *P. setiferus* to be 30% (Andrews et al., 1972; Lee and Lawrence, 1985); for *P. stylirostris* to be 35% (Colvin and Brand, 1977); for *P. monodon* to be from 40 to 50% (Lee, 1971; Alava and Lim, 1983); and, for *P. japonicus* to be from 52 to 60% (Deshimaru and Shigeno, 1972; Deshimaru and Yone, 1978); (2) the protein requirement of penaeids decreases as postlarvae grow into adults (Colvin and

Brand, 1977; Aquacop, 1978; Chen et al., 1985); (3) *P. vannamei* and *P. stylirostris* have a lower protein requirement for dietary protein than *P. monodon* when cultured in ponds (Aquacop, 1984); and (4) the decreased growth attributed to higher salinities can be partially reversed by increasing the protein level of the feed (Robertson et al., 1992b).

Deshimaru and Shigeno (1972) were the first to report that diets should have an amino acid composition similar to shrimp. The importance of protein quality in penaeid feeds has been emphasized (Shewbart et al., 1972; Forster, 1975; Fenucci and Zein-Eldin, 1976; Zein-Eldin and Corliss, 1976; Venkataramiah et al., 1978) and may be more critical to growth than the actual level of protein chosen for inclusion in the feed. In the laboratory, the kuruma prawn, *P. japonicus*, had best growth when fed a diet containing an amino acid profile similar to the short-necked clam (Deshimaru, 1981). Fish meals, shrimp meals, and squid meals have been used as major dietary protein sources (Fennuci and Zein-Eldin, 1976; Fennuci et al., 1980; Forster and Beard, 1973). However, dietary constituents of vegetable origin also have been evaluated as possible ingredients of dried shrimp diets. *P. aztecus* growth was satisfactory when fed diets whose main protein fraction was plant protein for experiments conducted in the laboratory (Venkataramiah et al., 1975). Also, soybean (Lawrence et al., 1987) and wheat (Taiwan Fisheries Research Institute, 1990) have been satisfactory in shrimp feeds. Several authors have concluded that the source of protein for shrimp diets is important and should be carefully chosen (Sedgwick, 1979; Alava and Lim, 1983; Lee et al., 1984).

The objectives of this study were to determine the growth and survival responses of juvenile *P. setiferus* and *P. vannamei* to animal versus plant protein in formulated feeds containing 25% protein in laboratory tanks and pens in earthen ponds and to evaluate the relative contribution of dried diets and natural productivity in earthen ponds to the nutritional requirements of shrimp.

Materials and Methods

Two experiments using laboratory tanks and four experiments using pens in earthen ponds were conducted. The laboratory system consisted of rectangular, flat bottom, fiberglass tanks with internal dimensions of 83 cm x 83 cm x 36 cm and a seawater volume of 250 l. This system was part of a 50-metric ton recirculating system designed to maintain constant culture conditions in the absence of natural productivity. Water exchange in the individual tanks was approximately 100% per day with a photoperiod set at 12:12 h light:dark cycle. The pond system consisted of bottomless cylindrical pens with a height of 1.14 m and a cross-sectional area of 1 m^2. The bottomless pens allowed the shrimp free access to the substrate and the flora and fauna associated with it. This pen system has been previously described (McKee et al., 1989). Normal pond management strategies for a semi-intensive shrimp pond culture system for south Texas was used (Lawrence et al., 1983b; Smith and Lawrence, 1990). Dissolved oxygen, temperature, and salinity were measured daily in both systems.

The ingredients and proximate analyses for the experimental feeds are given in Tables 1 and 2, respectively.

All feeds were formulated to contain 25% protein and had protein fractions of either all animal sources (1:0), all plant sources (0:1), or 3 intermediate ratios (animal protein:plant protein; 2:1, 1:1, and 1:2). The animal feedstuffs were fish and shrimp-head meals at a 1:1 protein ratio. The plant feedstuffs were rice bran, wheat bran, and soybean meal, at a 1:1:2 protein ratio, respectively. The methods used for proximate analyses were as follows: protein using a micro-Kjeldahl procedure (Barnes, 1959); lipid determined gravimetrically using chloroform methanol extract (Bligh and Dyer, 1959); carbohydrate using the phenol-sulfuric acid method of Dubois et al. (1956) and ash and gross energy according to modifications of Association of Official Analytical Chemists method (Association of Official Analytical Chemists, 1984).

For preparation of the feeds, the dry ingredients and lipid source were mixed in a covered Hobart mixer (Hobart Corporation, Troy, Ohio) for 20 minutes. Hot deionized water was then added to the mixture, and a meat grinder with a 2 mm die was used to pellet the feeds. Alginate was used according to the method reported by Meyers (1981) to make the feed pellets stable in seawater. The feeds were dried by forced air at 50°C for about 12 h to a moisture content of 8 to 10%. Feeds were crumbled to desired size and stored in an air-conditioned room at 15°C until used.

For the laboratory system, the shrimp were fed in excess (approximately 10% body weight/day) 4 times/day at 6-h intervals. For the pond system, the shrimp were fed twice per day (at 0800 and 1800 h) using a previously published feed rate schedule (Chamberlain et al., 1981). The duration of all experiments was 28 days.

Juvenile *P. setiferus*, having initial sizes of 0.8 g and 1.5 g, and *P. vannamei*, having initial sizes of 0.7 and 1.7 g, were used. Mated *P. setiferus* females were sourced in the Gulf of Mexico, near Corpus

Table 1. Ingredient Composition of Experimental Feeds[a]

Ingredients	Experimental Feed, Animal:Plant Protein Ratio[b]				
	1:0	2:1	1:1	1:2	0:1
Shrimp-Head Meal[c]	27.8	18.5	13.9	9.3	—
Fish Meal[d]	18.6	12.4	9.3	6.2	—
Rice Bran[e]	—	11.3	16.9	22.5	33.8
Wheat Bran[f]	—	10.7	16.0	21.3	32.0
Soybean Meal[f]	—	8.7	13.1	17.4	26.2
Corn Starch[g]	39.0	26.8	19.7	13.2	0.2
Cellulose[g]	7.1	4.1	3.6	2.6	0.3
Constants[h]	7.5	7.5	7.5	7.5	7.5

[a]Values are % of feed on an as fed basis.
[b]Animal protein consists of an 1:1 ratio of shrimp meal:fish meal on a protein basis; plant protein consist of a 1:1:2 ratio of rice bran: wheat bran:soybean meal on a protein basis.
[c]Blum and Bergeron, Houma, LA.
[d]Zapata-Haynie Co., Houston, TX.
[e]Riviana Foods, Inc., Houston, TX.
[f]Continental Grains, Houston, TX.
[g]ICN Nutritional Biochemicals, Cleveland, OH.
[h]Each diet contains: 0.50% cholesterol[g], 1.00% lecithin[g], 2.00% vitamin package[g], 1.00% mineral supplement[g], 2.00% alginate[i], and 1.00% sodium hexametaphosphate[j].
[i]Kelco, San Diego, CA.
[j]Calgon Water Softner, Pittsburgh, PA.

Table 2. Proximate Composition of Experimental Diets[a]

Item	Experimental Diet, Animal:Plant Protein				
	1:0	2:1	1:1	1:2	0:1
Protein[b]	28.0 ±0.3	27.6 ±1.4	27.1 ±0.8	26.8 ±0.9	28.3 ±0.8
Lipid[b]	5.2 ±0.1	5.1 ±0.1	5.1 ±0.1	4.9 ±0.3	5.2 ±0.2
Carbohydrate[b]	40.3 ±0.9	38.7 ±0.8	38.3 ±0.8	39.7 ±0.4	39.7 ±0.3
Ash[b]	12.0 ±0.8	11.9 ±0.2	10.5 ±0.9	10.9 ±0.6	11.5 ±0.7
Gross energy[b]	3.8 ±0.2	4.1 ±0.1	3.9 ±0.6	3.8 ±0.1	3.7 ±0.3
Fiber[c]	10.0	10.0	9.7	10.0	10.0

[a]Values are % of diet (dry weight bases), except gross energy, which is kcal/g.
[b]Values are mean ± S.E.M. of 3 replications for each dried diet by proximate analysis (as formulated).
[c]Values estimated from published data (National Research Council, 1983).

Table 3. Dissolved Oxygen, Temperature, and Salinity Values During Experiments

Tanks or Pens	Initial Stocking Density	Dissolved oxygen (ppm)		Temperature (C)		Salinity (ppt)
		a.m.	p.m.	a.m.	p.m	
		For Experiments Involving *Penaeus setiferus*				
Tanks	29/m²	6.2±0.1		28.6±0.1		28±1
Pens	15/m²	5.2±0.2	9.0±0.3	24.6±0.6	28.0±0.6	32±1
Pens	30/m²	4.2±0.3	8.4±0.3	24.6±0.6	27.8±0.6	32±1
		For Experiments Involving *Penaeus vannamei*				
Tanks	20/m²	5.3±0.2		25.0±0.5		35±1
Pens	15/m²	5.6±0.1	8.1±0.2	27.3±0.2	31.1±0.3	32±1
Pens	20/m²	5.2±0.2	9.5±0.3	23.1±0.5	25.8±0.6	35±1

Values are mean ± S.E.M. of 28 observations.

Christi, Texas (latitude 27°40'N, longitude 97°20'W), whereas *P. vannamei* were matured and mated in the laboratory operated by Texas A&M University at Corpus Christi. Both species were spawned in the laboratory, and the nauplii were reared to 5-day old postlarvae at the larviculture facility operated by Texas A&M University at Galveston, Texas, and to juvenile shrimp in ponds at the Texas A&M University facility at Corpus Christi, Texas.

For the pen experiments, shrimp were stocked outside and inside the pens at identical initial stocking densities. The shrimp outside the pens were fed similarly to the pen groups with the exception that the shrimp were presented a 30% protein commercial diet.

Nutritional response of the shrimp to the experimental feeds was evaluated using the following parameters: (1) shrimp weight gain (final mean wet weight-initial mean wet weight); (2) percent growth [(final mean wet weight-initial mean wet weight)/ initial mean wet weight] x 100; (3) survival [(initial number of animals-final number of animals)/initial number of animals] x 100; and (4) final biomass (final wet weight of shrimp /m²).

Conceptually, the nutrients required for the increase in shrimp biomass for the experiments conducted in ponds could either come from the presented feeds or from natural productivity. An estimation of the percent of the increase in shrimp biomass from nutrients obtained from natural productivity can be made using the following equation: final biomass minus initial biomass of unfed shrimp (growth from nutrients from natural productivity only) divided by final biomass minus initial biomass of fed shrimp (growth from nutrients from presented feed and natural productivity) times 100.

Growth and survival responses were tested for difference through analysis of variance (ANOVA) with 0.05 as the level of significance. Responses expressed as percentages were transformed and tested likewise. Significant levels of differences between parameters were tested with Duncan's New Multiple Range comparison (DMNR) test (Ott, 1977).

Results

Water quality data for all experiments are given in Table 3. A minimum of 4.2 to 6.2 ppm dissolved oxygen was observed about 1 h after sunrise. Salinities ranged from 28 to 35 ppt. For the experiments conducted in the ponds, the mean minimum and maximum temperatures ranged from 23 to 26 to 27 to 31 °C when *P. vannamei* was used and 25 to 28 °C when *P. setiferus* was used. For the experiments conducted in the laboratory, the mean temperature was 25 and 28 °C for *P. vannamei* and *P. setiferus*, respectively.

All experimental feeds were formulated to contain 25% protein, 5% lipid, 11% ash, and 10% fiber. Proximate analysis showed that the desired lipid and ash was obtained, but the protein level in the

Table 4. Survival, Growth, and Final Biomass for *Penaeus setiferus* and *Penaeus vannamei* Reared in Tanks

Feed (Animal:Plant Protein Ratio)	Percent Survival (%)	Weight Gain (gm)	Percent Growth (%)	Final Biomass (gm/m²)
For *Penaeus setiferus* at 29/m² stocking density.				
1:0	95±2 [a]	2.1±0.1 [a]	265±10 [a]	79.9±1.9 [a]
2:1	98±2 [a]	1.6±0.1 [b]	200± 6 [b]	68.2±2.1 [b]
1:1	96±2 [a]	1.5±0.1 [b]	188± 6 [bc]	64.0±1.9 [bc]
1:2	94±3 [a]	1.4±0.1 [b]	175± 9 [c]	60.0±1.7 [c]
0:1	99±1 [a]	0.6±0.1 [c]	75± 5 [d]	40.2±1.6 [d]
For *Penaeus vannamei* at 20/m² stocking density.				
1:0	95±2 [a]	3.1±0.1 [a]	182±28 [a]	91.2±4.8 [a]
1:1	93±4 [a]	2.9±0.1 [a]	171±17 [a]	85.6±3.4 [a]
0:1	94±3 [a]	2.3±0.1 [b]	135± 9 [b]	75.2±3.7 [b]

Values are means ± S.E.M. of 5 replications.
Values with common letters are not statistically different ($p < 0.05$) for different diets. Statistical comparison is for the same parameter.
The initial mean ± S.E.M. weight was 0.80±0.01 gm and 1.7±0.1 gm for *P. setiferus* and *P. vannamei*, respectively.

experimental feeds varied from 26.8 to 28.3% with a mean of 27.56%. Thus, the average protein level was above the formulated value. However, there were no significant differences for the protein, lipid, carbohydrate, ash, and gross energy levels for the five experimental feeds.

For the laboratory experiments, there was no significant effect on survival for either species fed any of the feeds (Table 4).

There was a significant decrease in growth for *P. setiferus* whereas the lower growth was not significant for *P. vannamei* fed the feed containing a 1:1 ratio of animal to plant protein versus a feed containing all animal protein. The growth for both species was significantly reduced for shrimp fed feeds containing only plant protein versus feeds containing an 1:1 ratio of plant to animal protein.

Similar to the laboratory experiments, there was no significant effect on survival for either *P. vannamei* (86 to 90% and 73 to 79% for experiments initially stocked at 15/m² and 30/m², respectively) or *P. setiferus* (80 to 86% and 90 to 95% for experiments initially stocked at 15/m² and 20/m², respectively) fed any feed in earthen ponds (Tables 5 and 6).

There were no significant changes in weight gain, percent growth and final biomass for *P. vannamei* initially stocked into pens in ponds at 15/m² and 30/m² and fed any of the feeds (Table 5). However, the unfed *P. vannamei* had significantly less weight gain, percent growth, and final biomass than all of the fed groups. Similar to *P. vannamei*, there were no significant changes in weight gain, percent growth, and final biomass for *P. setiferus* initially stocked at 15/m² and fed any of the feeds (Table 6). However, when the initial stocking density was increased to 30/m², the weight gain, percent growth, and final biomass all were significantly less for shrimp fed the feed containing all plant protein versus feeds containing all or some animal protein. Similar to *P. vannamei*, unfed *P. setiferus* had significantly less weight gain, percent growth, and final biomass than any fed group.

For the experiments conducted in pens, shrimp also were initially stocked outside the pens at the same density. For all four experiments conducted in earthen ponds the percent survival, weight gain, percent growth, and final biomass were not significantly different from shrimp fed the feed containing all

Table 5. Survival, Growth, and Final Biomass for *Penaeus vannamei* Reared in Pens in Earthen Ponds

Feed (Animal:Plant Protein Ratio)	Percent Survival (%)	Weight Gain (gm)	Percent Growth (%)	Final Biomass (gm/m²)
At 15/m² stocking density, 0.7 gms initial weight				
1:0	83±9[a]	5.2±0.4[a]	743±18[a]	73.5±1.9[a]
2:1	80±8[a]	5.1±0.2[a]	730±12[a]	69.6±2.1[a]
1:1	81±9[a]	5.2±0.3[a]	743±20[a]	71.7±1.7[a]
1:2	86±4[a]	4.8±0.2[a]	686±13[a]	71.0±1.5[a]
0:1	85±6[a]	5.0±0.3[a]	714±12[a]	72.7±1.6[a]
Fed[c]	83	5.1	723	71.7
Unfed	83±5[a]	4.2±0.2[b]	600±22[b]	61.0±3.1[b]
At 20/m² stocking density, 1.7 gms initial weight.				
1:0	93±4[a]	3.0±0.3[a]	176±12[a]	87.4±3.8[a]
2:1	92±4[a]	3.1±0.2[a]	182±19[a]	88.3±1.9[a]
1:1	95±2[a]	3.0±0.1[a]	176±14[a]	89.3±2.9[a]
1:2	94±1[a]	3.1±0.1[a]	182±11[a]	90.2±2.2[a]
0:1	93±4[a]	3.0±0.2[a]	176±13[a]	87.4±3.5[a]
Fed[c]	93	3.0	178	88.1
Unfed	92±9[a]	2.4±0.2[b]	141±11[b]	75.4±3.9[b]

Values are means ± S.E.M. of 7 replications.
Values with common letters are not statistically different ($p < 0.05$) for different feeds. Statistical comparison is for the same parameter.
[c]Values represent means for the groups fed whose values are not significantly different for the same parameter.

animal protein and for shrimp stocked outside the pens fed a 30% protein commercial feed.

The estimation of percent contribution of natural productivity to the nutritional requirements for both *P. setiferus* and *P. vannamei* decreased from 76 to 69% and 82 to 77%, respectively, with increased stocking density, 15/m² to 30/m² and 15/m² to 20/m², respectively, and increased harvest biomass, 79.5 gm/m² to 127.1 gm/m² and 71.9 gm/m² to 88.1 gm/m², respectively (Table 7).

It is interesting that with similar harvest biomasses of 71.9 gm/m² and 88.1 gm/m² for *P. vannamei* and 79.5 gm/m² for *P. setiferus* that the estimated percent contribution of natural productivity to *P. vannamei* requirements is higher (82 and 77%) than for *P. setiferus* (76%).

Discussion

The salinity and dissolved oxygen values for all the experiments conducted in the laboratory and pond system are desirable for shrimp growth and survival (Clifford, 1985). Although Clifford also recommended that 23°C was the lowest temperature acceptable for growth, because of temperature effect on shrimp growth (Zein-Eldin and Griffith, 1969), the different temperatures ranging from 23 to 25°C versus 27 to 31°C for two different experiments using pens for *P.*

Table 6. Survival, Growth, and Final Biomass for *Penaeus setiferus* Reared in Pens in Earthen Ponds

Feed (Animal:Plant Protein Ratio)	Percent Survival (%)	Weight Gain (gm)	Percent Growth (%)	Final Biomass (gm/m²)
At 15/m² stocking density, 1.5 gms initial weight.				
1:0	88±2[a]	4.6±0.2[a]	306±18[a]	80.5±1.9[a]
2:1	86±2[a]	4.8±0.2[a]	320±12[a]	81.3±2.1[a]
1:1	90±2[a]	4.4±0.2[a]	293±20[a]	79.7±1.7[a]
1:2	88±3[a]	4.5±0.2[a]	300±13[a]	79.2±1.5[a]
0:1	88±1[a]	4.3±0.2[a]	286±12[a]	76.6±1.6[a]
Fed[d]	88	4.5	301	79.5
Unfed	88±4[a]	3.5±0.3[b]	233±18[b]	66.0±1.7[b]
At 30/m² stocking density, 1.5 gms initial weight.				
1:0	79±5[a]	3.8±0.1[a]	253±13[a]	125.6±6.3[a]
2:1	78±3[a]	4.2±0.2[a]	280±15[a]	133.4±3.9[a]
1:1	75±3[a]	4.0±0.2[a]	267±12[a]	123.8±6.4[a]
1:2	79±4[a]	3.8±0.1[a]	253±12[a]	125.6±7.2[a]
Fed[d]	78	4.0	263	127.1
0:1	77±5[a]	3.3±0.1[b]	220± 8[b]	110.0±2.5[b]
Unfed	75±8[a]	3.0±0.1[c]	200± 5[c]	101.3±2.5[c]

Values are means ± S.E.M. of 7 replications.
Values with common letters are not statistically different ($p < 0.05$) for different feeds. Statistical comparison is for the same parameter.
[d]Values represent means for the groups fed whose values are not significantly different for the same parameter.

vannamei and the different temperatures for the experiments conducted with *P. vannamei* versus *P. setiferus* makes it difficult for comparison of growth between experiments and species.

A 25% protein level was selected for the experimental feeds since 22 to 28% protein levels are used for commercial shrimp production in Ecuador (personal communication, A.L. Lawrence, Texas A&M University, Port Aransas, TX, 78373) and it is below the reported required protein level for *P. setiferus* and *P. vannamei*. Lee and Lawrence (1985) reported that *P. setiferus* grew faster in indoor tanks when fed 30, 38, and 50% protein rations as opposed to a 22% protein feed and Andrews et al. (1972) reported optimal dietary protein level for *P. setiferus* to be in the range of 28 to 32%. The recommended protein level for *P. vannamei* has been reported to be 30% (Colvin and Brand, 1977; Smith et al., 1985). Although the actual protein level determined for the prepared feeds was 26.8 to 28.3% and higher than predicted, these levels are still limiting for maximum growth for these two species.

Conceptually, for feeds containing inadequate amounts of protein required for maximum growth, as the amount of plant protein increases and the quality of the feed for shrimp growth decreases there should be a further decrease in growth. Using feeds containing 28% protein, *P. setiferus* in aquaria grow faster as the proportion of animal to plant protein increased (Chen et al., 1985). Data in this paper also indicates that, as the plant protein is increased in the feed, growth of *P. setiferus* is decreased in the laboratory.

Table 7. Estimated Percent Contribution of Natural Productivity[a] to Nutritional Requirements of *Penaeus setiferus* and *P. vannamei* in Earthen Ponds

Species	Initial Stocking Density		
	15/m²	20/m²	30/m²
setiferus	76%[a]	N.D.[b]	69%[a]
vannamei	83%[a]	77%[a]	N.D.[b]

[a]Values in percent contribution of natural productivity as estimated using the following equation: final biomass minus initial biomass of unfed shrimp divided by final biomass minus initial biomass of fed shrimp times 100.
[b]Values not determined.

The data also suggest that this is true for *P. vannamei*. This suggests that essential amino acids are limiting in the experimental feeds and, as plant protein is increased, they become more limiting resulting in decreased growth.

Growth experiments conducted in pens in earthen ponds show that there was no decreased growth when the animal protein in the experiment feed was reduced by half for *P. setiferus* and when all the animal protein was replaced by plant protein for *P. vannamei*. In both cases, this indicates that natural productivity was being utilized by both species. Other scientists have also made this same conclusion (Schroeder, 1983; Maguire and Leedow, 1983; Maguire and Bell, 1981). These data also indicate that lower quality feeds can be used for commercial production of shrimp if natural productivity is being utilized by shrimp in an earthen pond.

The estimation of percent contribution of natural productivity from the data obtained in this study indicate that there was a percent contribution of 67 to 82% for *P. vannamei* stocked at 15/m² and 20/m² with harvest biomasses of 71.9 gm/m² to 88.1 gm/m². Anderson et al. (1987) and Parker et al. (1989) obtained a similar estimated percent contribution of natural productivity of 53 to 77% and 44 to 86% for assimilation of organic carbon by *P. vannamei* for similar stocking densities and harvest biomasses using a different experimental approach, stable carbon isotope tracer techniques. This similarity indicates that the method used in this paper can be used for estimating the contribution of natural productivity to the nutritional requirements of shrimp in earthen ponds.

Conceptually, as the stocking density and harvest biomass increase in an earthen pond, the percent contribution to the nutritional requirements of shrimp would decrease if all other factors are the same. The data reported in this paper for both species support this concept. Further, using higher stocking densities with greater harvest biomasses than used in this study, a lower estimated percent contribution of natural productivity to the nutritional requirements of *P. vannamei* (39 to 42%, Robertson et al. 1992c) and *P. setiferus* (about 68%, Robertson et al., 1992a) was obtained for *P. vannamei* (77 to 83%) and *P. setiferus* (69 to 76%) as reported in this study.

The data in the present study also indicate that *P. setiferus* requires a higher protein level in their diet than *P. vannamei* for two reasons. First, the growth of *P. setiferus* was significantly reduced in the laboratory when 33% of the animal protein was replaced with plant protein whereas *P. vannamei* growth was not significantly reduced when 50% of the animal protein was replaced with plant protein. Secondly, there was a significant reduction in *P. setiferus* but not *P. vannamei* growth in pens in earthen ponds when all of the animal protein was replaced with plant protein. Chen et al. (1985) also concluded that *P. setiferus* requires more animal protein in its diet than *P. vannamei*.

The data reported in this study indicate that the contribution of natural productivity to the nutritional requirements of shrimp in ponds must be considered in formulating cost-effective feeds for commercial production of shrimp in ponds.

Conclusions

1. A pen system in earthen ponds can be used to evaluate pond culture requirements for shrimp.
2. An increasing level of plant protein in feeds containing inadequate levels of total protein decreases growth when fed to *P. setiferus* and *P. vannamei*.
3. *P. setiferus* requires more animal protein in their diet than *P. vannamei*.
4. Feed and natural productivity both significantly contribute to the nutritional requirements of shrimp in earthen ponds.
5. The greater the stocking density and harvest biomass the less the percent contribution of natural productivity to shrimp nutritional requirements in an earthen pond.
6. Lower quality feed and/or lower feed rate can be used to culture shrimp in earthen ponds when natural productivity is be consumed by shrimp.
7. Only laboratory research in the absence of natural productivity is inadequate for determining

absolute nutritional requirements for a cost-effective feed for commercial production of shrimp in earthen ponds.

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