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ISSN 0792 - 156X

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PUBLISHER:

Israeli Journal of Aquaculture - BAMIGDEH -
Kibbutz Ein Hamifratz, Mobile Post 25210,
ISRAEL
Phone: + 972 52 3965809
http://siamb.org.il

The Israeli Journal of Aquaculture - Bamidgeh, IJA_64.2012.782, 7 pages

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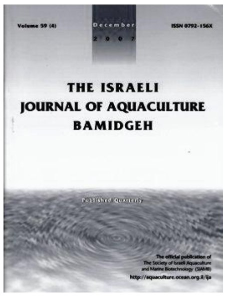

Level of L-ascorbyl-2-monophosphate-Mg as a Vitamin C Source in Practical Diets for the Asian sea bass, *Lates calcarifer*

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(Received 25.12.11, Accepted 29.1.12)

Key words: sea bass, feed efficiency, vitamin C, wound healing

Abstract

The stable vitamin C derivative, L-ascorbyl-2-monophosphate-Mg (AMP), was used as a supplement in practical diets for Asian sea bass, *Lates calcarifer*. Optimum growth, liver ascorbic acid (AA) saturation, and wound healing were determined. Sea bass (78.9±0.4 g) were fed a maintenance diet without vitamin C supplement for 25 days, then distributed into fifteen 1500-l oval fiberglass tanks at 30 fish each and fed one of five practical diets containing 0, 50, 100, 200, or 400 AMP mg/kg diet for 14 weeks. Fish fed the AMP-free diet exhibited clinical signs of vitamin C deficiency and significantly poorer final average weight, specific growth rate, protein efficiency ratio, feed conversion ratio, and hematocrit level ($p<0.05$). The level of AA in the sea bass brain increased as the level of dietary AMP increased. The AA concentration in the liver was similar at all AMP dietary levels. Body calcium of sea bass fed the AMP-free or 50 mg diets was significantly lower ($p<0.05$) than in fish fed the other diets. AMP enhanced wound healing regardless of supplement level, but fish fed the highest dietary AMP (400 mg/kg diet) exhibited histopathological changes in the liver. The dietary level of 50 mg AMP/kg diet was adequate for optimum growth, liver AA saturation, and prevention of clinical signs of vitamin C deficiency in 80-220 g sea bass. However, 100 mg AMP per kg diet was necessary for optimum body calcium.

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Introduction

Vitamin C is a very important micronutrient but is unstable in water and in the conditions in which fish feeds are ordinarily processed and stored. Stable derivatives of vitamin C have been tested as supplements in fish diets with noted variations in bioavailability. L-ascorbyl-2-monophosphate-Mg (AMP) has vitamin C activity in many aquaculture species (Lin and Shiau 2005; Catacutan et al., 2011). The vitamin C requirement varies with the size, age, and growth rate of fishes (NRC, 1983).

The Asian sea bass (*Lates calcarifer*) is a popular fish species for aquaculture and its vitamin C requirement has been demonstrated. Sea bass of about 5 g fed a practical diet without vitamin C had a reduction in growth rate after six weeks, exhibited clinical signs of vitamin C deficiency, and had pathological changes compared with fish fed AMP (Phromkunthong et al., 1997). In similar size European sea bass (*Dicentrarchus labrax*), tissue ascorbic acid (AA) varied with the dietary level and source of vitamin C such as monophosphate or polyphosphate derivatives (Alexis et al., 1999; Fournier et al., 2000). Vitamin C is important in wound healing in tilapia (Jauncey et al., 1985) and catfish (Erazo-Pagador and Din, 2011). In 56 g rainbow trout (*Oncorhynchus mykiss*), the AA level in the skin and muscle of experimentally inflicted wounds correlated with the dietary intake and healing proceeded more rapidly when higher dietary AA levels (150-1000 mg AA/kg dry feed) were fed (Wahli et al., 2003).

This study aimed to determine growth efficiency, liver AA saturation, and wound healing in 80-g sea bass (*L. calcarifer*) fed different dietary levels of AMP in practical diet formulations.

Materials and Methods

Fish. Sea bass fry (*Lates calcarifer*) from the SEAFDEC/AQD hatchery in Iloilo, Philippines, were reared in several 650-l fiberglass tanks with aerated sand-filtered sea water flowing at a rate of 2-4 l/min. Fry were fed *Artemia* for two weeks and gradually weaned during three weeks using a combination of *Artemia* and a dry pelleted maintenance diet (44% protein) until the fish subsisted on the pelleted feed alone. Prior to distribution in experimental tanks, five fish were sampled and the livers were dissected, fixed in Bouin's solution, and stored in 70% alcohol for later processing to determine initial liver histology.

Diets. Five experimental diets were formulated to contain graded levels of L-ascorbyl-2-monophosphate-Mg (AMP; 93% purity, Showa Denko K.K., Japan) with an estimated dietary energy content of about 18.5 MJ/kg dry diet. The dietary levels of AMP were 0, 50, 100, 200, and 400 mg per kg diet, with ascorbic acid (AA) equivalent to 0, 21.6, 43.2, 86.3, and 172.6 mg, respectively. The protein sources in the basal diet included Peruvian fishmeal, squid meal, *Acetes* sp., and defatted soybean meal (Table 1). Rice bran was reduced by the amount of AMP added to the diets. Diets were prepared as described by Catacutan et al. (2011). Briefly, the dough-like mixture was extruded twice at room temperature using a 5 mm diameter die, oven dried at 70°C for 2-3 h, and stored at 15-18°C. About 8-10 batches of feed were prepared throughout the study. Proximate analyses of the diets were carried out according to AOAC (2000). In a previous experiment, a practical diet containing 100 mg AMP/kg dry diet and steamed for 5 min retained AMP at 95% after one month of storage at room temperature (28-32°C).

Growth trial. A total of 450 fish (78.9±0.4 g) that had been fed a maintenance diet without vitamin C supplementation for 25 days were stocked at 30 individuals per tank in fifteen 1500-l oval fiberglass tanks with aerated sand-filtered sea water flowing at 2-4 l/min. Fish were sampled every 30 days, weighed in bulk, and the feeding rate was adjusted, accordingly. Initially, sampled fish were anesthetized with 100 ppm MS-222 (Sigma Chemical Co.); the dose was increased to 300 ppm as the fish increased in weight. The feeding rate was 5.5% of the body weight at the start of the trial and 3.5% at its end. Feed was given twice daily, at 09:00 after the tanks were cleaned and at 16:30. The growth experiment terminated at 14 weeks and the final average weight, survival rate, specific growth rate (SGR), protein efficiency ratio (PER), feed conversion ratio (FCR), and hepatosomatic index (HSI) were determined. Survival (%) was

Table 1. Composition of the basal diet.

Ingredient (g/kg dry diet)	
Peruvian fishmeal	480
Bread flour	200
Defatted soybean meal	80
Rice bran	70
Acetes sp.	70
Cod liver oil	38
Squid meal	20
Vitamin mix[1]	12
Mineral mix[2]	20
Lecithin	5
Dicalphos[3]	5
Chemical composition (% dry matter basis)	
Moisture	4.2
Crude protein	46.5
Crude fat	9
Crude fiber	2.2
Ash	12
Nitrogen free extract	27.4
Dietary energy (MJ/kg dry diet)[4]	19.2

[1] mg/kg dry diet: betacarotene 4.9; cholecalciferol 2.4; DL-alphatocopherol 164; menadione 27; paraaminobenzoic acid 164; biotin 2.4; Ca-pantothenate 114.5; choline chloride 3000; cyanocobalamine 0.037; folic acid 0.6; inositol 1640; niacin 327; pyridoxine 16; riboflavin 82; thiamin 24.5; cellufil 6434
[2] g/kg dry diet: K_2 HPO_4 5.0; KCl 2.5; $NaH_2PO_4.2H_2O$ 7.5; Ca $(H_2PO_4)_2.H_2O$ 5
[3] Contains 18% P, 22% Ca, 1% F
[4] computed as 23.7 MJ, 17.2 MJ, and 39.5 MJ per kg for protein, carbohydrate, and fat, respectively

calculated as 100(final no. of fish/initial no. fish) and condition factor (K) as 100(wt/length^3).

Vitamin C and hematocrit analysis. At the end of the trial, fish were sampled to determine vitamin C concentrations in brain and liver tissues, and samples were taken for liver histology, body Ca (by gravimetric method, AOAC 962.01, 2000), and hematocrit level. AA in the liver and brain were analyzed by HPLC using a reversed-phase column (C 18, 4.6 x 250 mm) following the methods of Koshio et al. (1997) and Sakakura et al. (1998). A Shimadzu HPLC system (Japan) was used that consisted of a column oven (Shi-madzu CTO-6A, at 40°C), a detector (Shimadzu SPD-6AV, UV 257 nm), a pump (Shimadzu LC-9A), and a chromatopak (Shimadzu C-R4A).

Fish hematocrit was determined by the microhematocrit method of Hesser (1960). Blood was withdrawn from the caudal artery of three fish from each aquarium into heparinized capillary tubes and centrifuged at 11,500 rpm for 5 min at room temperature (about 25°C). The remaining fish were maintained in their respective tanks for the wound healing experiment.

Wound healing and histopathological procedures. Fish from the terminated growth experiment were used in the wound healing experiment. A small wound approximately 2 cm long and 2-3 mm deep was cut at the upper part of the lateral line on the middle part of each anesthetized fish. Wounded fish were immediately returned to their respective tanks, fed their respective diet, and periodically sampled for histological analysis of wounds by sacrificing three fish per treatment 7 h, 24 h, and 2, 4, 7, 10, 14, and 21 days after wounding.

Livers from fish at the beginning of the trial, livers from three fish per treatment after the growth experiment (3 fish/treatment), and wound tissues were sampled for histology. Samples were fixed for 24 h in Bouin's fixative and decalcified in Frank Ruichilo's solution for 5-7 days, embedded in paraffin, sectioned (4-5 um), stained with hematoxylin and eosin (Humason, 1972), and examined under a light microscope.

Statistical analysis. Data were analyzed using analysis of variance and Duncan's Multiple Range Test in the SAS package for IBM-PC (SAS, 1998). Means were compared and differences between treatments were considered significant at $p<0.05$.

Results

Fish weight approximately tripled during the 14-week trial (Table 2). Fish without dietary AMP supplementation were dark and had a soft body texture. Most had eroded fins and hemorrhagic eyes that were not exhibited by AMP-fed fish. These fish also moved and swam more slowly than fish fed dietary AMP and their final average weight, SGR, PER, FCR, HSI, and hematocrit level were significantly poorer than in fish fed diets containing AMP, regardless of the dietary level. Body calcium of sea bass fed the AMP-free or 50 mg diets was significantly lower ($p<0.05$) than in fish fed the other diets. Growth of fish fed the AMP-free diet dropped after the second sampling (60 days). Survival rates (86.7-100%) and condition factors (2.29±0.01) did not differ between treatments.

Tissue analysis revealed that brain and liver AA of fish fed the 50 mg diet were significantly higher than in fish fed the AMP-free diet. The sea bass brain had more AA than the liver and the level increased as the percent of dietary AMP increased. The concentration of AA in the liver was remarkably similar in fish fed all levels of AMP.

Table 2. Growth, feed efficiency, hepatosomatic index (HSI), hematocrit, body calcium, and levels of ascorbic acid in sea bass fed diets containing different levels of L-ascorbyl-2-monophosphate (AMP) for 14 weeks (means±SEM).

	Diet (g AMP per kg dry diet)				
	0	*50*	*100*	*200*	*400*
Initial avg wt (g)	79.1±0.3	79.8±0.4	78.7±0.5	79.0±0.3	78.7±0.4
Final avg wt (g)	173.3±6.2[b]	222.0±7.9[a]	219.2±7.2[a]	219.2±3.7[a]	211.2 ±5.2[a]
SGR (%/day)[1]	0.80±0.04[b]	1.06±0.04[a]	1.04±0.03[a]	1.04±0.02[a]	1.00±0.02[a]
PER[2]	0.37±0.02[b]	0.54±0.02[a]	0.53±0.02[a]	0.55±0.02[a]	0.49±0.01[a]
FCR[3]	5.8±0.4[a]	4.0±0.1[b]	4.0±0.2[b]	3.9±0.1[b]	4.2±0.1[b]
HSI (%)[4]	2.1±0.1[b]	2.2±0.1[a]	2.2±0.1[a]	2.2± 0.1[a]	2.2± 0.1[a]
Hematocrit (%)	28.0±3.0[b]	38.6±5.3[a]	41.0±1.0[a]	40.34±3.1[a]	43.0±1.5[a]
Body calcium (% dry wt)	3.76± 0.17[b]	3.75±0.20[b]	4.38±0.18[a]	4.49±0.11[a]	4.53±0.22[a]
Ascorbic acid (ug/g of wet tissue)					
Liver	70.9±4.1[b]	108.4±9.3[a]	108.36±2.4[a]	102.2±16.6[a]	107.3±8.1[a]
Brain	119.3±7.2[d]	182.4±3.8[c]	225.4±5.8[b]	233.3±11.4[b]	316.9 ±0.8[a]

Values in a row with the same superscripts do not significantly differ ($p>0.05$).
[1] Specific growth rate = 100(ln avg final wt - ln avg initial wt)/no. days
[2] Protein efficiency ratio = wt gain/protein fed
[3] Feed conversion ratio = dry wt feed/wet wt gain
[4] Hepatosomatic index =100(liver wt/fish body wt)

Liver of fish fed the 400 mg diet exhibited histopathological differences from the start of the feeding experiment (Fig. 1a). Vacuolization was noted around the pancreatic tissues (Fig. 1b). In the wound healing experiment, the incisions in the fish muscles had lost their epidermal tissue overlay in all treatments at 7 and 24 h after wounding (Fig. 2a) but, in fish fed the supplemented diets, the wound had an increased number of fibroblasts at days 2 and 4 with epidermal thickening and melanin pigment distributed in a newly restored dermal layer (Fig. 2b). In fish fed the AMP-free diet, dense fibroblasts were seen in wounds only at day 7. Wound healing progressed well in all fish fed diets with AMP, showing numerous granulation tissues, a well-defined border between the incised and normal musculature, and normal architecture at day 14. The dermis, epidermis, and muscle tissues were normal in wounds of fish without dietary AMP only at day 21 (Fig. 2c).

a b

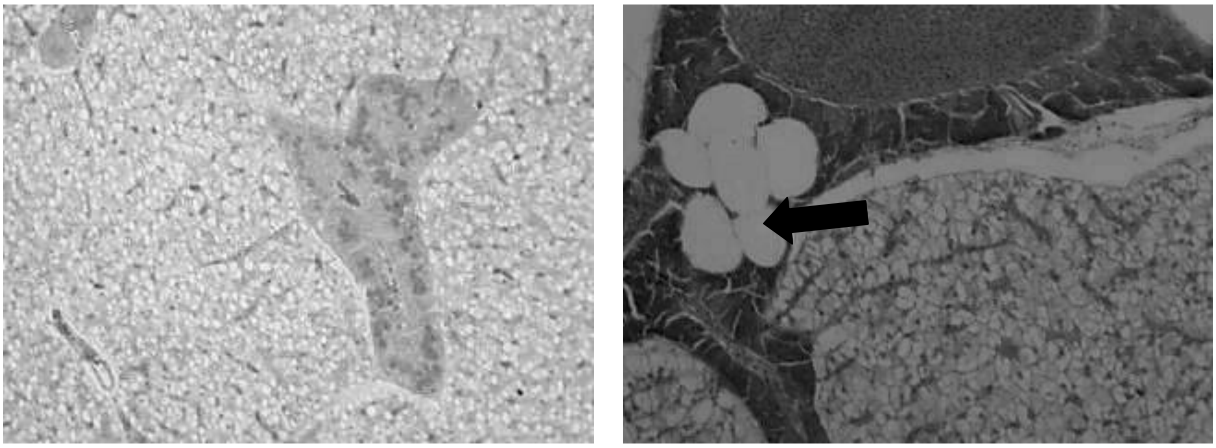

Fig. 1. Normal liver structure of sea bass (a) at the start of feeding (H&E 10X) and (b) after 14 weeks of feeding a diet supplemented with 400 ppm L-ascorbyl-2-monophosphate-Mg; arrow shows vacuolization (H&E 40X).

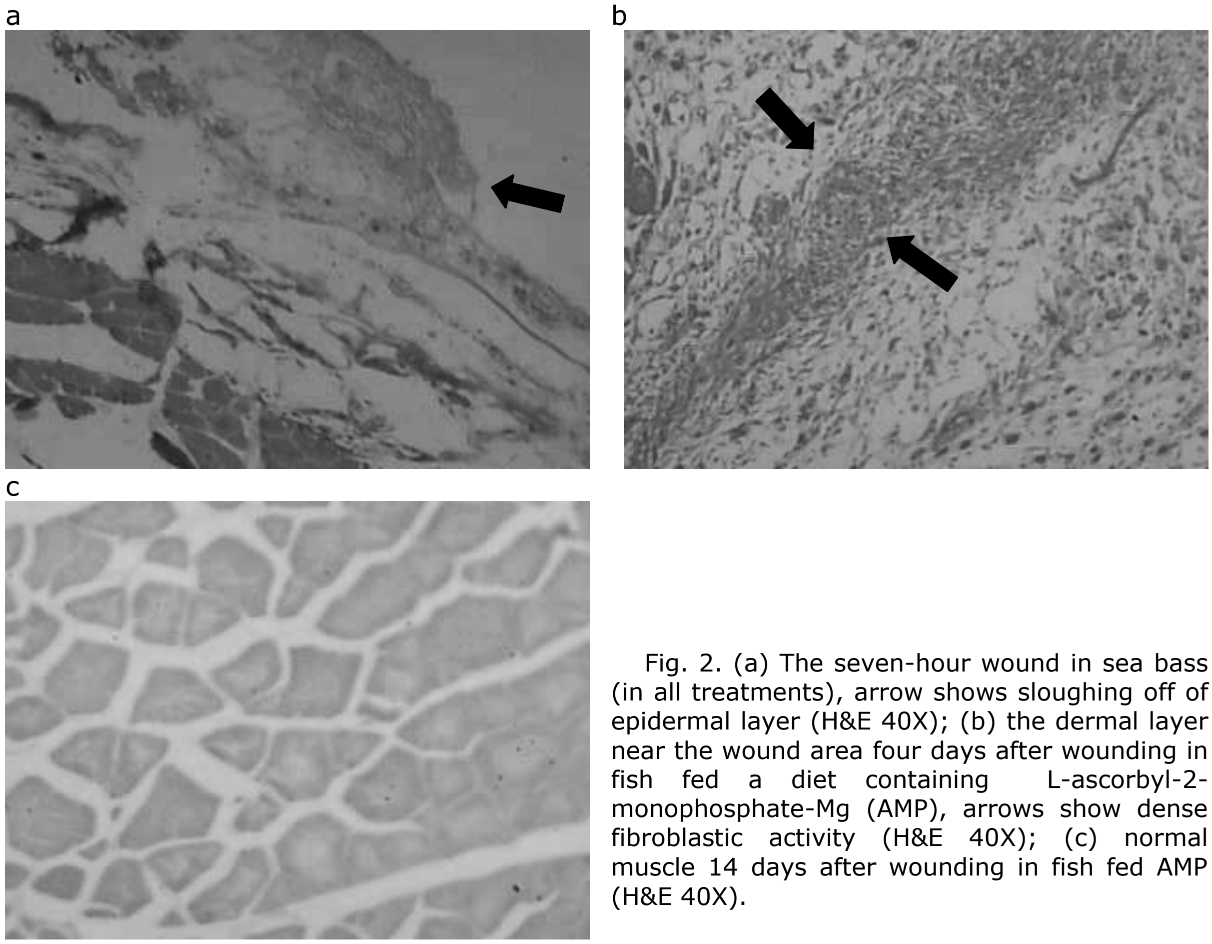

Fig. 2. (a) The seven-hour wound in sea bass (in all treatments), arrow shows sloughing off of epidermal layer (H&E 40X); (b) the dermal layer near the wound area four days after wounding in fish fed a diet containing L-ascorbyl-2-monophosphate-Mg (AMP), arrows show dense fibroblastic activity (H&E 40X); (c) normal muscle 14 days after wounding in fish fed AMP (H&E 40X).

Discussion

The phosphate form of vitamin C used in this study is stable and bioavailable as a source of vitamin C in aquaculture species (Teshima et al., 1993; Phromkunthong et al., 1997). In the present study, sea bass fed practical formulations without a dietary vitamin C supplement for 14 weeks had the poorest growth efficiency, lowest hematocrit level, and notable vitamin C deficiency symptoms. Without dietary vitamin C supplementation, fish are anemic and have low hematocrit levels (Lim et al., 2000). This could partly explain the poor growth efficiency of sea bass fed the AMP-free diet. The efficacy of AMP as a source of vitamin C for sea bass is indicated by the significantly improved growth efficiency and lack of vitamin deficiency symptoms in fish fed even the lowest level of AMP supplementation (50 mg/kg diet). This dietary level of AA (21.6 mg/kg diet) is within the reported vitamin C requirement for growth in Asian and European sea bass, 5-31 mg AA eq/kg diet (Gouillou-Coustans and Kaushik, 2001). In channel catfish (*Ictalurus punctatus*), dietary vitamin C levels of 30 and 60 mg/kg diet, respectively, are adequate for maximum weight gain and sufficient for wound repair and prevention of signs of deficiency (Lim and Lovell, 1978).

Although wounds of all fish were healed at day 21, there were more fibroblasts in lesions of AMP-fed fish during the first 4 days than in fish fed the AMP-free diet, indicating the importance of AMP as a source of vitamin C in wound healing. In tilapia, delay in wound healing is attributed to reduced collagen synthesis when fish are fed a diet without supplementary vitamin C (Jauncey et al., 1985). Collagen synthesis increased in embryos and larvae of sea bass (*Dicentrarchus labrax*) and sea bream when a high dose of coated L-ascorbic acid (2000 mg/kg) was added to the broodstock diet (Terova et al., 1998). In the present study, the highest dietary level of AMP showed no advantage over lower levels in effecting a faster rate of wound healing in sea bass.

The practical feed ingredients in the basal diet in this study enabled survival of sea bass for 14 weeks in addition to the 25 days of acclimation on a maintenance diet unsupplemented with vitamin C. Possibly, the endogenous vitamin C in the practical diet formulation was sufficient to sustain survival of sea bass. A practical diet containing 20% red drum muscle contains about 6 ppm vitamin C (Aguirre and Gatlin, 1999). For the size of sea bass in our study, the survival rate was not affected by the basal diet containing 48% fishmeal, whether or not supplemented with AMP. Likewise, growth and survival are not affected in other fish species when practical feed ingredients are used with or without vitamin C supplementation (Matusiewicz et al., 1995; Henrique et al., 1998).

Vitamin C, an important anti-oxidant, was more abundant in the brain of sea bass than in the liver. The concentration of AA in the brain increased with increasing dietary vitamin C whereas the concentration of AA in the liver reached saturation at 108 ug/g with the lowest AMP supplementation (50 ppm or 21.6 ppm AA). This differs from some fish species where liver AA saturation is not attained even at high dietary vitamin C levels (Matusiewicz et al., 1995; Fracalossi et al., 1998). The concentration of liver AA is a good index of the ascorbic acid status in Asian sea bass and a more stringent response criterion for determining the vitamin C requirement of juvenile European sea bass (Fournier et al., 2000).

For Asian sea bass, the dietary supplement of 400 ppm AMP showed no difference over lower AMP levels in terms of growth, feed efficiency, wound healing, hematocrit level, HSI, and liver AA saturation. AMP may be toxic to sea bass; red snapper fed a diet containing the highest AMP supplement (540 mg AMP/kg diet) displayed significantly poorer growth and feed efficiency, and had histological changes in the liver and kidney (Catacutan et al., 2011).

Sea bass fed the diet containing 50 mg AMP attained the maximum weight and liver AA saturation, without vitamin C deficiency symptoms. However, the level of calcium in their carcass was significantly lower and similar to that of fish fed the AMP-free diet. Thus, the supplementary level of 50 ppm AMP (21.6 ppm AA) might be sub-optimum for sea bass. AMP is 25-35% pure and costs about US$8/kg. Using this product in commercial sea bass feed at an inclusion rate equivalent to 43 ppm AA/kg diet is beneficial and would increase profitability at a cost of less than 1 cent in the formulation.

The vitamin C requirement for growth and prevention of deficiency symptoms is lower than the quantity required to achieve tissue saturation (Gabaudan and Velrhac, 2001). Based on the conditions of this study, a dietary level of 50 mg AMP/kg practical diet was adequate for growth, liver AA saturation, wound healing, and prevention of clinical vitamin C deficiency symptoms in Asian sea bass. However, a dietary level of 100 mg AMP/kg was necessary for the optimum uptake of calcium.

Acknowledgements

The authors would like to acknowledge Dr. Veronica Alava and Dr. Relicardo Coloso for their valuable comments and Mr. Hernando Alcalde for the commendable technical assistance. This study was funded by SEAFDEC/AQD.

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