水研センター研報，第31号，31－41，平成22年
Bull. Fish. Res. Agen. No. 31, 31-41, 2010

Approach to Expand the Use of Japanese Anchovy as a Feed Material : Efficacy of Thiamine Supplementation

Noriko ISHIDA [*1], Kenji NAKANISHI [*2], and Hisashi MURATA [*3]

Abstract : Three experimental diets of thiamine supplemented (TA), non-supplemented (NTA) anchovy-based moist pellets and mackerel-based moist pellets (MM) were fed to one-year-old amberjack for 20 weeks. Thiamine supplementation to anchovy pellets prevented thiamine deficiency disease observed in NTA, such as reduced levels of growth performance, plasma cholesterol and total protein, muscle fat content and disease-resistance against *Streptococcus dysgalactiase*. Furthermore, TA fish achieved the same level of growth performance, disease-resistance and meat quality as the general feed observed in MM.

Key words : Japanese anchovy, feed material, thiamine, deficiency, supplementation

The Japanese anchovy (*Engraulis japonicus*) resource is considered to be extensive but not effectively utilized. In order to resolve this issue and eventually increase the anchovy catch, we focus here on its use as an aquaculture feed material.

Anchovy is definitely the cheapest among the fish available for use in moist pellets. There is a strong demand for a cheap and readily available feed material by amberjack (*Seriola dumerili*) farmers, however anchovy is not used as a moist pellet as there is concern about the development of thiamine deficiency. The effects of thiamine deficiency were studied on yellowtail (*Seriola quinqueradiata*) in the 1970s and the problem was shown to develop in conjunction with feeding anchovies (Ishihara *et al.*, 1974). However, there have been no previous studies on thiamine deficiency in amberjack.

To increase anchovy use in inexpensive moist pellets, we investigated the development and symptoms of thiamine deficiency by feeding anchovy to 0 age group amberjack (Nakanishi *et al.*, 2007). In this paper, by feeding anchovy-based moist pellets to growing one-year-old amberjack, we observed the development of the disease, the period before its start, and its symptoms, and determined an appropriate supplement level of thiamine. In addition, the growth performance and meat quality were compared between thiamine supplemented anchovy-based moist pellets and mackerel moist pellets, a general feed currently used in amberjack farms.

Materials and Methods

Experimental diets

The ingredients used in the experimental diets were shown in Table 1. Three experimental groups of fish were fed anchovy-based moist pellets (NTA), thiamine supplemented anchovy-based moist pellets (TA) and mackerel-based moist pellets (MM), respectively. The MM diet represents the currently used feed. NTA contained 80% raw anchovy, 20% commercial feed blend and cod liver oil to adjust to the same caloric value of the other diets. TA contained 0.0013% thiamine. MM contained 80% raw mackerel, 20% commercial feed blend and cod liver oil.

The anchovy and mackerel were purchased from

[*1] National Research Institute of Fisheries Science, FRA, Fukuura, Kanazawa, Yokohama 236-8648, Japan
Email address; larry@affrc.go.jp
[*2] Miyazaki Prefectural Fisheries Experimental Station, Miyazaki 889-2162, Japan
[*3] University of Miyazaki, 1-1, Gakuen Kibanadai-nishi, Miyazaki 889-2192, Japan

the amberjack farms in Miyazaki Prefecture. NTA and TA were prepared every four weeks and MM was prepared every eight weeks. The feeds were frozen at −20℃ before use. All the frozen experimental diets were thawed, refrigerated, and used up within three days. The proximate composition of raw anchovy and mackerel were determined in advance. Each experimental diet was adjusted to the same caloric value of 480 kcal/100 g by adding cod liver oil.

Fish and feeding trial

One-year-old amberjack were obtained from a fish farm (Miyazaki, Japan), that were transferred from China and reared starting in May 2006. Fish with an initial mean weight of 1,019 g were distributed to 2 × 2 × 2.5 m net cages. Each dietary treatment was comprised of 53 fish that were fed the experimental diets to satiation, once a day, 5 days/week for 20 weeks from June to November 2007. Bathing fish in freshwater to detach external worms and changing the cage nets to keep the living environment clean were conducted every week or every other week. The water temperature during the period was in the range 21–29℃.

Growth performance and sampling

Body weight and length of all the fish were measured initially and every four weeks. Six fish were sampled from each dietary group after 12 and 20 weeks and chemical analyses of the whole body, serum and plasma were conducted. Lipid peroxidation level, antioxidant activity, thiamine content in the liver and nutrient content in the dorsal muscle were determined.

Infection experiment

Ten fish from each dietary treatment were transferred to an indoor FRP fish tank (2 tons) after 12 and 20 weeks. Fish were injected intraperitoneally with *Streptococcus dysgalactiase* (1×10^8 CFU/fish), a major pathogen of amberjack in summer. *Streptococcus dysgalactiase* was incubated in THB medium at 37℃ for 24 h and diluted with physiological saline for injection. After 12 weeks, the first stage trial was conducted and the survival rate was observed for 14 days without feeding. After 20 weeks, the second stage trial was conducted and the fish were observed for 21 days.

Analysis methods

Proximate composition. Water contents in the diets and the fish tissues were calculated from the constant mass obtained by baking samples at 105℃. Lipid contents were measured according to the method of Bligh & Dyer (1959) using the gravimetric method. Lipid in the samples was extracted, evaporated, vacuumed and its weight measured. Crude protein content was measured as nitrogen using the Kjeldahl method. Ammonia nitrogen was formed by reacting the sample with sulfuric acid, absorbed to boric acid by the steam distillation technique and determined by neutralization analysis. Crude ash content was calculated from the constant mass obtained by incinerating samples at 600℃ in an electric furnace oven.

Thiamine concentration. The concentrations of free thiamine and its phosphate in the diets and livers were measured according to the high-performance liquid chromatography (HPLC) method of Ishii *et al.* (1979a, 1979b). Ten percent trichloroacetic acid was added to the samples, which were immediately homogenized and centrifuged. The centrifuged supernatant was transferred and extracted three times with ethyl ether. The extract was chromogenically reacted by adding 0.3 M cyanogen bromide. This solution was added to 2 N sodium hydroxide and used for HPLC analysis. A HPLC column obtained from Merck Co. was LiChrospher®-NH_2 (4 × 250 mm). The solvent system was acetonitrile:50 mM phosphate buffer (pH 8.4) at a ratio of 60 : 20, v/v. The spectrofluorometer was operated with an excitation wavelength set at 375 nm and the fluorescence wavelength at 430 nm. Thiamine concentration was expressed in μg per gram tissue weight.

α-Tocopherol concentration. The concentration of α-tocopherol in the diets and livers was measured according to the HPLC method of Yamauchi *et al.* (1991). Samples were saponified to obtain the unsaponifiable material. The derived

unsaponifiable material was extracted with n-hexane and analyzed by HPLC using a Zorbox-BP-NH_2 (4 × 150 mm) column (Wako Co.). The solvent system was n-hexane:isopropyl alcohol at the ratio of 200:1, v/v. The spectrofluorometer was operated with the excitation wavelength set at 295 nm and the fluorescence wavelength at 325 nm. For the internal standard material, 2,2,5,7,8-pentamethyl-6-hydroxychromane was used. The concentration of α-tocopherol was expressed in μg per gram tissue weight.

Ascorbic acid. The concentrations of ascorbic acid and dehydroascorbic acid in the diets and livers were measured in simultaneous quantification according to the HPLC method of Ito *et al.* (1995). Ascorbic acid and dehydroascorbic acid in the sample was extracted with 5% metaphosphoric acid and analyzed by HPLC. A HPLC column obtained from Shimadzu Co. was Shim-pack SCH-101H (7.9 ×300 mm). The mobile solvent was oxalic acid and 100 mM sodium hydroxide was used as a reaction solution. The ultraviolet spectrofluorometer was operated at 300 nm. The concentration of ascorbic acid and dehydroascorbic acid was expressed in μg per gram tissue weight.

Lipid peroxidation. The contents of triacylglycerol hydroperoxide (TGOOH) and phosphatidylcholine hydroperoxide (PCOOH) in the diets and livers were measured according to the chemiluminescence HPLC method of Ito *et al.* (2000). A HPLC column obtained from Shimadzu Co. was Shim-pack SIL(M) (250 × 4.6 mm). The mobile solvents was acetonitrile:methanol:water (5.5:4:0.5, v/v) and 1 μg/ml cytochrome c in 20 mM boric acid buffer containing 1% methanol (pH 10.5) was used as a chemilumigenic reagent. The active oxygen was expressed in nmol per gram tissue weight.

Thiobarbituric acid value (TBA value). The TBA value in the diets and livers was measured according to the steam distillation method of Ando & Yamauchi (1968). Malondialdehyde (MDA), lipid peroxide degradation product was reacted with thiobarbituric acid to produce red derivative. The red derivative (MA) was calculated by measuring absorbance of 532 nm. The active oxygen was expressed in μg MA per gram tissue weight.

Hematological variables and plasma biochemical property. Red blood cell content (RBC) was measured by the visual method. Hematocrit value (Ht) was obtained by centrifugation (1,200 rpm, 5 min) using hematocrit centrifuge tube. Hemoglobin concentration (Hb) was measured by cyanmethemoglobin method (Kawatsu, 1969). EC50 values of osmotic tolerance erythrocyte was expressed the NaCl concentration of 50% red cell hemolysis according to the methods as described previously in Takagi *et al.* (2006). Total plasma protein was measured using a plasma protein analyzer (Hitachi Co.). Plasma biochemical property was measured using a clinical chemistry automated analyzer (Arkray Co.).

Statistical analysis. Growth and feed performance, blood variables, osmotic tolerance in blood cell membranes, plasma biochemical analyses and hepatic lipoperoxidative levels were analyzed by ANOVA and the statistical significance of difference between treatment groups was assessed at the 5% level of probability by Tukey-Kramer test (Kramer, 1956). The results of the infection experiment were assessed by Fisher's exact test (Yanai, 2000).

Results

Experimental diets

The proximate compositions and thiamine concentrations of the experimental diets are shown in Table 1. In the proximate compositions, MM contained a higher crude protein content and less crude fat than NTA and TA. In the thiamine concentrations, NTA contained 0.2 mg/100 g fish, TA contained 1.7 mg/100 g fish and MM contained 0.7 mg/100 g fish. In terms of energy, all the diets had similar values. In the energy/crude protein ratio, MM showed the least value.

Feeding trial

Growth performance. The growth performance values for fish fed the experimental diets are shown in Table 2. No deaths from thiamine deficiency were seen during the trial. The NTA group showed

a remarkably lower specific growth rate (SGR) and feed conversion ratio (FCR) after 8 weeks and significantly lower final weight and condition factor at 20 weeks compared with the other groups. Thiamine was not added to MM, however the MM group showed constant growth, along with the lowest FCR and daily feed intake (DFI), and highest SGR. TA group also showed a constant growth rate and reached a similar final body weight to the MM group at 20 weeks.

Organ weights. The organ weights of the experimental fish are shown in Table 3. The NTA group showed the lowest liver and gut weights. On the other hand, no differences were seen between the TA and MM groups.

Hematological variables, osmolality tolerance of erythrocytes and plasma biochemical properties. The hematological variables, osmolality tolerance of erythrocytes and plasma biochemical properties in the experimental fish are shown in Table 4. At the end of the trial, the NTA group showed significantly a lower hemoglobin concentration (Hb) than the MM group. NTA group also showed the significantly lowest plasma total cholesterol and total protein levels.

Table 1. Composition and nutrient contents of experimental diets.

Diet:	NTA*1	TA*2	MM*3
Ingredient (%)			
Anchovy	80	80	
Mackerel			80
Commercial feed blend ※1	20	20	20
Cod-liver oil ※2	4.9～10.7	4.9～10.7	4.4～4.7
Thiamine	0	0.0013	0
Nutrient content (dry matter basis %)			
Crude protein	50.0	49.5	56.4
Crude fat	28.2	27.5	24.8
Sugar	11.1	12.4	8.8
Ash	10.7	10.5	10.1
Thiamine（mg/100g）	0.2	1.7	0.7
Energy（kcal/100g）	481.9	477.9	476.4
Calorie/Protein ratio※3	96.4	96.8	84.6

*1 Non-thiamine supplemented anchovy-based moist pellets

*2 Thiamine supplemented anchovy-based moist pellets

*3 Mackerel-based moist pellets

※1 Marubeni Nisshin Feed Co. Ltd.

※2 Yamakei-Sangyo Co. Ltd.

※3Calorie(kcal/kg) to protein(% protein) ratio.

Table 2. Growth performance of amberjack fed the experimental diets for 20 weeks.

	Body weight (g)		SGR※1	CF※2	FCR※3	Daily feed intake	Death
Diet	Initial	Final	(%)			(%)	(%)
NTA	1015	1642[a]	0.34	19.9[a]	6.3	1.54	1.9
TA	1034	2333[b]	0.58	22.7[b]	3.7	1.48	0.0
MM	1008	2361[b]	0.61	22.6[b]	3.0	1.40	3.8
ANOVA*4		**		**			

※1 Specific growth rate

※2 Condition factor

※3 Feed conversion ratio; g feed/g weight gain

※4 Result of two-dimensional correlation analysis.

** Significantly different (P<0.001)

Values are means ±standard deviation for the number of fish indicated.

Means with different superscripts within the same column are significantly different (P<0.05).

Table 3. Organ weights of amberjack fed the experimental diets for 20 weeks.

Diet	Liver (%) ※1	Gut (%) ※2	Spleen (%) ※3
NTA	0.6 [a]	3.9 [a]	0.16
TA	1.0 [b]	5.3 [b]	0.12
MM	1.1 [b]	5.4 [b]	0.15
ANOVA ※4	**	**	NS

※1 Liver weight (g) / Body weight (g) ×100

※2 Gut weight (g) / Body weight (g) ×100

※3 Spleen weight (g) / Body weight (g) ×100

※4 Result of two-dimensional correlation analysis.

** Significantly different (P<0.001)

NS, no significant difference

Values are means ±standard deviation for the number of fish indicated.

Means with different superscripts within the same column are significantly different (P<0.05).

Hepatic hydroperoxide levels and antioxidant activities. Hepatic hydroperoxide levels and antioxidant activities in the experimental fish are shown in Table 5. In the hepatic hydroperoxide level, the NTA group showed the significantly highest value. In terms of antioxidant activity, the NTA group showed lower ascorbic acid and dehydroascorbic acid at 12 weeks but there were no significant differences for α-tocopherol and γ-tocopherol among the groups.

Hepatic thiamine content. Hepatic thiamine contents in the experimental fish are shown in Table 6. In all groups, thiamine pyrophosphate (TPP) was dominant, accounting for 90% of the total thiamine. The NTA group showed significantly lower TPP and total thiamine than the other groups at 12 and 20 weeks. The TA and MM groups showed similar tendencies in thiamine content level and the total thiamine content of both groups showed a constant value of about 3 μg/g.

Table 4. Hematological variables, osmolality tolerance of erythrocytes, and plasma biochemical properties in the experimental amberjacks fed the experimental diets.

Diet	Red blood cell count (10^6/mm^3)	Hemoglobin concentration (g/dl)	hematocrit level (%)	MCV※1 (μm^3)	MCHC※2 (%)	MCH※3 (pg)	EC50※4 (NaCl %)	Plasma total cholesterol (mg/dl)	Plasma total protein (%)
Stage 1 (12 week)									
NTA	3.89	8.27	44.4	116.0	18.8	21.9	0.34	260	7.8
AM	4.17	8.53	49.2	118.5	17.4	20.6	0.33	278	8.4
MM	3.84	8.47	44.6	116.7	19.0	22.2	0.31	262	8.1
Pooled SEM	0.130	0.177	1.123	2.751	0.354	0.669	0.004	6.8	0.11
Probability	0.556	0.817	0.181	0.930	0.164	0.598	0.067	0.521	0.212
Stage 2 (20 week)									
NTA	3.46	7.71[a]	46.0	134.6	16.8	22.6	0.41	241 [a]	7.8 [a]
TA	3.40	8.47[b]	48.3	142.2	17.6	25.0	0.40	315 [b]	9.2 [b]
MM	3.85	8.60[b]	49.3	128.3	17.5	22.5	0.38	315 [b]	8.8 [b]
Pooled SEM	0.097	0.144	0.898	2.225	0.209	0.489	0.007	5.7	0.13
Probability	0.165	0.047	0.344	0.066	0.285	0.094	0.340	<0.001	0.002
Results of ANOVA※5									
Diet	NS	NS	NS	NS	NS	NS	*	**	NS
Period	*	NS	NS	**	*	*	**	**	**
Interaction	NS	NS	NS	NS	NS	NS	NS	**	**

Values are average of 6 fish.
Different superscript letters in the same column indicate a significant difference.
※1 Mean corpuscular volume
※2 Mean corpuscular hemoglobin concentration
※3 Mean corpuscular hemoglobin
※4 NaCl concentration at EC50 for osmolality tolerance of erythrocytes
※5 Two-dimensional correlation analysis by ANOVA
** $P < 0.01$
* $P < 0.05$
NS, No significant difference

Table 5. Lipid peroxidation level and antiooxidative activity in liver of experimental amberjacks fed the experimental diets.

Diet	TGOOH※1 (nmolAO/g)	PCOOH※2 (nmolAO/g)	TBARS※3 (μgMA/g)	ASA※4 (μg/g)	D-ASA※5 (μg/g)	T-ASA※6 (μg/g)	α-Toc※7 (μg/g)	γ-Toc※8 (μg/g)
Stage 1 (12 week)								
NTA	18.7	0.09	0.16	3.5	15.8	19.3	95.3	0.02
TA	16.6	0.09	0.16	4.6	14.8	19.5	95.3	0.04
MM	16.0	0.06	0.13	4.1	15.4	19.5	114.6	0.02
Pooled SEM	0.68	0.01	0.02	0.30	0.63	0.81	8.31	0.01
Probability	0.303	0.163	0.809	0.380	0.815	0.997	0.580	0.673
Stage 2 (20 week)								
NTA	35.7[a]	0.48	1.11	1.7	12.5	14.1[ab]	44.0[a]	0.05
TA	19.7[b]	0.19	0.43	1.5	10.6	12.1[a]	75.5[ab]	0.00
MM	20.4[b]	0.22	0.36	1.9	14.3	16.2[b]	99.4[b]	0.03
Pooled SEM	1.76	0.06	0.14	0.39	0.69	0.48	4.55	0.01
Probability	0.016	0.169	0.140	0.891	0.184	0.037	0.007	0.434
Results of ANOVA※9								
Diet	**	NS	NS	NS	NS	NS	*	NS
Period	**	**	**	**	**	**	*	NS
Interaction	*	NS	NS	NS	NS	NS	NS	NS

Values are average of 3 samples of 2 pooled fish.
Different superscript letters in the same column indicate a significant difference.
※1 Triacylglycerolhydroperoxide
※2 Phosphorylcholinehydroperoxide
※3 2-thiobarbituric acid reactive substance value
※4 Ascorbic acid
※5 Dehydroascorbic acid
※6 Total ascorbic acid
※7 α-Tocopherol
※8 γ-Tocopherol
※9 Two-dimensional correlation analysis by ANOVA
** $P < 0.01$
* $P < 0.05$
NS, No significant difference

Proximate composition of experimental fish. The proximate compositions of the experimental fish is shown in Table 7. At 12 weeks, the NTA group showed a significantly lower water content and significantly higher crude protein and crude fat content than the MM group. At 20 weeks, the NTA group showed the highest water content and lowest crude protein and crude fat levels among the three groups. The MM group showed higher crude fat and crude ash content, but those differences were small.

Infection experiment. The survival rates after being intraperitoneally injected with *Streptococcus* disease pathogen to the amberjacks fed the experimental diets are shown in Figure 1. At 12 weeks, no difference was seen among the three

Table 6. Hepatic thiamine concentrations in experimental amberjacks fed the experimental diets.

Diet	TH※1 (μg/g)	TMP※2 (μg/g)	TPP※3 (μg/g)	T-TH※4 (μg/g)
Stage 1 (12 week)				
NAM	0.09	0.11^{a}	1.35^{a}	1.55^{a}
TAM	0.12	0.18^{ab}	2.69^{b}	2.99^{b}
MM	0.14	0.27^{b}	2.61^{b}	3.03^{b}
Pooled SEM	0.014	0.020	0.067	0.086
Probability	0.422	0.035	<0.001	<0.001
Stage 2 (20 week)				
NAM	0.13	0.07^{a}	1.73^{a}	1.93^{a}
TAM	0.14	0.20^{b}	2.52^{b}	2.86^{b}
MM	0.12	0.17^{b}	2.62^{b}	2.91^{b}
Pooled SEM	0.01	0.01	0.03	0.04
Probability	0.528	0.013	<0.001	<0.001
Results of ANOVA※5				
Diet	NS	**	**	**
Period	NS	NS	NS	NS
Interaction	NS	NS	*	*

Values are average of 3 samples of pooled 2 fish.
Different superscript letters in the same column indicate significant difference.
※1 Free thiamine
※2 Thiamine monophosphate
※3 Thiamine pyrophosphate
※4 Total thiamine
※5 Two-dimensional correlation analysis by ANOVA

** $P < 0.01$

* $P < 0.05$

NS, No significant difference

groups. However, at 20 weeks, the NTA group showed a significantly lower survival rate than the other two groups.

Discussion

The symptoms observed in the NTA groups are considered to have been due to the deficiency of thiamine because the difference between NTA and TA was only the lack of thiamine addition. There were many differences among growth performance, biochemical analyses, and survival rates in the infection test between the NTA group from the other two groups. For the use of anchovy-based moist pellets, thiamine supplementation is considered to be absolutely necessary because the NTA group

Table 7. Proximate composition in muscle of experimental amberjacks fed the experimental diets.

Diet	Water content (%)	Crude protein (%)	Crude fat (%)	Crude ash (%)
Stage 1 (12 week)				
NAM	74.8^{b}	22.4^{a}	1.83^{a}	1.55
TAM	73.8^{ab}	22.8^{ab}	2.38^{ab}	1.56
MM	73.4^{a}	23.1^{b}	2.97^{b}	1.51
Pooled SEM	0.18	0.08	0.15	0.009
Probability	0.019	0.022	0.024	0.087
Stage 2 (20 week)				
NAM	75.4^{b}	21.9^{a}	1.64^{a}	1.52^{a}
TAM	71.5^{a}	22.8^{b}	4.75^{b}	1.52^{a}
MM	72.0^{a}	23.2^{c}	3.65^{b}	1.54^{b}
Pooled SEM	0.19	0.05	0.23	0.003
Probability	<0.001	<0.001	<0.001	0.015
Results of ANOVA※1				
Diet	**	**	**	NS
Period	**	NS	**	NS
Interaction	**	*	**	**

Values are average of 6 fish.

Different superscript letters in the same column indicate significant difference.

※1 Two-dimensional correlation analysis by ANOVA

** P <0.01

* P < 0.05

NS, No significant difference

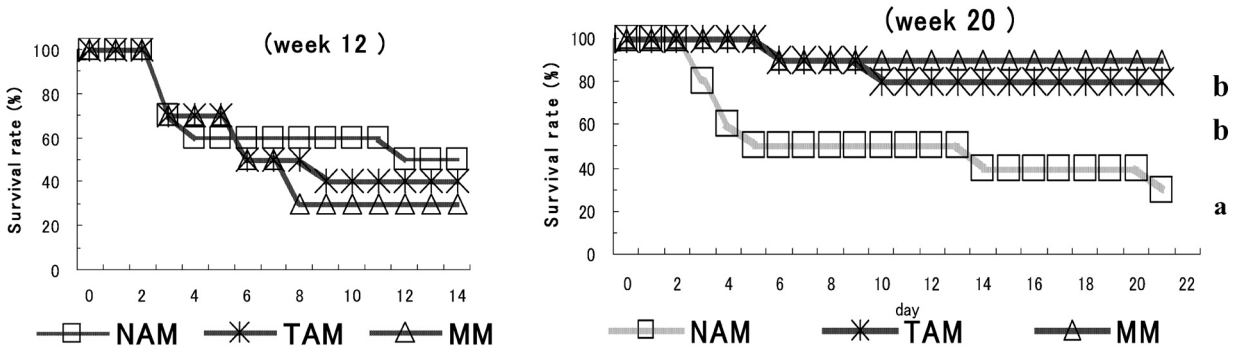

Fig. 1. The survival rate of amberjack fed the experimental diets after intraperitoneal injection at 12 weeks and 20 weeks of *Streptococcus dysgalactiae,* pathogenic to the fish. The result of infection experiment was assessed by Fisher's exact test. Different superscript letters in the same column indicate a significant difference.

at 20 weeks showed a significantly lower survival rate.

The TA group was similar to the MM group as a general feed used on commercially farms. The supplemented volume of thiamine of TA was 12.5 mg/100 g fish. The supplementation of thiamine at 12.5 mg/100 g fish is thought to prevent amberjacks fed anchovy from developing a thiamine deficiency. We also conducted another experiment with thiamine supplemented at 25 mg/100 g fish, double the concentration of the present experiment, and showed little difference between the results (not published).

On the other hand, the MM group results support the use of mackerel-based moist pellets on commercial farms. The MM was not supplemented with thiamine but the thiamine concentration of MM was similar to that of TA. In addition, the MM group showed the highest growth performance.

The TA group showed similar performance to the MM group with respect to growth performance and meat quality when the caloric values were adjusted to be equal. From the above results, we conclude that anchovy-based moist pellets should be used on commercial farms only when supplemented with thiamine at an adequate concentration.

References

Ando, N. and Yamauchi, K. 1968: Studies on oxidative rancidity in cooked meat I. A steam distillation method for the determination of TBA number of meat. *Jap. J. Zootech. Sci.,* **39**; 41–47.

Bligh, E.G. and Dyer W.J., 1959: A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.*, **37**, 911–917.

Ishihara, T., Yasuda, M., Kashiwagi, S., Akiyama, M., and Yagi. M., 1974: Studies on thiaminase I in marine fish VI. Preventive effect of thiamine on nutritional disease of yellowtail fed anchovy. *Bull. Jap. Soc. Sci. Fish.*, **40**, 775–781.

Ishii, K., Sarai, K., Sanemori, H., and Kawasaki, T., 1979a: Analysis of thiamine and its phosphate esters by high-performance liquid chromatography. *Anal. Biochem.*, **97**, 191–195.

Ishii, K., Sarai, K., Sanemori, H., and Kawasaki, T., 1979b: Concentrations of thiamine and its phosphate esters in rat tissues determined by high-performance liquid chromatography. *J. Nutr. Sci. Vitaminol.,* **25**, 517-523.

Ito, T., Murata, H., Yasui, Y., Matsui, M., Sakai, T., and Yamauchi. K., 1995: Simultaneous determination of ascorbic acid and dehydroascorbic acid in fish tissues by high-performance liquid chromatography. *J. Chromatogr.*, **667**, 355–357.

Ito, T., Kera, A., Murata, H., Yoshida, T., Sakai, T., Yamauchi, K., Yamazaki, Y., Yamaguchi, T., and Ukawa. M., 2000: Experimentally induced bacterial hemolytic jaundice of yellowtail and oxidative stress. Nippon Suisan Gakkaishi, **66**,

50–54.

Kawatsu, H., 1969: Studies on the anemia of fish III. An example of macrocytic anemia found in brook trout, *Salvelinus fontinalis*. Bull. Freshwater Res. Lab., **19**, 161–167.

Kramer, C.Y., 1956: Extension of multiple range test to group means with unequal numbers of replications. *Biometrics*, **12**, 307–310.

Nakanishi, A., Nakamura, M., Iwata, K., Kera, A., Murata H., and Yoshida. T., 2007: Development of fish feed for healthy farmed fish I: Low cost fish feed (in Japanese). Annual Rep. Miyazaki Pref. Fish. Exp. Station, 233–243.

Takagi, S., Murata, H., Goto, T., Hayashi, M., Hatate, H., Endo, M., Yamashita, H., and Ukawa M., 2006: Hemolytic suppression roles of taurine in yellowtail *Seriola quinqueradiata* fed non-fishmeal diet based on soybean protein. *Fish. Sci.*, **72**, 546–555.

Yamauchi, K., Murata, H., Ohashi, T., Katayama, H., Pearson, A. M., Okada, T., and Yamakura. T., 1991: Effect of dietary α-tocopherol in broiler skeletal muscles and subcellular membranes and its relationship to oxidative stability. Nippon Shokuhin Kogyo Gakkaishi, **38**, 545–552.

Yanai, H., 2000: Statcel : The useful add-in forms on Excel. OMS-publishing, Saitama.