THE EFFECT OF VITAMIN C (L-ASCORBYL MONOPHOSPHATE-Mg) ON THE DEFORMITY PERFORMANCE OF HUMPBACK GROUPER (*Cromileptes altivelis*) LARVAE

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(*Received 15 Januari 2010; Accepted 19 April 2010*)

ABSTRACT

This study was aimed to get a standard protocol for vitamin C application in enrichment of *Artemia* nauplii and to examine the impact of vitamin C on humpback grouper larval deformity performance. Vitamin C in the form of L-ascorbyl-2-monophosphate-Mg (AMP-Mg) was used for the enrichment and incorporated into *Artemia* nauplii at a given level of 0-1.5 g/L with enrichment period of 4, 6, and 24 hours for each level. The effect of AMP on larval deformity was tested using the following treatments: un-enriched both *Artemia* nauplii and commercial diet, un-enriched *Artemia* nauplii and experimental diet containing 2.5 g AMP-Mg/kg diet, enriched *Artemia* nauplii obtained from the protocol and commercial larval diet. Analysis of vitamin C content in *Artemia* nauplii was carried out using HPLC and larval deformities were observed using staining method, visual examination, or X-ray depending on larval age. The results showed that the best enrichment method of AMP in *Artemia* nauplii is achieved at the dose of 0.9 g/L for 6 h. The lowest percentage of abnormalities was found in larvae fed with enriched *Artemia* nauplii and commercial diets, suggesting that vitamin C has an important role in controlling larval deformities.

KEYWORDS: humpback grouper larvae, vitamin C, deformity

INTRODUCTION

Humpback grouper (*Cromileptes altivelis*) is one of important food fish widely cultured in Southeast Asia notably in Indonesia, the Philippines, and Taiwan. Seed production technology of this species has been successfully developed for more than a decade (Sugama *et al*., 2001; Kawahara *et al*., 2000), however, problems particularly incidences of larval deformities and larval mass mortalities are still experienced. Improvements in larval nutrition are then necessary to solve these problems.

Vitamin C (ascorbic acid, AA) is an essential vitamin in fish larval diets and has been shown to give a pronounced effect in reducing stress and increasing disease resistance (Merchie *et al*., 1995a; 1995b, 1997; Gapasin *et al*., 1998; Moe, 2004). Besides that, inadequate amount of dietary vitamin C results in a number of deformity signs, such as scoliosis, distorted/twisted gill filaments, opercular deformity, and short snout (Gapasin *et al*., 1998; Dabrowski, 1990; Soliman *et al*., 1986). The requirement of vitamin C in fish is only obtained from exogenous supply of vitamin C source as fish lack the enzyme L-gulonolactone oxidase (EC 1.1.3.8) which is responsible for synthesis of vitamin C *de novo* (Wilson, 1973). To satisfy the need, the supply of dietary vitamin C is usually given through the formulated diet or enrichment in live feed. Extensive studies of dietary vitamin C requirement have been done for juvenile and adult stages of several marine

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fish (Sealey *et al*., 1999; Wilson *et al*., 1973; Ai *et al*., 2004; Wang *et al*., 2003; Dabrowski *et al*., 1990) and the recommended values ranged from 20 to 50 mg of ascorbic acid kg^{-1} diet (NRC, 1993). Nevertheless, few data on vitamin C requirement for first feeding larvae particularly in improving larval deformity are available. Merchie *et al*. (1996) reported that ascorbic acid concentration in un-enriched *Artemia* that can reach up to 500 mg equivalent of ascorbic acid per gram dry matter was adequate to satisfy the nutritional requirement of larvae. Meanwhile, ascorbic acid concentration of approximately 1,400 µg/g DW in enriched *Artemia* was sufficient to improve growth and larval performance of African catfish (Merchie *et al*., 1997). Another study (Gapasin *et al*., 1998) reported that milkfish larvae fed with HUFA and ascorbic acid-enriched live food had significantly lower opercula deformity incidence and exhibited higher growth than those given un-enriched live food. Those results suggest that the requirement varies to some degree with fish species, size, diet and its preparations, the form of vitamin C and experimental conditions. However, there is no information about the effect of vitamin C on larval stage of groupers, particularly humpback grouper.

On the other hand, due to the highly unstable of vitamin C and its loss activity upon processing and storage, the stable forms of ascorbic acid derivatives were used; they are ascorbyl palmitate (AP), L-ascorbyl-2-monophosphate-Mg (AMP-Mg), L-ascorbyl-2-monophosphate-Na/Ca (AMP-Na/Ca), L-ascorbyl-2-polyphosphate (APP), ascorbyl-2-sulphate, etc. Of the derivatives, ascorbyl-2-monophosphate seems recognized to be very stable.

Thus, the present study was aimed to investigate the effect of vitamin C (in the form of AMP-Mg) to control deformities on humpback grouper larvae. Incorporating AMP-Mg into larval grouper was conducted through live food enrichment. For this purpose, *Artemia* nauplii enrichment with AA was conducted and further investigated as a tool to deliver vitamin C to the larvae.

MATERIALS AND METHODS

Artemia Enrichment

Decapsulated *Artemia* cysts (INVE, Great Salt Lake Prime Gold, Prime *Artemia*, Midvale, UT, USA) were cultured in white polycarbonate cones filled with 100 L of sea water (34 ± 1 ppt) with aeration system at 28°C–33°C. At 24 hours, newly hatched *Artemia* were removed from the hatching cones. Two liters of *Artemia* nauplii were sampled, three-times rinsed in distilled water, filtered and freeze dried for dry weight (DW) and AA analyses. After that, the rest of *Artemia* nauplii were cultured in 24 L of seawater with aeration for ± 12 h. After 12 h, *Artemia* nauplii were divided and placed into 12 of 2-L of seawater-filled plastic bottles for enrichment protocol treatments. Treatments were conducted by enriching the nauplii with L-ascorbyl-2-monophosphate-Mg (AMP-Mg) at the level of 0, 0.6, 0.9, 1.2, and 1.5 g/L and enrichment period of 4, 6, and 24 h. To ensure the presentation of appropriate particle size for *Artemia* nauplii, AMP was blended for 30 s in seawater before added to *Artemia*. Enriched *Artemia* nauplii were removed and washed as previously described. *Artemia* nauplii samples were freeze-dried for 24 h and prepared for AA analysis.

Total AA is detected using High-Performance Liquid Chromatography (HPLC) with Lichrocart 250-4 column and mobile phase 0.05 M KH_2PO_4 pH 2.8, flow rate 0.5 mL/min., at room temperature. The product was detected using UV detector at 245 nm (Shiau & Hsu, 1994). Factorial statistical analysis was conducted using the MSUSTAT program (MSUSTAT). Values of ($P<0.05$) were considered significantly different.

Larval Culture

Eggs of humpback grouper at a density of 100 eggs/L were stocked into three $5m^3$-concrete tanks filled with seawater (salinity 34±1 ppt). During the first 10 days, the larvae were reared without water exchange. Larval rearing feeding regime followed the method described in Table 1.

At day 20^{th}, the larvae were started fed with the following treatments: (A) un-enriched both *Artemia* nauplii and commercial larval diet (control); (B) un-enriched *Artemia* nauplii and vitamin C-enriched experimental diet (containing 2.5 g AMP/kg, prepared in the laboratory; provided in Table 2); and (C) vitamin C-enriched *Artemia* nauplii (based on the result of the enrichment protocol experiment) and unenriched commercial larval diet.

Samples of larvae were taken started on day 30^{th} and collected every 10 days for stain-

Table 1. Feeding regime to humpback grouper larvae

Food	2	3	8	15	20	25	30	35	40	45	50
Nannochloropsis	----------										
Rotifer		----------									
Artemia nauplii				▲	----------						
Artificial diet				----------							
				Start vitamin C							

Table 2. Composition of experimental larval grouper diet

Ingredients	%
Casein	30.95
Fish meal	32.00
Squid liver meal	8.00
Mysid meal	6.00
Vitamin mixture	1.30
Mineral mixture	2.50
Dextrin	5.00
Squid oil	6.00
Carrageenan	8.00
Vitamin C (AMP-Mg)	0.25
Total	**100**

ing. The staining procedure followed the method described by Potthoff (1983). Samples of 90-days and 5-months old juveniles were taken for visual examination and X-ray analysis. At the end of the experiment, the juveniles were harvested and calculated to quantify their deformity.

RESULTS AND DISCUSSION

Artemia Enrichment

Ascorbate level in *Artemia* nauplii given in different concentrations of AMP-Mg and enrichment period is shown in Figure 1. The endogenous level of AA in un-enriched *Artemia* nauplii was 713.1 µg g^{-1} DW. In Figure 1(a), it is clearly seen that with increasing concentration of AMP-Mg at each enrichment period, the ascorbate level of *Artemia* nauplii significantly increased ($P<0.05$) and reached a maximum level at 0.9 g AMP-Mg/L enrichment. Interestingly, the period of AMP-Mg enrichment also influenced the endogenous AA level of *Artemia* nauplii. Short period of enrichment into *Artemia* nauplii exhibited the increased endogenous AA level from 0.72% to 1%, 1.14%, 1.05%, and 1.4%, respectively in each AMP-Mg concentration. Prolonged enrichment to 6 h showed a similar trend of AA level except for that at enrichment dose of 1.5 g AMP-Mg/L.

Unlike those in 4 and 6h of enrichment period, the endogenous AA level of 24 h-enriched *Artemia* nauplii decreased in each concentration. The highest ascorbate level of *Artemia* was attained at 6 h of enrichment period. This suggested that the best enrichment method of AMP-Mg in *Artemia* nauplii was achieved at a dose of 0.9 g/L for 6 h ($P<0.05$) and this result was used as the protocol for humpback grouper larval rearing. The result in the present study was different from that reported by Smith *et al*. (2004). Their study showed that the maximum enrichment using ascorbyl-2-phosphate was attained at rates of 8 1.2 g/L for 24 h giving a 60-fold increase in *Artemia* nauplii AA levels. The discrepancy is

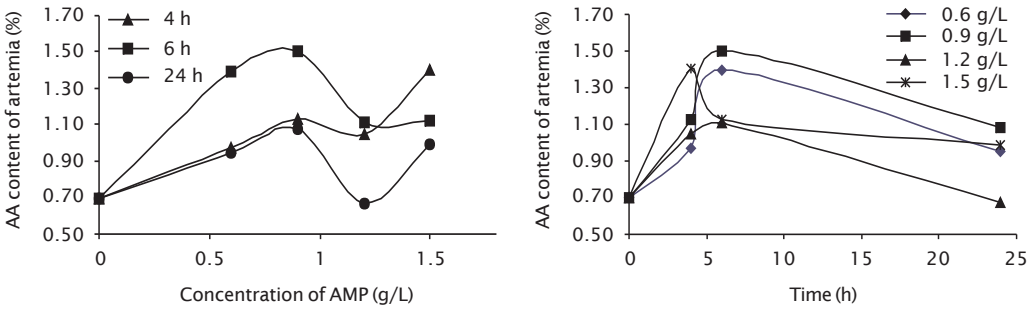

Figure 1. Ascorbic acid content in *Artemia* nauplii enriched with AMP-Mg in the given treatments: (a) AMP concentration (0-1.5 g/L), (b) AMP enrichment period (4, 6, and 24 hours)

possibly due to differences in *Artemia* size used for enrichment, the form of vitamin C derivatives, and experimental conditions. Of all, it can be concluded that AMP-Mg could be assimilated as AA in *Artemia* nauplii in a dose and period dependant.

Larval Performance

The deformity performance of humpback grouper larvae determined using staining method is given in Figure 2. It is seen that deformities in humpback grouper larvae were still found and humpback grouper larvae fed with un-enriched *Artemia* nauplii and commercial diet (control diet) exhibited the highest percentage incidence of deformities during 50 days of culture. In addition, larvae fed with un-enriched *Artemia* and AA-enriched experimental diet gave lower deformities than those in fed control diet, while larvae fed with AA-enriched *Artemia* and un-enriched commercial diet gave the lowest. This suggested that vitamin C played a role in controlling larval abnormality.

Based on the staining method, the different trial groups presented a variety of deformities. The most frequent deformities found were vertebral deformities occurred at the backbone which could not be observed visually (Figure 3).

Vertebral deformities observed consisted of lordosis, scoliosis, kyphosis, and abnormal on the backbone (Figure 3A-E). Deformities in fusion on dorsal spine and epurals, and branching of the dorsal proximal radial were also found during the trials. Although feed had contained AA supplementation, deformities were still found; this may be attributed to the insufficient requirement of AA concentration for tissue and bone matrix of the larvae or to the reason that AA supplementation did not totally eliminate

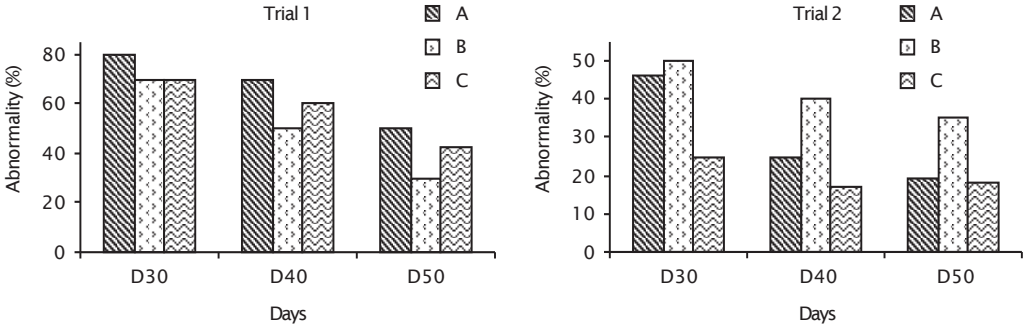

Figure 2. Percentage deformity of humpback grouper larvae (D30-D50, by staining method) fed with un-enriched both *Artemia* nauplii and commercial diet (A), larvae fed with un-enriched *Artemia* nauplii and AA-enriched experimental diet (B), larvae fed with AA-enriched *Artemia* nauplii and un-enriched commercial diet (C)

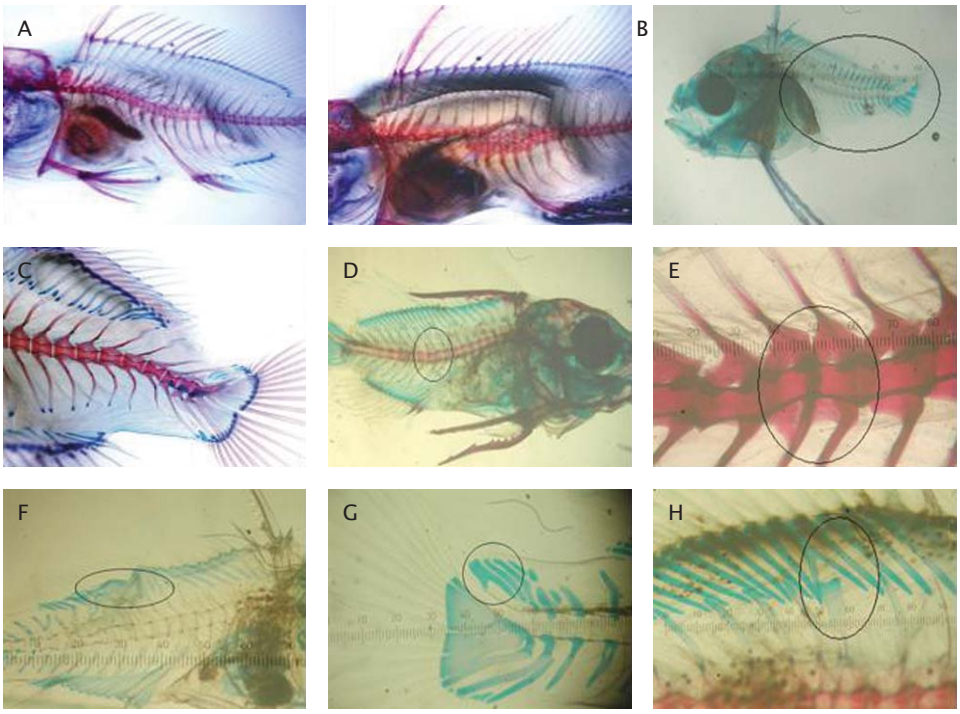

Figure 3. Main skeletal deformities developed in humpback grouper larvae (stained specimens): A. lordosis, B. scoliosis, C. kyphosis, D and E. abnormal on back bone, F. fusion on dorsal spine, G. fusion on epurals, H. branching of the dorsal proximal radial

abnormality in the larvae. The AA concentration in fish larvae fed with different diets was not measured in the present study.

After three months of culture, deformity of the juvenile was measured visually and a similar trend to those with staining method was observed (Figure 4). However, the result in the trial 1 of 90-day-old larvae was on the contrary to those in the trial 2 of 90-day-old larvae and in the staining specimens. This may be ascribed to the eggs coming from different broodstock or different spawned-egg period contributing to different quality of larvae which deformity was not apparent during early larval stage. Visual examination was also conducted to 150-day-old juveniles and a similar trend as those in the staining results was obtained.

Visually, deformities found in the juveniles were mostly open operculum (opercula deformity), short body, bent head, and discharged epithelia. A similar finding that an opercula deformity observed in ascorbate deficient milkfish larvae was reported by Gapasin *et al*. (1998). They reported that the opercula deformity incidence was significantly lower in the larvae fed with HUFA + vitamin C-enriched rotifers and *Artemia* than those fed with HUFA-enriched or un-enriched rotifers and *Artemia*. Another study by Fraser & de Nys (2010) reported that deficiency of vitamin C induced spinal deformities like 'broken back' syndrome and lordosis and opercula deformity in barramundi juvenile. The addition of vitamin C (in the form of free ascorbic acid) significantly reduced the spinal deformities and completely lowered the opercula deformity incidence but not affect the incidence of jaw deformities in the juvenile.

X-ray examination was only applied to 150-day-old samples to visualize the bone/skeletal structure of juveniles. In accordance with that in the staining result, the deformities observed dominantly occurred on the back bone (Figure 5).

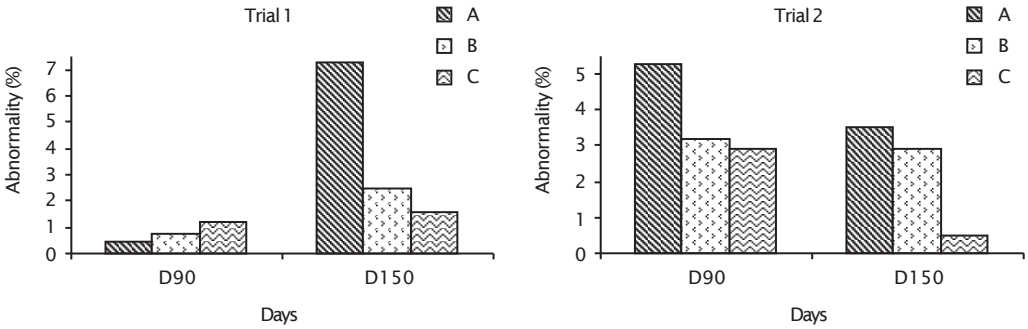

Figure 4. Percentage deformity of humpback grouper larvae (D-90-D-150, visual examination) fed with un-enriched both *Artemia* nauplii and commercial diet (A), larvae fed with un-enriched *Artemia* nauplii and AA-enriched experimental diet (B), larvae fed with AA-enriched *Artemia* nauplii and un-enriched commercial diet (C)

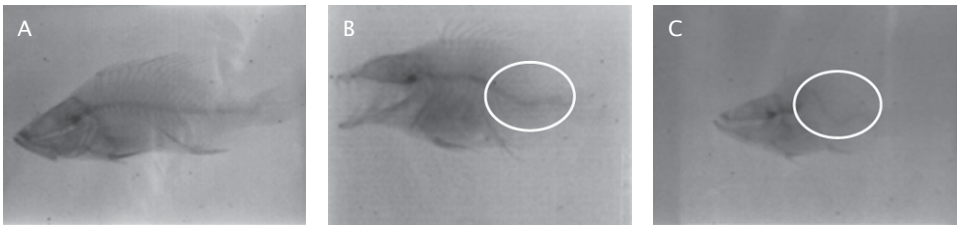

Figure 5. X-ray analysis of 150-day-old humpback grouper juveniles: A. normal, B. lordosis, C. scoliosis

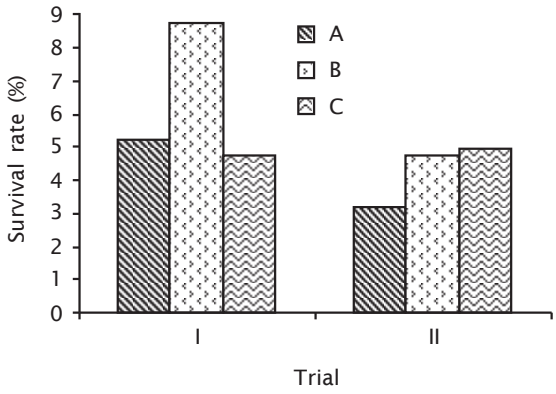

Figure 6. Percentage survival of 150-day-old humpback grouper fed with un-enriched both *Artemia* nauplii and commercial diet (A), larvae fed with un-enriched *Artemia* nauplii and AA-enriched experimental diet (B), larvae fed with AA-enriched *Artemia* nauplii and un-enriched commercial diet (C)

Interestingly, after 5 months (150 days) of culture, survival rate of the larvae did not differ among the treatment groups in each trial (Figure 6), suggesting that vitamin C did not affect the survival rate of the larvae. The finding was in accordance with the previous results reported by Gapasin *et al*. (1998). Cahu *et al*. (2003) also reviewed that the enrichment ascorbic acid did improve neither the survival nor the growth.

In the current study, it is clear that vitamin C supplementation in larval diet was highly important and revealed low percentage of deformities. The results were in accordance to the previous studies both for larval and juvenile of marine species (Merchie *et al*., 1995b, 1996, 1997; Gapasin *et al*., 1998; Moe *et al*., 2004; Dabrowski *et al*., 1990; Soliman *et al*., 1986; Wilson, 1973; Sealay *et al*., 1999; Wilson *et al*., 1973; Ai *et al*., 2004; Wang *et al*., 2003). The requirement of this micronutrient varied to different species. In addition, Dabrowski *et al*. (1996) reported that larval stages need a higher level of vitamin C than juvenile stages of African catfish. The study of Fraser & de Nys (2010) suggested that vitamin C had a role in ontogeny of skeletal deformities in barramundi larval.

CONCLUSIONS

1. The optimum enrichment method of L-ascorbyl-2-monophosphate in *Artemia* nauplii was at a dose of 0.9 g/L for 6 h.
2. Humpback grouper larvae fed with vitamin C-enriched feed exhibited low percentage of deformity, indicating that vitamin C has an important role in controlling abnormality of *C. altivelis* larvae.

ACKNOWLEDGEMENT

This study was financially supported by Australian Centre for International Agricultural Research (ACIAR) under the Research Institute for Mariculture (RIM) Gondol and ACIAR collaborative project. The authors acknowledge I Komang Suarsana for the excellent technical assistance.

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