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CHARACTERIZATION OF THIAMINE DEFICIENCY IN CHANNEL CATFISH (*Ictalurus punctatus*, RAFINESQUE) FED HEATED AND NON-HEATED CATFISH PROCESSING WASTE

Arsenio Sabañgan Camacho

Certificate of Approval:

R. Allison, Associate Professor, Fisheries and Allied Aquacultures

R. T. Lovell, Chairman Associate Professor Fisheries and Allied Aquacultures

J. S. Dendy, Professor Fisheries and Allied Aquacultures

M. E. Lisano, Assistant Professor, Zoology-Entomology

H. F. Tucker, Associate Professor, Animal and Dairy Sciences

Paul F. Parks, Dean Graduate School

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Arsenio Sabañgan Camacho

A Dissertation
Submitted to
the Graduate Faculty of
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in Partial Fulfillment of the
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Degree of
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VITA

Arsenio S. Camacho, son of Arsenio C. Camacho and Amparo D. Sabañgan, was born January 23, 1945, in Baliwag, Bulacan, Philippines. He attended Caloocan Elementary School from 1951-1957. He attended Torres High School, Manila, from 1957-1961. He went to senior high school in Pewaukee, Wisconsin, U.S.A. from 1961-1962 as an exchange student under the American Field Service International Scholarship Program. He entered the University of the Philippines as a Science Talent Search grantee of the National Science Development Board (NSDB) and received the degree of Bachelor of Science in Zoology in May, 1966. After two years of teaching in the College of Fisheries, University of the Philippines, he entered the Graduate School of Arts and Sciences, University of the Philippines as an NSDB graduate scholar and completed all course requirements for a Masters' degree in Zoology, in 1970. In 1971, he was selected to receive fish culture training in Auburn University, under a NEC-AID scholarship program in connection with the Philippine Inland Fisheries Project.

He married Elizabeth, daughter of Pascual E. Jose and Cirila Apresto in December 19, 1968. They have two children, Maria Lenora and Juan Carlos.

DISSERTATION ABSTRACT

CHARACTERIZATION OF THIAMINE DEFICIENCY IN CHANNEL CATFISH (*Ictalurus punctatus*, RAFINESQUE) FED HEATED AND NON-HEATED CATFISH PROCESSING WASTE

Arsenio Sabañgan Camacho

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Channel catfish fingerlings, stocked at 6,000 per acre in 0.005-acre concrete tanks were fed 32% protein, nutritionally adequate, moist fermentation-preserved feeds containing raw or heated catfish waste for 120 days. Fish were also fed diets prepared in dry, pelleted form containing catfish waste or fish meal.

Fish fed the fish meal diet gained approximately 20% more than those fed any of the rations containing catfish waste. Gain by fish fed preheated waste in a moist, fermentation-preserved diet was equal to that by fish fed the waste in a dry, pelleted feed, indicating that moist, fermentation-preserved feeds are suitable for feeding catfish.

Slightly poorer growth response by fish fed the raw waste diet indicated that catfish waste possibly contains a heat labile anti-nutritional factor. A subsequent feeding trial was conducted in which channel catfish fingerlings were fed diets containing raw or heated waste which were sufficient in all nutrients except thiamine. Fish fed rations containing either raw or heated waste to which no thiamine was added exhibited a syndrome associated with thiamine deficiency.

Chemical tests showed that catfish skin and viscera contained a heat labile anti-thiamine factor but its activity was low. Low pH of 4.5 associated with fermentation did not inactivate the anti-thiamine factor.

These studies indicate that waste from catfish is severely deficient in thiamine. If the waste is not heated prior to mixing in a fish feed, the thiamine supplement should be fed in a separate feed.

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I. INTRODUCTION

Recovery of wastes from catfish processing plants, which comprises approximately 40% of the total weight entering the plant, represents a potential source of protein for use in fish and livestock feeds. In 1970 the total weight of catfish processed in 18 processing plants located in the states of Alabama, Mississippi, Arkansas, Tennessee and Georgia amounted to about 8,950,000 pounds. Unfortunately, none of these processing plants has large enough volume of production to afford the necessary facilities for drying the waste (heads, skins and viscera) into meal. In most instances the waste has no practical value, in fact its disposal may even be a liability to the processor. One method for utilizing the waste is feeding it to fish or animals in a non-dried form. Feeding unprocessed waste presents several problems, namely, preservation and the possibility of anti-nutritional factors in the raw waste. These problems, in addition to an evaluation of the protein quality of catfish waste, were evaluated by feeding moist and dry diets containing appreciable amounts of raw and heat processed waste to channel catfish.

The initial phase of the research concerned a feeding trial in which catfish waste, in raw or heat processed forms,

was compared with commercial fish meal for growing channel catfish to harvestable size. Test rations contained one-third catfish waste, on a dry matter basis, and two-thirds dry ingredients, to produce 32% protein, isocaloric, nutritionally complete feeds. One test diet was dried and pelleted, another contained the raw waste and was prepared in moist form, another contained pasteurized waste and was fed in moist form, and the fourth ration was a dry-pellet diet of similar composition except that the animal protein came from commercial fish meal. The moist diets were placed in hermetically sealed containers and fermented for preservation.

Relatively poorer growth response of the catfish to the raw waste diet led to another phase of investigation which concerned the cause of the growth repression. Laboratory studies were conducted to determine the presence of an anti-nutritional enzyme, specifically thiaminase, in the raw waste. Feeding trials with channel catfish fingerlings in aquaria were used to evaluate anti-thiamine activity of raw waste and heat processed waste, each with supplemental thiamine fed simultaneously or separately. Chemical assays were made for anti-thiamine factors in the raw and heated waste. Thiamine activity in moist diets containing raw and heated waste was measured in fresh and fermentation-preserved samples.

II. REVIEW OF LITERATURE

Nutritional Value of Catfish Waste

Based on samples collected from a number of sources throughout all seasons of the year, Lovell (1973) reported that, on a moisture-free basis, catfish waste contained 42% crude protein, 35% fat, 5.4% calcium and 2.8% phosphorus. A recent publication (National Research Council, 1973) indicated that the content of lysine, methionine, cystine, isoleucine and threonine are lower in catfish waste than in fish meal.

Catfish fat was an excellent source of energy in catfish feeds and did not adversely affect flavor of fish consuming the feed which contained up to 7.3% catfish fat (Lovell, 1973).

In a recent work, Koonsoongnoen (1974) compared the nutritional value of catfish waste with that of fish meal, meat and bone meal, and casein. Protein efficiency ratio (PER) values indicated that catfish waste protein was about twice as valuable as meat and bone meal but was slightly inferior to fish meal and casein.

The nutritional value of catfish waste in practical-type swine feeds was initially evaluated with laboratory rats (Lovell, 1971). The diets contained 70% wet catfish waste,

30% corn, and vitamin and mineral supplements, which on a dry matter basis, contained 16% crude protein. The diets were preserved by fermentation and freezing and fed in moist form. Growth data showed that the PER was greater for the catfish waste ration than from a control swine ration. A feeding trial with swine by Lovell and Tucker (1973) showed that catfish waste can substitute for meat and bone meal as a source of animal protein in productive swine rations.

Utilization of Catfish Processing Waste

Processing of catfish includes removal of the head, viscera, and skin, and other activities such as packaging and storing, to yield a product that is attractive to a discriminating consumer (Lee, 1973). The procedures used by several processing plants are almost the same, certain differences being in the extent of mechanization and arrangement of equipment (Lee, 1973).

In 1964 there was only one major catfish processing plant in operation in the United States (McCoy and Sherling, 1973). By 1971 the list of major catfish processing plants in the southeastern United States included 21 major firms, of which seven were in the state of Alabama (Adrian, McCoy and Sherling, 1971). A commercial processor is defined arbitrarily as one who has the capability to produce 5,000 pounds of finished product per 8-hour shift day.

The estimated annual volume of five catfish processors in 1970 was about 17 million pounds (Greenfield, 1970).

Fish waste (heads, skin, and viscera) constitutes 40-45% of the live weight of fish processed and represents 6 to 7 million pounds of byproduct which could be processed into animal feed ingredients, protein hydrolysates, commercial hormones, and industrial oils.

Seasonality of catfish production and the scattered location of the catfish processing plants cause commercial drying of the waste into meal to be of questionable feasibility. Because of the large installation cost of commercial drying system, one practical use for catfish waste may be as an ingredient in moist feeds for swine or fish as suggested by Lovell (1973). He recommended that processing waste be heated, ground and mixed with dry ingredients to yield a 16% protein ration for swine. For catfish feeds containing processing waste that are to be fed back to fish in the same production system, the waste may not require heating before feeding.

Anti-Nutritional Factors in Fish Waste

A. Historical Review--The incorporation of raw fish in diets fed to foxes resulted in severe outbreaks of a paralyzing and fatal disease. This was first noted by Green in 1832 on the fur farm of J. S. Chastek of Glencoe, Minnesota. From the very start, it was shown that the so-called "Chastek paralysis" syndrome was neither food poisoning nor a transmissible disease. Green and Evans (1940), and Green, et al. (1941) published descriptions of the disease as being

characterized mainly by anorexia, general weakness, progressive ataxia, spastic paraplegia and death within 48-72 hours after onset of neurologic symptoms. In these early studies, and subsequently by Evans, et al. (cited in Yudkin, 1949), the pathology of the disease was described. Gross examination of the heart showed massive degeneration with cloudy swelling, whereas microscopic examination revealed large nucleoli and focal necrosis in myocardial fibers, proliferation of connective tissue around the vascular tree, and hemorrhages. The liver showed degeneration. Most significant, however, were the lesions of the central nervous system. The lesions, characterized by irregular dilatation or varicose deformations with marked proliferation of the endothelial cells and adventitia, were found ventral to the floor of the fourth ventricle, in the quadrigeminal plate, in certain nuclei ventral and lateral to the aqueduct of Sylvius, in the thalamus, and in certain areas of the cerebral cortex. Microscopic examination revealed that the lesions were bilaterally symmetrical and did not extend to the white matter.

This disease in foxes was shown to be a result of the destruction of thiamine in the diet before being absorbed from the gastrointestinal tract (Green, et al., 1941). Since more severe disorders resulted when raw fish was mixed with the ration than when the fish was fed separately or with supplements of vitamin B_1, it was concluded that the active

principle in the fish reacted with the dietary thiamine to either destroy or inactivate it (Green, et al., 1941, 1942). Green and Evans (1940) reported dramatic recovery in foxes already in the paralytic stage of the disease when they were fed milk, liver juice and small amounts of yeasts.

Thiamine deficiency was also produced in chicks by Spitzer, et al. (1941) by incorporating 25% raw whole carp in the diet. Schneberger (1941) observed that in order to maintain vigor in trout, it was necessary to add fresh meat and cystalline B_1 to their diet in which canned carp was utilized. Typical thiamine deficiency symptoms in rainbow trout fed diets containing raw carp were alleviated by injection of cystalline B_1 or by addition of dried brewers yeast to the diet. Wolf (cited in Yudkin, 1949) and Wolf (1952) described a disease of brook, brown and rainbow trouts caused by diets containing raw fish. The affected fish exhibited loss of balance causing fish to lie on their sides at the bottom of the container. When disturbed the fish exhibited great excitability, dashing about violently. These side swimmers eventually weakened, lost their appetite, and died. Woodbury (1942) described similar symptoms in the case of hatchery-reared rainbow trout which were fed a diet composed mainly of canned carp with fresh meat supplement.

Smith and Proutt (1944) fed a diet containing raw carp to cats. The deficiency symptoms, which showed in 23 to 40 days, were anorexia, weight loss, staggering gait due to

ataxia of hind legs, increasing weakness, circus movement, head ventroflexion, loss of righting reactions, convulsions and death. Harrington (1954) reported that thiaminase-induced B_1 avitaminosis resulted when tadpole madtoms (Schilbeodes mollis) were fed exclusively on carp eggs. The fish began to get sick and die as early as the 17th day, with a majority of the fish dying within 30 days.

Since 1942 work on the anti-thiamine active principle has involved the methods of extraction, assay, and chemical properties and the nature of its reaction with thiamine. The physiological function of the enzyme has not been established to any extent.

B. Distribution--The occurrence of a thiamine destructive principle in fish and other organisms has been confirmed by either feeding them to subject animals and observing whether the symptoms of the deficiency could be produced or by determining the amount of thiamine destruction through an in vitro incubation of the vitamin with extracts of the animal or plant material to be tested. Deutsch and Hasler (1943), using an in vitro method on whole fish viscera, examined 31 species of fish in the Great Lakes region for presence of a thiaminase. Fifteen of 22 freshwater species contained the enzyme, whereas none of the nine marine species examined showed the activity. Wolf (cited in Yudkin, 1949) reported the presence of thiaminase in a

salt-water fish, the Atlantic herring (Clupea harrengus) from feeding experiments with trout.

Lieck and Agren (1944) examined 30 species of fish caught in Swedish Lakes, the Baltic Sea and the Kattegat. The presence of a thiamine-inactivating factor was demonstrated in ten species, nine of which belonged to the carp family. In an investigation concerning the possible use of certain trash fish in the Long Island Sound area, Yudkin (1945) did not observe thiaminase activity in four marine fish. Neilands (1947) reported the presence of thiaminase in the stomach contents of some white and yellow perch but recognized that this finding could be attributed to the fact that the enzyme might have been present in the food of these animals. Deolalkar and Sohonie (1954) found the enzyme in six species of fish from freshwater, brackish water, and salt water environments in India. Lee, et al. (1955) conducted chemical assays for several shrimp trawl trash fish in the northern Gulf of Mexico and found high levels of thiaminase in the razor belly and moray eel. Hilker and Peter (1966) examined various species of fish in Hawaiian waters where 21 of 30 species tested contained thiaminase. Extensive listings of marine and freshwater organisms containing thiaminase are presented by Lee (1948), Harris (1951) and Greig and Gnaedinger (1971).

The first quantitative study of the distribution of thiaminase in several organs in carp was performed by

Sealock, et al. (1943). The spleen was found to contain the greatest concentration, amounting to as much as 25 activity units per gram; the liver, pancreas, gastro-intestinal tract and gills contained 1.5-8.7 units per gram; the kidney and blood, 0.8-1.4 units; the ovaries contained 0.6 units while the heart, testes, brain, gall bladder, bile and eyes contained less than 0.3 unit per gram. The swim bladder and muscle did not show any thiaminase activity. A similar work was undertaken for certain species of Japanese fish by Fujita, et al. (cited in Fujita, 1954). They found that the greatest concentration of the enzyme was in the kidneys, liver and spleen.

C. Enzyme Hypothesis--Thiaminase behaves as a typical enzyme in that it is thermolabile, protein in nature, exhibits optimum pH and temperature for its activity, shows velocity constants and is subject to both activation and inhibition by selected chemical compounds. Spitzer, et al. (1941) incubated raw or cooked carp entrails with thiamine hydrochloride and attempted to recover the added vitamin by means of the thiochrome method. They showed that the destruction of thiamine occurred in the feed mixture, the inactivation being proportional to the length of time it is in contact with the raw entrails. In this experiment they also tested the heat lability of the factor. Woolley (1941) obtained extracts of fresh carp tissue that inactivated thiamine *in vitro*, using 10% NaCl solution. Aqueous extracts

had only 25% of the activity of the whole carp suspensions. He postulated that the factor is an enzyme which destroyed thiamine or protein which combined with thiamine to render it unavailable. Sealock, et al. (1943) prepared a stable, desiccated powder from acetone-extracted carp viscera from which they isolated the active principle with 10% NaCl. Maximum disappearance of thiamine occurred at pH 9.1 with corrections for loss due to alkaline hydrolysis. Activation of the principle was greatest at 68 C whereas raising the temperature to 100 C for 30 minutes completely inactivated the principle. They found that the disappearance of thiamine was approximately proportional to the amount of extract used and fitted more or less the kinetics of a first-order reaction.

Proof of the enzyme hypothesis was demonstrated by Krampitz and Woolley (1944) who isolated and characterized the degradation products from thiamine subsequent to action of a factor in carp tissue. The reaction which was confirmed microbiologically was essentially a hydrolytic cleavage of the thiamine molecule between the pyrimidine and thiazole rings, resulting specifically in the formation of 4-methyl-5-hydroxyethylthiazole and 2-methyl-4-amino-5-hydroxymethylpyrimidine. The formation of hydrogen ion in the cleavage of thiamine by the fish principle was subsequently demonstrated by Sealock and Livermore (1944). Krampitz and Woolley (1944) further noted that most of the thiamine destroying

potency of carp extract was lost upon dialysis against distilled water and that the activity of the extract was restored with the addition of the dialysate. The principle in the dialysate was found to be heat stable, whereas that in the undialyzable fraction was heat labile.

Lieck and Agren (1944) showed that the inactivation of thiamine by carp extract is not an oxidation process since there was no oxygen uptake noted on monometric experiments and there was no reduction of methylene blue in the experiment carried out in Thunberg tubes. Agren (1945a) observed that the enzymatic inactivation of thiamine appeared to be a reductive reaction requiring glutathione, cysteine and vitamin C. In this process thiamine was split into two halves. In another paper Agren (1945b) reported twenty-fold purification of thiaminase without considerable loss of activity. He postulated that the enzyme appeared to belong to a group of albumins with an isoelectric point close to pH 5.0.

Inhibition of the enzyme was shown by Sealock and Goodland (1944) upon the addition of cupric sulfate, zinc chloride, ferric chloride, and cysteine. They also showed that compounds resembling the thiamine molecule, namely, O-aminobenzyl and β-aminoethyl-4-methyl thiazolium chloride were strongly inhibitory.

Meanwhile, it was shown that the analog m-aminobenzylthiazolium salts failed to inhibit the enzymatic degradation of thiamine and actually caused increased

destruction or activation (Sealock and Livermore, 1949). Sealock and Davis (1949) stated that the activating effect of m-aminobenzyl 4-methyl thiazolium chloride is due to its combination, through its amino group, with the 5-methylene group of the pyrimidine moiety as the latter is split from thiamine during the reaction. They also showed that other amines, namely, m-nitroaniline and m-aminobenzoic acid are strong activators of the reaction.

D. Thermostable Factor--More recent literature concerning the anti-thiamine factor has demonstrated that the nature of thiamine inactivation by fish extracts is not entirely enzymatic in nature. This development parallels earlier findings with plant species. Bhagvat and Devi (cited in Lee, 1948) found that the oil-seed plants, ragi and cottonseed, have a predominantly heat stable-fraction which showed no loss of activity on autoclaving at 15 psi for 15 minutes or boiling for one-half hour. They concluded that the reaction was not enzymatic because of the stability of the active principle to heat. Meanwhile, the anti-thiamine factor of ferns was found to be composed of a thiaminase and thermostable factor which can be separated by repeated precipitation with acetone and dialysis at low temperature (Fujita, 1954).

The presence of a thermostable anti-thiamine factor or factors in fish was first reported by Somogyi and Kundig (1963). They reported that a thermostable factor,

crystallized from carp viscera appeared to be a large molecule containing amino acids but was not a typical protein. In the course of their analyses for anti-thiamine activity in various Hawaiian salt water fish Hilker and Peter (1966) noted that a thermostable factor in fish muscle was generally present in all species containing enzymatic activity. From a series of tests undertaken to study the effects of temperature and pre-boiling on the anti-thiamine activity of skipjack tuna muscle preparation, Hilker and Peter (1968) found that incubating the homogenates at temperatures above 75 C for a period of time enhanced the activity and concluded that the reaction was not enzymatic. In a subsequent paper by Tang and Hilker (1970) the anti-thiamine activity in skipjack tuna preparation was also found to be heat stable. Rutledge and Ying (1972) reported that boiling the cephalothorax of crayfish for as long as 30 minutes and oven-drying at 100 C for 24 hours failed to destroy all anti-thiamine activity.

III. MATERIALS AND METHODS

Phase 1

The first phase was a practical feeding test comparing 1) raw catfish waste fed in moist, fermentation-preserved diet, 2) pre-heated catfish waste fed in moist, fermentation-preserved diet, 3) catfish waste fed in a dry, pelleted diet, and 4) fish meal fed in a dry, pelleted diet, for growing channel catfish to harvestable size in concrete ponds. Chemical and microbiological methods were used to evaluate fermentation as a method of preservation of moist fish feeds.

Experimental Ponds--The experiment was conducted in 0.005-acre concrete ponds (9 × 24 × 2.5 feet) at the Fisheries Research Unit of the Auburn University Agricultural Experiment Station. Sixteen ponds, each with an independent water supply and drain system were utilized. Water in each pond was aerated using a 1/3 horsepower submersible pump equipped with a Venturi attachment made of polyvinyl-chloride pipe fittings. Timers were installed to provide aeration from 9 p.m. to 9 a.m. daily. Twice a month all ponds were overflowed overnight in an attempt to flush planktonic scums that accumulated periodically during the growth season. Two 1/2 horsepower submersible pumps were held in reserve for

supplementary use whenever a pond showed an oxygen level of 2 p.p.m. or less. Oxygen and temperature readings were obtained daily at dawn using a YSI Model 54 oxygen meter. The pH readings were obtained during the first month of the experiment.

Fish--The channel catfish used in the experiment were the Auburn strain and measured 5 to 7 inches in total length when stocked in the experimental ponds. Prior to stocking, the fish were treated in 200 p.p.m. of formalin for one hour, for external parasites. As a precautionary measure against the high pH readings (9.0-10.0) of the water in the concrete ponds, 100 ml of concentrated sulfuric acid was added to each pond approximately 24 hours before stocking. Immediately prior to stocking, the fish were anaesthesized with quinaldine to minimize stress during sorting and weighing. Thirty fish were stocked in each pond. As another safety measure against alkaline conditions in the water, 50 p.p.m. of $CaSO_4$ was added to each pond during the first week after the fish were stocked.

Experimental Diets--Four diets containing 32% crude protein were each fed to channel catfish fingerlings in four randomly assigned ponds. Before offering the experimental diets, the fish were conditioned for three weeks by feeding a dry pelleted catfish feed at the rate of 1.5% of fish weight per day.

The diets were formulated to contain the optimal levels of all nutrients necessary for the growth of channel catfish (Table 1). In diets 1-3, protein was provided from ground catfish waste and in diet 4 marine fish meal supplied an equal amount of protein. The remainder of the proteins in all diets was furnished primarily by equal proportions of soybean meal and peanut meal. Feed grade wheat flour was the source of carbohydrate and it allowed for good water stability of the moist feeds. The percentage of catfish waste used in the diets provided more fat and inorganic material than did the amount of fish meal used; consequently diet 4 was supplemented with lard and dicalcium phosphate to make it equal to the other diets on a protein, fat and ash basis. Based on chemical analyses, the following compositions of catfish waste and commercial fish meal were used in formulating the diets:

Nutrient	Catfish Waste	Fish Meal
Protein (% of dry matter)	48.9	64.8
Fat (% of dry matter)	33.5	10.6
Ash (% of dry matter)	16.0	14.3

A commercial vitamin premix containing all the necessary vitamins for the growth of channel catfish was used in all diets. Salt, added as a preservative (for the fermented feeds) and carboxy-methyl-cellulose (referred to hereafter as CMC) as a binder, were incorporated in all diets.

TABLE 1--Composition of experimental diets used to compare the nutritional value of catfish waste (CFW) with commercial fish meal as fish feeds.

Ingredients	Experimental Diets			
	1	2	3	4
	%	%	%	%
Catfish waste	60.00	60.00	60.00	
Commercial fish meal				15.17
Soybean meal	8.5	8.5	8.5	8.5
Peanut meal	8.5	8.5	8.5	8.5
Wheat flour	18.7	18.7	18.7	18.7
Lard				5.11
Dicalcium phosphate				1.04
Salt	2.0	2.0	2.0	2.0
Binder (carboxy-methyl-cellulose)	2.0	2.0	2.0	2.0
Vitamin mix[a]	0.42	0.42	0.42	0.42
Water				38.56
Protein[b]	36.9	36.1	34.7	34.5
Ether extract[b]	5.9	8.3	10.0	10.8
Ash[b]	9.1	13.7	13.1	11.9
Ca[b]	0.8	1.0	0.9	1.0
P[b]	1.0	1.3	1.2	1.2

[a] Vitamin mixture (mg/kg diet): vitamin A (30,000 I.U./g), 183.3; micro D_3, 73.7; alpha tocopherol, 119.9; ascorbic acid, 550; vitamin B_{12}, 41.6; riboflavin, 124.8; menadione sodium bisulfite, 13.7; calcium pantothenate, 187; niacin, 198; thiamine hydrochloride, 22; pyridoxine, 5.5; folic acid 5.5; biotin, 0.44; choline chloride, 3300; ethoxyquin, 198.

[b] Average values as percentages of dry matter, determined by chemical analyses of the diets.

Catfish waste was obtained from the Farm Fresh catfish processing plant in Greensboro, Alabama. The waste was ground by passing through a 1/4-inch plate and then a 1/8-inch plate in a meat grinder. The percentages, by weight, of heads, skins and viscera in the waste were 57%, 15%, and 28%, respectively. After grinding, the waste was either heat processed or put directly into frozen storage until mixed with the dry ingredients in the different diet preparations.

Diet 1, the moist feed containing raw catfish waste, was prepared by first pre-mixing salt, binder (CMC) and vitamins. This mixture was then added to the remaining dry ingredients, soybean meal, peanut meal and wheat flour, in a cement mixer and mixed for 30 minutes. The dry ingredients were then mixed by hand with the raw, ground catfish waste and subsequently passed two times through a Hobart grinder with a 1/8-inch plate. Finally, the wet mixture was hermetically sealed in double plastic bags in 300 to 400 gram quantities and allowed to ferment at room temperature.

Preparation of diet 2, the moist feed containing heated waste, was the same as that of diet 1 except that the ground waste was heated in a double-aluminum boiler until the cold point reached a 185 F and was held at that temperature for 10 minutes. The waste was then cooled quickly and mixed with the dry ingredients. The moist mixture was

inoculated with 10% of a previously fermented feed before sealing in plastic bags.

Diet 3, the dry pelleted feed containing catfish waste, was mixed the same way as diet 1 except that following extrusion from the Hobart grinder, the moist material was spread on trays to dry at 130 F overnight in a large oven. The dried mixture was then ground in a hammer mill and mixed with vitamins, CMC, and salt in the cement mixer. The diet was subsequently pelleted (3/16-inch diameter) in an Astro-Pelleter pelleting machine.

In the preparation of diet 4, the dry pelleted fish meal diet, dicalcium phosphate, wheat flour, peanut meal, soybean meal and fish meal were all mixed in the cement mixer for 30 minutes. To this was added lard, then enough water to bring the moisture content equal to that of the moist diets. After passing through the Hobart grinder twice, the wet mixture was dried, reground and, after adding the vitamins, CMC and salt, was pelleted in a similar manner as diet 3.

Feeding Procedure--The rations were offered six days a week. Daily feed allowances on a dry matter basis, were based on fish weight. The percentage allowance per day changed as fish increased in size as shown below:

Average fish weight (lb)		Dry matter fed per day (per cent of fish weight)
less than 0.5	-	3.0
0.5-0.75	-	2.5
0.75-1.25	-	2.0
more than 1.25	-	1.5

The moist feeds (diets 1 and 2) were removed from the bags, weighed, extruded through a 3/16 plate in a Hobart grinder, and laid on elevated wire screens to dry. A forced draft from an ordinary electric fan was blown over the feed for a minimum of 5 hours at ambient temperature and relative humidity conditions. The surface-dried extrusions were broken into one-inch length pieces before offering to the fish. All diets were fed on an equal dry matter basis. Four ponds, one from each treatment were sampled once a month to assign feed allowances. Bi-weekly adjustments of feeding rate, between monthly samplings, were based on an assumed S conversion of 1.5.

The fish were fed for a 120-day experimental period.

Feed Analysis--Proximate analyses of the diets were performed according to AOAC recommendation (Horwitz, 1965). •Water stability of the moist diets was determined by placing a weighed amount of extruded moist feed into still water on a wire basket for 15 minutes and measuring the amount of dry material retained in the basket during this time.

Since CMC is a relatively expensive ingredient, a series of diets were formulated to test the water stability of the diets, without binders or with replacement binders. The value of salt as a preservative in the moist diets was also investigated.

Feed Preservation. To evaluate fermentation as a method of preservation for moist feeds, samples of diets 1 and 2 were obtained periodically and analyzed for pH, lactic acid and putrefactive deterioration. The method of Ling (1951) was used for lactic acid analysis. In the determination of lactic acid concentration, 3 g (wet weight) of sample was added in sufficient solvent (1.0% NA_2HPO_4) to bring the volume to 100 ml. The flasks were placed in a mechanical shaker for 15 minutes, after which 10 ml of the suspension was diluted to 100 ml with solvent. Twenty-five ml of the mixture was transferred to a 125 ml Erlenmeyer and the following reagents were added rapidly and while stirring: $BaCl_2$, 10 ml; 0.66N NaOH, 10 ml; and $ZnSO_4$, 5 ml. The mixture was then filtered with suction through a Whatman No. 40 filter paper. Following the same procedure, a reagent blank was run with 25 ml of distilled water. Ten ml of the clear filtrate was pipetted into a clean, dry test tube to which was added a 1.0 ml of freshly prepared $FeCl_3$ solution, diluted 1:4 with water. Optical density values were read at 425 millimicrons.

Putrefactive deterioration was determined by smelling the samples and observing the amount of liquefaction that had occurred.

Fermentation Rates of Lactic Acid-Producing Bacteria and Naturally Occurring Bacteria--Stock pure cultures of lactic acid producing bacteria, *Pediococcus cerevisiae* and *Lactobacillus casei* were streaked on brain heart infusion (BHI) plates and incubated for 48 hours. Isolate colonies were transferred to BHI slants and incubated for 48 hours. The organisms were washed from the slants with 5 ml of 0.85% sterile saline, decanted into sterile tubes and mixed thoroughly. One ml of the resulting mixture was transferred to culture bottles containing tryptic soy agar (TSA) and incubated for 24 hours. Cells subsequently harvested were standardized at 600 millimicrons with optical density of 0.80. One ml of the resulting standard suspension was inoculated into each of 6-g samples of sterilized number 2 experimental diet for starter cultures. Starter cultures for each of the two lactic bacteria were incubated for 4 days at 32 C and then mixed thoroughly with 120-g portions of moist number 2 diet. A portion of the moist diet was also inoculated with 6-g of naturally fermented moist diet so that the preparations inoculated with the pure cultures of bacteria could be compared with one containing only naturally occurring bacteria. All samples were hermetically sealed in plastic pouches and stored for various periods at 32 C.

Determinations for pH, lactic acid production, and odor and texture changes were made at 1, 2, 4, 8, 16 and 30 day intervals.

Phase 2

A second feeding trial was conducted to determine whether or not anti-thiamine factors in catfish waste might suppress growth of catfish consuming diets containing the waste, and to ascertain the effect of heat processing on the possible anti-thiamine factors. Chemical methods were also used to measure anti-thiamine activity in the wastes. At the end of the long term experiment fish showing abnormalities were divided in half, and one half of the fish was continued on the original diets for an additional 20 days while the other half was fed these diets plus supplemental thiamine. The purpose of this "recovery" phase was to determine whether or not adding thiamine to the diet would correct the deficiency. Fish from each treatment were sampled for hematocrit readings and for examination for internal anomalies.

Experimental Units--The experiment was conducted with 18, 15-gallon glass aquaria (24" × 12" × 12") in a wet laboratory in the Fisheries Building at the Auburn University. Each aquarium had a continuously-flowing water supply, an air supply, and a siphon hose that removed the feces and other

waste material. Water temperature was maintained at 28 C. The aquaria were scrubbed once a week.

Experimental Fish--Fingerling channel catfish of the Auburn strain, 2-3 inches long and weighing 3-4 grams on the average, were used in the feeding trial. They were taken from pond S-10 and held in metal troughs with running water for 2 days, then treated for external parasites in 100 p.p.m. formalin for one hour. After 3 days, 50 fish were transferred to each of 18 aquaria and fed nutritionally complete fish feed, at a rate of 1% of body weight daily, for a 2-week acclimation period. The fish were recounted and reweighed prior to the actual beginning of the experimental period.

Experimental Treatments--Six experimental dietary treatments were used. The treatments, as indicated below, were nutritionally complete diets containing raw or heat-treated catfish waste and diets deficient only in supplemental thiamine with raw or heat-treated catfish waste. The efficacy of supplying thiamine in diets fed separately from those containing catfish waste was evaluated. The following outline summarizes the six treatments:

Treatment No.	Waste	Thiamine in Primary Diet	Thiamine in Supplemental Diet
1	Heated	No	No
2	Heated	Yes	No
3	Heated	No	Yes
4	Raw	No	No
5	Raw	Yes	No
6	Raw	No	Yes

The basal composition of the primary diets and of the supplemental diets is presented in Table 2. The purpose of the supplemental diets was only to supply thiamine separately from the catfish waste, in treatments 3 and 6.

The primary diets were prepared in the same manner as the moist diets described in Phase 1, except the diets were preserved by freezing instead of fermentation.

The supplemental diets were formulated using purified protein (vitamin-free casein), dextrin, alphacel, corn oil, and cod-liver oil. Commercial vitamin and mineral premixes were used to supply all the other necessary nutrients for optimal growth. Carboxy-methyl-cellulose was used as binder.

All diets were kept in the frozen condition (-28 C) until used for feeding.

Feeding Procedure--The six treatments were assigned at random to the 18 aquaria. The purified diets were fed in the morning at 1% of the body weight and the primary diets

Table 2--Composition of primary and supplemental diets fed to channel catfish in aquaria to test for the presence of anti-thiamine factors in catfish waste.[a]

Ingredients	Percentage
Primary diets:	
Catfish waste, raw or heated	60.00
Soybean meal	10.50
Peanut meal	10.50
Corn starch	16.40
Vitamin mix, with or without thiamine[b]	0.60
Carboxy-methyl-cellulose	2.0
Supplemental diets:	
Vitamin-free casein	37.0
Dextrin	33.0
Purified cellulose	18.0
Cod-liver oil	3.0
Corn oil	3.0
Carboxy-methyl-cellulose	2.0
Vitamin mix, with or without thiamine[b]	1.0
Mineral mix[c]	3.0

[a] Protein level 32%.

[b] Vitamin mixture (mg/kg diet): vitamin A (30,000 I.U./g), 187; vitamin D (40,000 I.U./g), 11; alpha tocopherol, 160.0; ascorbic acid, 550; vitamin B_{12}, 22; riboflavin, 33; menadione sodium bisulfite, 7.9; pantothenic acid, 192.5; niacin, 101.2; pyridoxine, 5.5; folic acid, 1.1; biotin, 0.44; choline chloride, 3300; ethoxyquin, 299.2.

In preparations containing thiamine, 22 mg/kg of diet was used.

[c] U.S.P.XIV (1950) Nutritional Biochemicals Corporation.

were fed in the afternoon, at 2% of body weight on a dry matter basis.

The fish were weighed once each month and feed allowances, on a dry matter basis, were based on average weight per treatment. Bi-weekly adjustments of feeding rates were based on an assumed feed conversion of 1.4.

The diets were thawed from the frozen state and extruded through a Hobart grinder with a 1/8-inch plate. They were laid on elevated wire screens and dried to about 10% moisture under forced ventilation for a minimum of 6 hours at room temperature. The dried diets were broken into smaller pieces by hand and further reduced in particle size with a Waring Blendor. The thawed feeds were kept under refrigeration until fed.

In the latter part of the feeding trial the drying condition was reduced to 30 minutes to yield a product, dry only on the surface, with an approximate dry matter content of 65%.

Chemical Assay for Anti-thiamine Activity--The assay method was similar to that of Rutledge and Ying (1972). Heads, skins, viscera, and body muscle from freshly slaughtered channel catfish were ground separately in the Hobart grinder, using a 1/8-inch plate, and frozen until analyzed later during the week.

Duplicate 4.5 g samples (wet weight) from each anatomical group were each homogenized with 145.5 ml of cold 5%

KCl for one minute at high speed in a Waring Blendor. The homogenate was centrifuged at 1,500 r.p.m. for 5 min. The supernatant was filtered through gauze and the residue discarded.

Ninety-seven ml of each extract and 3 ml of a standard thiamine hydrochloride solution (0.02 mg/ml) were incubated separately in 37 C water bath for 10 min. before mixing. At 0 time, the thiamine solution was added to the extract and two 3-ml aliquots were transferred to 25-ml volumetric flasks containing 12 ml of hot 0.1 N HCl. The rest of the extract plus standard thiamine was returned to the water bath for an incubation period of 30 min. After the addition of 7 ml of 5% KCl, the 25 ml volumetric flasks were heated for another 10 min. in boiling water to coagulate the protein, cooled rapidly to room temperature under tap water, and diluted to 25 ml with 2.5 M sodium acetate. One of the flasks served as a standard and the other as a blank. At the end of 30 minutes, two additional 3-ml aliquots were treated in like manner.

From the 25 ml flasks, 5 ml aliquots were placed in 250 ml separatory funnels. One ml of potassium ferricyanide solution (1 ml of 1% potassium ferricyanide with 24 ml of 15% NaOH) was added to each separatory funnel, except the blank to which 1 ml of 15% NaOH was added. Fifteen ml of isobutanol was added and each separatory funnel was shaken vigorously for 2 min. and allowed to stand for 5 min. to

separate the liquid phases. The aqueous layer was discarded and the butanol layer was shaken with approximately 1.5 g anhydrous sodium sulfate.

The butanol layer in each separatory funnel was decanted into cuvettes and the fluorescence was read with a Model 111 Turner Fluorometer, equipped with a 365 mu primary filter and 450 mu secondary filter.

The fluorescent measurements were initially determined as ug thiamine hydrochloride in 5 ml assay solution and subsequently converted to mg thiamine hydrochloride per 100 g of diet (wet weight).

Chemical Analysis of Thiamine Content--The six diets that were fed to the fish in the aquaria were analyzed for thiamine content using the fluorometric method described above. The assay procedure was run in exactly the same manner, except that the 30 min. incubation period was eliminated. All diets were analyzed one hour after removal from frozen storage. Diets containing raw waste and heated waste with thiamine supplement were analyzed after samples were kept at room temperature for 12 hours and after fermentation and subsequent storage in a sealed bag at 32 C for one week.

IV. RESULTS AND DISCUSSION

Phase 1

Growth Responses

Evaluation of the four test rations over a 120-day period in terms of percentage gain, percentage of fish greater than 14 inches in total length, yield per acre, and feed conversion are presented in Table 3. Fish fed diet number 4 which contained fish meal gained more and had better feed conversion than did fish fed any of the diets containing catfish waste. The superior growth response to the diet containing fish meal was probably due in part to higher contents of several amino acids. Fish meal is higher in lysine, methionine, cystine, isoleucine and threonine content than is catfish waste (National Research Council, 1973). Also, superior growth responses from diets containing fish meal has been attributed to the presence of so-called unidentified growth factors (UGF) (Sparre, 1965).

Diets 2 and 3 containing pasteurized catfish waste and dried catfish waste, respectively, were superior to diet 1 containing raw catfish waste. This finding indicated the likely presence of an anti-nutritional factor in the raw waste. Another possible factor was that subsequent chemical

TABLE 3--Survival, weight gain and feed conversion of channel catfish fed four experimental diets containing catfish waste or commercial fish meal over a 120-day period.

Diet	Pond No.	Production per Acre (lbs)	Percentage Recovery	Percentage Gain	Average Gain per Fish (lbs)	Percentage 14" or Larger	Feed Conversion
1	89	2,749	100	262	0.33	0	3.0
	93	3,649	100	363	0.48	10	2.1
	84	4,209	100	402	0.56	13	1.8
	80	5,168	100	483	0.71	0	1.4
	Average	3,944		377	0.52	6	2.1
2	90	3,399	97	311	0.44	10	2.3
	94	4,587	100	455	0.63	13	1.6
	79	5,707	100	577	0.81	33	1.2
	83	5,027	100	498	0.70	20	1.4
	Average	4,680		460	0.64	19	1.6
3	91	4,304	100	433	0.58	17	1.7
	95	4,837	100	498	0.67	27	1.5
	82	4,987	100	462	0.68	33	1.4
	78	-	-	-	-	-	-
	Average	4,709		464	0.65	25	1.5
4	92	6,081	100	602	0.87	50	1.1
	96	5,612	100	632	0.81	37	1.2
	77	6,024	100	595	0.86	47	1.1
	81	5,999	100	668	0.87	50	1.1
	Average	5,929		624	0.85	46	1.1

analyses of the four test rations showed that the diet containing raw waste was appreciably lower in energy (Table 1).

The performance of the fish on the moist, fermented diet containing pasteurized waste (Diet 2) was equal to the dry, pelleted feed containing catfish waste (Diet 3). This indicated that moist feeds were as suitable for feeding catfish as were conventional, pelleted feeds. It also demonstrated that fermentation-preserved feeds were palatable and nutritious for catfish.

Overall survival of fish during the 120-day feeding trial was 94%. The temperature and dissolved oxygen readings during the experimental period are presented in Table 4. It is shown that during the hottest part of the growing season, when the mass of fish was near 4,000 pounds per acre, the aeration system effectively maintained oxygen levels at no less than 4 p.p.m. at dawn. The pH of the water was generally 9 to 10 at mid-afternoon.

Maximum feeding allowance was about 100 pounds of dry feed per acre per day. During August it was not uncommon to observe dense concentrations of phytoplankton which rose to the surface. In one pond a sudden plankton die-off caused 80% loss of fish. Immediately after the fish kill, dissolved oxygen was less than 1 p.p.m. and the water which previously had been greenish color turned brown. After that incident, the aerators were operated on a full day schedule and feeding time was shifted to the morning. Whenever a

TABLE 4--Dissolved oxygen and water temperatures observed over a 120-day period. Values are averages of three consecutive daily observations around the designated periods.

Date	Diet 1		Diet 2		Diet 3		Diet 4	
	Temp. (°C)	Oxygen (p.p.m.)	Temp. (°C)	Oxygen (p.p.m.)	Temp. (°C)	Oxygen (p.p.m.)	Temp. (°C)	Oxygen (p.p.m.)
June 11, 1972	25	5.8	25	6.4	25	5.9	25	6.0
July 7, 1972	25	6.3	25	5.6	25	6.2	25	6.1
July 28, 1972	27.5	4.2	27.5	4.2	27.5	4.6	27.5	4.3
August 15, 1972	26	5.2	26	5.5	26	5.9	26	5.3
August 29, 1972	28	5.1	28	5.0	28	5.3	28	4.8
Sept. 15, 1972	26	5.9	26	5.5	26	5.8	26	5.4
Sept. 29, 1972	28	4.3	28	4.5	28	4.6	28	4.2
Oct. 15, 1972	23	6.4	23	6.9	23	7.0	23	6.9
Oct. 27, 1972	17	9.0	17	9.2	17	9.5	17	7.0

pond showed a dissolved oxygen reading less than 2 p.p.m. in the morning, a half horsepower pump was installed and operated for a full day to complement the system of aeration. On Oct. 7, 1972, three weeks before the termination of the experiment, a fish kill occurred in pond 80. The event was attributed to oxygen depletion due to mechanical failure of the aerating system.

Preservation by Fermentation

Table 5 shows pH, lactic acid, and water stability determinations for the fermented, moist diets. The moist diets, which contained 35-40% moisture, apparently were satisfactory substrates for rapid fermentation. Within 48 hours significant lactic acid production had occurred and pH had essentially reached its minimum level of 4.4 to 4.6. The odor of normal fermentation can be described as pleasant; one which is sweet-sour or resembles the mild aroma of fine cheese. This characteristic odor was observed to replace the original fishy odor in the experimental diets within 48 hours.

Diets containing pasteurized waste had a firm consistency which did not undergo any mark deterioration at the end of 60 days. The diets with raw waste had partially liquified at the end of the 60 days, indicating some degree of proteolytic activity. In improperly sealed samples surface mold growth occurred and some putrefaction was evidenced by strong ammoniacal odors. In properly sealed

TABLE 5--Lactic acid, pH, and water stability of fermentation-preserved, moist fish diets containing catfish waste.[a]

Diet	Days Preserved	Lactic Acid %	pH	Water Stability %[b]	Sensory Quality
Raw waste,[c] CMC, 2%, Salt, 2%	0	0	6.8	88	Satisfactory
	2	1.7	4.6	88	Satisfactory
	6	3.3	4.5	82	Satisfactory
	10	4.0	4.5	73	Satisfactory
	30	5.3	4.7	79	Satisfactory
	60	5.4	4.8	73	Satisfactory
	200[d]	6.7	4.9	81	Satisfactory
Heated waste,[c] CMC, 2%, Salt, 2%	0	0	6.9	84	Satisfactory
	2	1.7	4.5	83	Satisfactory
	6	2.2	4.6	77	Satisfactory
	10	2.5	4.7	74	Satisfactory
	30	2.6	5.1	63	Satisfactory
	60	2.8	5.2	56	Satisfactory
	200[d]	6.6	4.9	82	Satisfactory

TABLE 5--Continued

Diet	Days Preserved	Lactic Acid %	pH	Water Stability %[b]	Sensory Quality
Raw waste,[c] CMC, 2%, Salt, 0	0	0	6.7	93	Satisfactory
	2	2.0	4.6	94	Satisfactory
	6	3.3	4.5	92	Satisfactory
	10	5.3	4.5	91	Satisfactory
	30	5.6	4.5	91	Satisfactory
	60	5.6	4.5	93	Satisfactory
	200[d]	6.6	4.9	84	Satisfactory
Heated waste,[c] CMC, 2%, Salt, 0	0	0	6.8	83	Satisfactory
	2	1.7	4.6	81	Satisfactory
	6	2.5	4.6	75	Satisfactory
	10	3.3	4.5	71	Satisfactory
	30	3.5	4.5	54	Satisfactory
	60	6.7	4.6	48	Satisfactory
	200[d]	7.3	5.0	84	Satisfactory

TABLE 5--Continued

Diet	Days Preserved	Lactic Acid %	pH	Water Stability %[b]	Sensory Quality
Raw waste,[c] Salt, 2%, CMC, 0	0	0	6.7	84	Satisfactory
	2	2.3	4.7	85	Satisfactory
	6	3.7	4.5	82	Satisfactory
	10	4.3	4.5	79	Satisfactory
	30	5.0	4.7	78	Satisfactory
	60	5.3	4.8	81	Satisfactory
Heated waste,[c] Salt, 2%, CMC, 0	0	0	6.8	60	Satisfactory
	2	3.0	4.4	62	Satisfactory
	6	3.2	4.5	52	Satisfactory
	10	3.5	4.5	52	Satisfactory
	30	3.8	4.5	19	Satisfactory
	60	4.8	4.6	13	Satisfactory

TABLE 5--Continued

Diet	Days Preserved	Lactic Acid %	pH	Water Stability %[b]	Sensory Quality
Raw waste,[c] CMC, 0, Salt, 0	0	0	6.8	94	Satisfactory
	2	2.3	4.5	94	Satisfactory
	6	3.8	4.5	92	Satisfactory
	10	4.0	4.5	90	Satisfactory
	30	6.2	4.5	80	Satisfactory
	60	6.3	4.6	81	Satisfactory
Heated waste,[c] CMC, 0, Salt, 0	0	0	6.8	54	Satisfactory
	2	2.0	4.4	56	Satisfactory
	6	2.7	4.5	27	Satisfactory
	10	2.7	4.5	21	Satisfactory
	30	3.8	4.5	8	Satisfactory
	60	3.8	4.6	6	Satisfactory

TABLE 5--Continued

Diet	Days Preserved	Lactic Acid %	pH	Water Stability %[b]	Sensory Quality
Heated waste,[d] Kelp meal, 2%, Salt, 0	0	0	6.9	68	Satisfactory
	2	4.0	4.5	67	Satisfactory
	6	4.7	4.5	84	Satisfactory
	10	4.7	4.5	86	Satisfactory
	30	4.7	4.6	83	Satisfactory
	60	4.5	4.6	88	Satisfactory
	200	8.0	4.9	84	Satisfactory
Heated waste,[d] Boiled rice, 8%, Salt, 0	0	0	6.8	59	Satisfactory
	2	6.3	4.3	59	Satisfactory
	6	6.4	4.2	53	Satisfactory
	10	6.6	4.2	42	Satisfactory
	30	7.3	4.3	46	Satisfactory
	60	7.3	4.5	48	Satisfactory
	200	6.6	4.9	44	Satisfactory

TABLE 5--Continued

Diet	Days Preserved	Lactic Acid %	pH	Water Stability %[b]	Sensory Quality
Heated waste,[d] Boiled rice, 16%, Salt, 0	0	0	6.8	52	Satisfactory
	2	2.6	4.7	51	Satisfactory
	6	6.0	4.2	59	Satisfactory
	10	6.7	4.2	49	Satisfactory
	30	6.3	4.5	33	Satisfactory
	60	6.0	4.4	24	Satisfactory
	200	8.0	4.8	1.0	Satisfactory
Heated waste,[d] Gelatinized wheat flour, 18%, Salt, 0	0	0	6.8	36	Satisfactory
	2	4.6	4.3	34	Satisfactory
	6	4.3	4.2	27	Satisfactory
	10	4.5	4.2	24	Satisfactory
	30	4.7	4.2	22	Satisfactory
	60	4.7	4.5	18	Satisfactory
	200	6.0	4.9	18	Satisfactory

[a] The diets contained 60% wet catfish waste, 8.5% soybean meal, 8.5% peanut meal, 18.7% wheat flour. Moisture content was 40-44%.

[b] Percentage of dry material retained in a wire basket after immersion in still water for 15 minutes.

[c] Samples were stored at room temperature (26-28 C).

[d] Samples were stored at 32 C.

containers, the interior of samples containing either raw or pasteurized waste had no indication of putrefaction, and color and consistency were essentially unchanged.

Diets which were incubated at 30 C for a period of 200 days had putrefaction only on the surface of the samples. The material appeared normal in the interior and the odor was typical of the fermented product. Samples with preheated rice were more liquid in consistency but the aroma of normal fermentation was still evident.

Results presented in Table 5 indicate that the moist feed containing pasteurized waste had poor water stability when carboxy-methyl-cellulose (CMC) was omitted from the formula. In contrast, binder was of no benefit when the preparation contained raw waste. Apparently the nonheated protein in the raw waste provided binding property which the protein in the heated waste did not have. The data in Table 5 show that kelp meal, added at the rate of 2% of the ration, holds promise as a cheaper binding material. The use of preheated (gelatinized) rice or wheat as substitute binders gave poor water stability.

Salt is frequently used in fermented products to prevent the growth of proteolytic microorganisms while allowing the lactic bacteria to grow. In this study, fermentation rate and keeping quality were not impaired in the preparation which had no salt.

Table 6 shows the data comparing fermentation rates in a moist diet inoculated with pure cultures and species of naturally occurring bacteria. Based on lactic acid production and pH change, the samples which were inoculated with previously fermented material containing the "wild" bacteria, fermented at the fastest rate. Samples which were not inoculated, and which depended only on the presence of naturally occurring organisms ranked second in the rate of acid production and rate of pH decline. These data indicate that deliberate introduction of pure strains of lactic acid bacteria is of doubtful value. In the manufacture of sauerkraut, pickles and green olives, the addition of pure culture organisms has not been found necessary or advantageous (Frazier, 1967). Nilsson and Rydin (1965) obtained superior products by the simple addition of a mixture of enzymatically active malt meal and barley meal to ground herring. Likewise, the preparation of many fermented marine products in Japan as described by Tanikawa (1971) does not utilize pure cultures. However, good batches of fish ensilage were produced using pure cultures according to the reports of Tarr, et al. (1953), Krishnaswamy, et al. (1965) and Roa (1965).

Phase 2

Growth Responses

Results of the 120-day aquarium feeding experiment in which catfish fingerlings were fed raw and heated catfish waste, with or without thiamine supplements are shown in

TABLE 6--Lactic acid production and pH changes in moist, fermentation-preserved fish diet inoculated with pure strains and naturally occurring lactic bacteria.[a]

Days Preserved	INOCULUM							
	Pediococcus cerevisiae		*Lactobacillus casei*		Week-old fermented feed		None	
	Lactic Acid %	pH	Lactic Acid %	pH	Lactic Acid %	pH	Lactic Acid %	pH
0	0	6.9	0	6.8	0	6.8	0	6.9
1	trace	5.3	trace	5.4	1.3	4.8	trace	5.2
2	2.3	5.2	2.0	5.3	4.0	4.6	2.3	4.7
4	3.7	4.8	2.7	5.1	5.7	4.5	4.6	4.4
8	4.0	4.8	2.5	5.0	5.7	4.5	4.0	4.3
16	4.4	4.8	3.0	5.0	5.5	4.5	4.0	4.3
30	5.0	4.9	4.7	4.9	6.3	4.6	6.6	4.6

[a] The diets contained 60% wet catfish waste, 10.5% soybean meal, 10.5% peanut meal, 16.4% corn starch, 2% carboxy-methyl-cellulose and vitamin premix. Storage temperature was constant at 32 C.

Table 7. Analysis of variance as a completely random design showed highly significant treatment mean differences ($P < 0.01$) in growth rates among the six treatments. Subsequent analysis of the factorial effects showed that the differences in the average gain per fish could be attributed to both heat treatment and thiamine supplementation. Fish fed the ration containing heated waste with no thiamine in the primary or supplemental diet (Treatment 1) and those fed the ration containing raw waste with no thiamine in the primary or supplemental diet (Treatment 4) made significantly poorer gains than fish fed the other diets. There was no significant difference ($P < 0.05$) in growth responses with rations containing heated waste with thiamine in the primary or supplemental diet (Treatments 2 and 3) and the ration containing raw waste with thiamine in the primary diet (Treatment 5). The highest ($P < 0.05$) gain was by the fish fed the raw waste ration with thiamine fed in the supplemental feed (Treatment 6). Apparently, the extensive heating, at 132 C for 30 minutes at 30 p.s.i., damaged the nutritional value of the catfish waste in some manner. Since a complete vitamin supplement was added to the rations, the damage may have concerned amino acids.

It was expected that the thiamine in the diet mixtures containing raw catfish waste would be inactivated; however, the high rate of weight gain by the fish fed the raw waste ration with thiamine in the primary diet

TABLE 7--Survival, weight gain, and feed conversion of channel catfish given six experimental treatments containing catfish waste, with or without thiamine supplement, over a 120-day period.

Treatment No.	Waste	Thiamine in Primary Diet	Thiamine in Supplemental Diet	Tank No.	Avg. Gain per fish, g	Survival %	Percentage Gain	Feed Conversion	Abnormal Fish
1	Heated	No	No	2	13.04	98	286	2.44	Yes
				17	12.90	98	265	2.39	
				6	16.88	100	341	1.87	
				Avg.	14.27	99	297	2.23	
2	Heated	Yes	No	4	29.90	100	702	1.07	No
				13	29.26	100	659	1.10	
				1	30.10	98	609	1.08	
				Avg.	29.75	99	657	1.08	
3	Heated	No	Yes	9	27.04	100	593	1.17	No
				10	27.68	100	586	1.14	
				12	28.88	98	643	1.09	
				Avg.	27.87	99	607	1.13	
4	Raw	No	No	14	16.43	98	351	1.79	Yes
				3	16.40	100	380	1.76	
				18	17.78	100	408	1.62	
				Avg.	16.87	99	406	1.72	

TABLE 7--Continued

Treat-ment No.	Waste	Thiamine in Primary Diet	Thiamine in Supple-mental Diet	Tank No.	Avg. Gain per fish, g	Sur-vival %	Per-centage Gain	Feed Conver-sion	Ab-normal Fish
5	Raw	Yes	No	8	30.80	100	611	1.05	No
				16	29.46	100	679	1.10	
				15	31.08	100	634	1.05	
				Avg.	30.45	100	641	1.07	
6	Raw	No	Yes	5	32.58	100	662	1.07	No
				7	36.48	100	757	0.96	
				11	34.18	100	642	1.02	
				Avg.	34.41	100	687	1.02	

(Treatment 5) indicated that thiamine was not appreciably destroyed by exposure to the raw waste. However, when the fish fed the raw waste ration received thiamine in a supplemental diet, separate from the diet mixture containing the raw waste (Treatment 6), they showed slightly higher gains indicating that perhaps a small amount of the thiamine fed in the raw waste diet mixture was destroyed.

The only fish showing nutrient deficiency anomalies were those fed the rations with no thiamine in either the primary or supplemental diet. The deficiency syndrome, described later in this report, indicated that the deficient nutrient was thiamine. The recovery phase of the feeding trial confirmed that thiamine deficiency was responsible for the suppressed growth of the two groups of fish. The growth curves in Figure 1 and 2 show that the deficient fish in Treatments 1 and 4 made dramatic growth responses when given thiamine in their rations. This improvement was found statistically significant ($P < 0.05$). About a week after thiamine was added to the rations the extreme excitability and erratic swimming disappeared and there was improvement in feed consumption as evidenced by the growth responses. Observation and stomach analyses indicated that the fish not fed supplemental thiamine did not eat as much as those receiving supplemental thiamine. Mortality was higher in those fish not on the thiamine-supplemented diets.

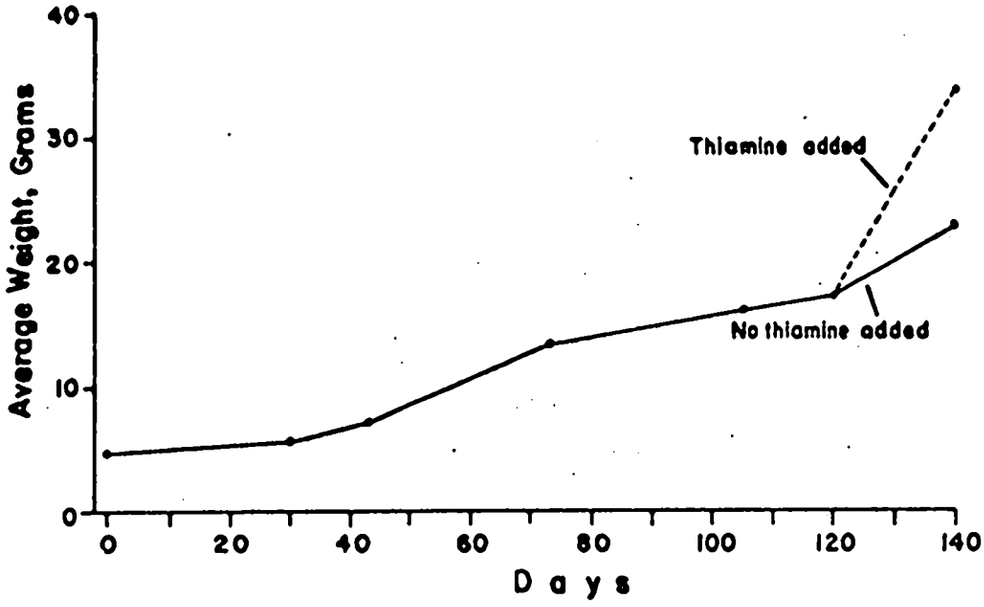

Fig. 1--Growth curve of channel catfish fingerlings on Treatment 1 before and after thiamine was added to the ration of one-half of the fish,

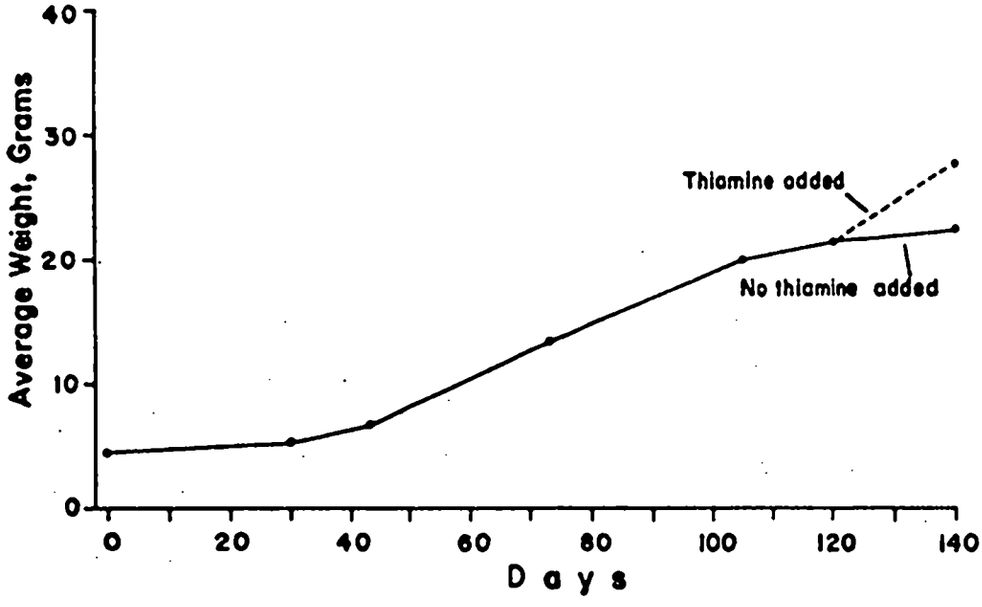

Fig. 2--Growth curve of channel catfish fingerlings on Treatment 4 before and after thiamine was added to the ration of one-half of the fish.

A plausible explanation for the failure of raw catfish waste to inactivate thiamine in the diet mixture may be that the diets were frozen (-28 C) immediately after preparation and held at low temperatures (2 C) until shortly before they were fed to the fish. The low temperature may have limited the activity of the anti-thiamine factor. This explanation is especially feasible if an anti-thiamine factor in catfish waste has a low level of activity.

Chemical analyses of thiamine contents of the diets (Table 8) indicate that both of the primary diets containing thiamine (2 and 5) support normal growth of the fish. Thiamine contents of primary diets 1, 3, 4 and 6, which contained no additional thiamine were very low. When diets containing thiamine were allowed to set at room temperature for 12 hours the thiamine activity depreciated significantly when raw waste was in the mixture (Table 8). There was essentially no change in thiamine content in the diet containing heated waste during the same period of time. This finding, plus the positive results of analyses of catfish skins and viscera for anti-thiamine activity (Table 9), confirm the proposal that had the rations containing thiamine and raw catfish waste in the same mixture been allowed to incubate for a long period of time at room temperature prior to feeding, the fed fish would likely have shown a thiamine deficiency.

When thiamine supplemented primary diets, containing raw or heated waste, were subsequently allowed to ferment for

TABLE 8--Thiamine content (mg/100 g) of the moist, primary diets used in the aquarium feeding study, determined immediately after the frozen diet was thawed, after setting 12 hours at 25 C and after fermentation in a plastic bag for 7 days at 32 C.

Primary Diet	Form of Waste	Condition of Diet		
		Immediate Thaw	12 Hours 25 C	7 Days Fermentation at 32 C
1	Heated	0.04	-	-
2	Heated	1.30	1.35	1.38
3	Heated	0.04	-	-
4	Raw	0.03	-	-
5	Raw	1.27	0.89	0.11
6	Raw	0.03	-	-

TABLE 9--Anti-thiamine activity in various parts of raw and heated catfish processing waste and muscle.

Part	Collection	Anti-thiamine Activity[a]	
		Raw	Heated
Head	1	0.00	0.00
	2	0.00	0.00
	3	0.00	0.00
	Avg.	0.00	0.00
Skin	1	278.00	0.00
	2	262.00	0.00
	3	257.00	0.00
	Avg.	266.00	0.00
Viscera	1	135.00	0.00
	2	167.00	0.00
	3	198.00	0.00
	Avg.	167.00	0.00
Muscle	1	0.00	0.00
	2	0.00	0.00
	3	0.00	0.00
	Avg.	0.00	0.00

[a] Anti-thiamine activity is expressed as micrograms of thiamine destroyed per 100 grams of tissue per 30 minutes.

7 days at 32 C, chemical analyses showed further reduction of thiamine in the diet containing raw waste with no appreciable change in the diet containing heated waste (Table 8). This indicates that the reduction in pH, to approximately 4.5, failed to inactivate the anti-thiamine factor in catfish waste. This supports the contention that, in the feeding trial discussed in Phase 1 of this report, the catfish fed the fermentation-preserved feed containing raw catfish waste gained less weight than the fish fed the fermented feed containing heated waste because of destruction of thiamine in the raw waste feed.

Table 9 shows the degree of anti-thiamine activity in heads, skin, viscera and muscle. The rate of anti-thiamine activity was estimated to be 167 and 266 micrograms thiamine hydrochloride destroyed per 100 grams (wet weight) per 30 minutes, for fresh samples of viscera and skin, respectively. No anti-thiamine activity was detected in the heated samples, indicating that thiamine destruction was apparently enzymatic. The high amount of anti-thiamine activity in the skin and the absence of activity in the head were unexpected and explanations are not available.

Thiamine Deficiency Syndrome in Channel Catfish

The growth rate of fish receiving the six dietary treatments were of the same magnitude until the 10th week of feeding. Within this period all of the fish readily accepted the feed and usually consumed their full ration in

less than two minutes. By the 11th week of the experiment fish receiving the rations without thiamine in the primary or supplemental diets (Treatments 1 and 4) began to lose appetite and ate poorly. The first mortality occurred in Treatment 1 where a fish showed hemorrhage at the base of the dorsal and caudal fins. Examination for parasites was negative. It was further observed that most of the fish on the thiamine-free rations had a light colored appearance.

By the 15th week of the experiment the fish receiving Treatments 1 and 4 began to show signs of unusual excitability, dashing about violently when the aquaria were approached. This sudden burst of excitement was followed by some of the fish swimming on their side, upside down, turning over, or taking a vertical position in the aquaria, as illustrated in Figure 4. Some fish which exhibited an obvious loss of equilibrium when excited were able to recover and swim normally again after a period of time. Others could not recover and struggled about the aquarium in an uncoordinated manner and appeared to gasp for air at the surface of the water. Sometimes the abnormal fish were found lying lethargic at the bottom of the aquarium or congregated around air stones. In some instances, when startled, the deficient fish would jump out of the aquarium. When handled, they exhibited strong muscular spasms and the outlines of the myotomes appeared distinctly along their sides. In some cases they showed characteristic dorso-ventral arching of

the body. The few fish which died were examined and found to have hemorrhagic areas on the gills and at the bases of the ventral fins.

Lesions in the liver and spleen were the only consistent internal abnormalities observed in the thiamine deficient fish. There were variable occurrences of clubbed gills, hyperemia in the intestine and gas in the stomach. There were no clinically discernible lesions found in the gall bladder, intestinal tract, posterior kidney or brain of the deficient fish.

The liver of the deficient fish appeared pale and splotched in contrast with a normal reddish-brown color of the liver of fish on complete diets. Histologic sections of the livers of the normal and thiamine deficient channel catfish are shown in Figures 5-8. The histological appearance of normal liver was described by Gaines (1972) as a loose aggregate of lobules containing epithelial cells with numerous pancreatic-like cells surrounding the blood vessels (Figure 5). In the liver of the thiamine deficient fish the organization of cells was undefined with extensive vacuolization and fatty infiltrations. The blood vessels and peripheral areas of the liver contained high concentrations of erythrocytes indicating hemorrhage (Figures 6 and 8).

A normal spleen, whose vital function is concerned with both hemopoiesis and filtration, in the channel catfish is a small bright red oval organ located dorsal to the

intestine (Gaines, 1972) (Figure 9). The spleen in the deficient fish was crenulated and showed abnormally dark pigmentation. Histologic sections showed abnormal distribution of erythrocytes around the red and white pulp indicative of widespread hemorrhage (Figure 10). The crenulated margin of the spleen showed abnormal bumps filled with erythrocytes, indicating hemorrhage (Figure 12). Extensively damaged spleens may account for the low hematocrit values obtained for the thiamine deficient fish on treatments 1 and 4 (Table 10).

Loss of equilibrium, spastic swimming and related abnormal behavioral symptoms of thiamine deficiency in channel catfish described here agree with the observations by Wolf (cited in Yudkin, 1949) in trout and are suggestive of neurologic disturbance. Similar disturbances were observed in other fishes (McLaren, et al., 1947; Halver, 1954; Phillips and Brockway, 1947; Dupree, 1966; Hashimoto, et al., 1970) and in rats, mice, cats, dogs, swine and monkeys (Follis, 1948).

TABLE 10--Hematocrit values for 10 channel catfish from each of 6 treatment groups fed diets containing catfish waste, with or without thiamine supplement, over a 120-day period.

TREATMENT NUMBER					
1	2	3	4	5	6
34	46	37	24	43	53
33	54	32	28	48	53
32	53	48	31	53	45
34	55	39	31	41	45
35	46	41	28	46	40
28	47	50	28	54	48
35	49	45	24	48	45
32	40	49	25	50	52
30	44	38	29	52	51
33	42	48	34	49	55
33	48	43	28	48	49
AVERAGES					

V. SUMMARY

Channel catfish fingerlings, stocked at the rate of 6,000 per acre in 0.005-acre concrete tanks were fed 32% protein, nutritionally adequate, moist feeds containing raw or heated catfish waste for 120-days. Fish were also fed diets prepared in dry, pelleted form containing either catfish waste or fish meal as the source of animal protein. The moist diets were fermented for preservation. Relatively poor growth of fish fed raw catfish waste stimulated a subsequent aquarium feeding trial to determine whether or not raw catfish waste contained a heat labile anti-thiamine factor. Catfish fingerlings were fed semi-purified diets containing raw or heated catfish waste with or without various thiamine supplementations for 120 days. The study revealed the following:

1. Catfish processing waste as a source of protein in production rations for channel catfish was approximately 80% as effective as marine fish meal for producing weight gain.

2. The fish fed the fermentation-preserved, moist diet containing heated catfish waste gained as much as those fed a diet of similar composition except in dry, pelleted form.

3. Fish fed the moist, fermented diet containing raw waste showed significatnly poorer growth than those receiving the heated waste diet. The growth repression was suspected to have been caused by a thiamine destructive factor in the raw catfish waste.

4. Moist fish feeds were preserved by fermentation for up to 200 days.

5. A binder was not necessary in feed preparations containing raw catfish waste, but was necessary when the waste was pre-heated. Kelp meal incorporated as a binder at the rate of 2% in feeds containing heated waste provided acceptable stability. Kelp meal is considerably cheaper than carboxy-methyl-cellulose.

6. Fermentation rate and preservation quality of moist diets, containing either raw or heated catfish waste, were not impaired in the absence of salt in the diet.

7. The use of pure strains of lactic acid bacteria for fermentation was not found to be advantageous.

8. Aquarium studies showed that semi-purified diets containing 60% of either raw of heated catfish waste were extremely deficient in thiamine. Catfish fingerlings fed these diets exhibited a thiamine deficiency syndrome characterized by anorexia and neurological disturbances. The fish were easily excited and showed loss of equilibrium while swimming. Lesions of the liver and spleen were the only consistent internal abnormalities observed.

9. Assays for anti-thiamine activity in separate samples of head, skin, viscera and muscle of channel catfish indicated the presence of a heat labile anti-thiamine factor in the skin and viscera, but not in the head or muscle.

10. Quantitative evaluations showed that the thiamine destruction activity in catfish waste is relatively low compared with that reported for other fishes. Prolonged exposure of thiamine to raw catfish waste was necessary for appreciable destruction.

11. The low pH of 4.5 associated with fermentation preservation of moist fish feeds did not inactivate the anti-thiamine factor in channel catfish waste.

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