ビタミンC源であるアスコルビン酸-2-モノリン酸Mg2+がイシダイの産卵に及ぼす効果

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著者 Yong, A.S.K.
瀬岡, 学
大川, 有紀
高岡, 治
滝井, 健二
熊井, 英水
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Spawning Performance of Striped Knifejaw, *Oplegnathus fasciatus* Fed Graded Levels of Ascorbyl-2-monophosphate Mg$^{2+}$ as Vitamin C Source

Annita Seok Kian Yong$^1$, Manabu Seoka$^1$, Yuki Ohkawa$^2$, Osamu Takaoka$^2$, Kenji Takii$^1$, * and Hidemi Kumai$^1$

**Abstract:** This study aimed to improve spawning performance of striped knifejaw using ascorbyl-2-monophosphate Mg salt (APM) as a dietary ascorbic acid (AsA) source. Five experimental diets, a control diet 1 without APM and four test diets 2-5 with 500, 1,000, 3,000 and 6,000 mg AsA/kg diet in an equivalent basis of APM, respectively, were prepared using a semi purified fish meal basal diet. Each diet was fed to duplicate broodfish groups consisting of 4 females and 2 males, having mean body weight of 587 and 595 g, respectively, once a day for 21 weeks, from April 2006. Dietary APM promoted earlier onset of spawning and induced a tendency of improving egg quality; diet 2 had higher tendencies in egg production, buoyancy, hatching rate and larval survival activity index. Dietary APM significantly correlated to AsA levels in eggs. Groups fed diets 4 and 5 tended to induce higher abnormal larve than groups fed diets 1-3. These results revealed that the broodfish of striped knifejaw effectively utilized APM as a dietary AsA source and promoted the early onset of spawning and performance; but high dietary APM might cause ill effects on egg quality.

**Key words:** Ascorbyl-2-phosphate Mg$^{2+}$; Striped knifejaw; Spawning performance; Ascorbic acid

Vitamin C, ascorbic acid (AsA), is an important and vital water soluble nutrient as internal antioxidant and reducing agent. Animals are able to biosynthesize AsA through a metabolic pathway from glucose. However, most of teleost fish have limited ability to biosynthesize AsA that is essential for maintaining good health and normal growth. As compared with other micronutrient requirement in small quantity, fish required AsA in a huge range of quantity, depending upon development stage, physiology status, species specific, interaction with other nutrients and culture environment (Ishibashi 1994; Luck et al. 1995; Terova et al. 1998; Dabrowski and Ciereszko 2001).

Apart from being important in the biosynthesis of collagen, AsA has been also reported to improve fish reproductive performance (Sandnes et al. 1984; Ishibashi 1994; Dabrowski and Ciereszko 2001). Striped knifejaw, *Oplegnathus fasciatus* is an important aquaculture fish species in Japan, Korea and Taiwan. However, study related to its AsA requirement is scarcely available. Ikeda et al. (1988) showed that striped knifejaw may have low ability to synthesize up to their requirement of AsA. Wang et al. (2003) showed that striped knifejaw juvenile (3.9 g initial body weight) required 118 mg AsA/kg diet in the form of APM for maximum growth. Ishibashi (1994) also indicated higher egg AsA level were obtained from striped knifejaw broodfish fed diet fortified with AsA-Ca salt at the level of 3,000 mg AsA/kg diet than 250 mg AsA/kg diet. With considerable vast biological functions of AsA, high AsA dose has been suggested to give beneficial and pharmacological effects for mammals (Luck et al. 1995), and it has been well debated to improve reproductive performance and health over the decades in fish (Blom and

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$^1$Fisheries Laboratory, Kinki University, Uragami, Wakayama 649-5145, Japan.

$^2$Fish Nursery Center, Kinki University, Uragami, Wakayama 649-5145, Japan.

*Corresponding author: Kenji Takii Tel.: +81-735-58-0116 Email: takii@nara.kindai.ac.jp
Dabrowski 1995; Terova et al. 1998). Blom and Dabrowski (1995) had recommended 8 times higher AsA requirement level than the National Research Council guideline of 50 mg/kg diet for improving the survival of the embryo survival and enhancing the tissue AsA levels in rainbow trout (Oncorhynchus mykiss). Terova et al. (1998) also reported that the adequate AsA level for normal of growth in sea bass (Dicentrarchus labrax) and sea bream (Sparus aurata) may not be sufficient for broodfish during reproductive season. These suggest that the broodfish may require larger amount of AsA for inducing reasonable reproductive function and performance. Thus, this study aimed to clear the effect of AsA fortification and a suitable dietary AsA level for reproductive performance of striped knifejaw broodfish using ascorbyl-2-monophosphate-Mg²⁺ salt (APM) that has little eliminate AsA activity during a dietary preparation, in contrast to AsA-Ca (Takii et al. 1999).

**Material and methods**

**Test diet**

The APM was used as a dietary AsA source (Phospitan-C, 43% ascorbic acid activity; Showa Denko Co. Ltd., Tokyo, Japan). We prepared five test diets; a control diet 1 without APM and diets 2-5 supplied with APM at levels of 500, 1,000, 3,000 and 6,000 mg AsA equivalent basis/kg diet (Table 1). Based on the APM level detected in each test diet and 43% ascorbic activity in APM; the AsA level in each diet was calculated equivalent to 0.52, 441, 853, 2,429 and 4,630 mg AsA/kg diet in test diets 1-5, respectively. White fish meal and pollack liver oil was used as protein and lipid sources, respectively. Feeding stimulants, a mixture of taurine, proline, serine and threonine (Takaoka et al. 1995), and a vitamin mixture excluding AsA and a mineral mixture (Halver 1957) were added at 1, 4 and 5%, respectively. Ingredients were weighed and mixed well before adding 30% water. The dough mixture was then passed through 10 mm die hole of an experimental pellet machine. Experimental diets were newly prepared biweekly and stored at −20°C until use. The proximate compositions of test diets were not significantly different among test diets. The mean values of proximate analysis were showed in Table 1.

**Fish and feeding trial**

The feeding trial was conducted at Fisheries Laboratory, Kinki University, Uragami, Wakayama. Ten groups, consisting of 6 striped knifejaw, 4 females to 2 males of 3-year-old broodfish having mean body weight of 587 g and 595 g, respectively, were procured from Fish Nursery Center, Kinki University, Uragami and randomly distributed into a 1.5 m³ tank and acclimated to rearing conditions for 10 days before the commencing of feeding trial.

During the feeding trial, 17 April-6 September 2006, each test diet was fed to duplicate groups of broodfish once daily (10:00 am) for 21 weeks. Flow-through filtered seawater system with 5 l/min was conducted throughout the feeding trial. In the initial 10 weeks of the trial, water temperature was controlled at 18–20°C. Then, water temperature was gradually increased to 22–23°C in 4 days duration and maintained until the end of the trial. Spawned eggs from each group were sampled from each group throughout the spawning period. Egg fecundity and quality were evaluated by methods cited previously (Yong et al. 2007). AsA analysis was conducted on egg samples. At the end of feeding trial, blood samples also colleted by heparined syringe from the caudal fin.
Chemical assay and statistical analysis

AsA assay was performed using a high performance liquid chromatography (LaChrom, Hitachi, Tokyo, Japan) according to Kodaka et al. (1985) with slight modification. Samples were homogenized with 5% metaphosphoric acid and centrifuged at 3000 rpm at 4°C for 10 min. After derivatization with 2-6 dicholorophenol-indophenol and 2,4-dinitrophenylhydrazine, the mixture was incubated at 50°C for 90 min. After cooling the reaction mixture, ethyl acetate was added, mixed and centrifuged at 3000 rpm. The resultant supernatant obtained after the centrifugation was injected into HPLC with a partisil column (silica 5 μm, 4.6 × 200 mm, GL Sciences, Tokyo, Japan). Mobile phase ethyl acetate: hexane: acetic acid (5: 4 : 1) was applied at the flow rate of 1.0 ml/min, and absorbance at 495 nm was monitored as AsA peak.

The dietary APM level was determined by HPLC using a reversed phase column (Wakosil 5C18, 6 × 150 mm, Wako Pure Chemical, Osaka, Japan). Mobile phase was 0.1 M KH₂PO₄ solution at pH 2, and flow rate was adjusted 0.5 ml/min. For the extraction of APM, samples were homogenized with 5% metaphosphoric acid and centrifuged at 3000 rpm for 10 min at 4°C. The resultant supernatant was injected into the HPLC system.

Proximate analysis of the test diets was performed in triplicates according to modified AOAC (1984) methods, moisture (105°C), crude protein (micro-Kjedahl), crude lipid (Soxhlet extraction with diethyl ether) and crude ash (550°C). Dietary sugar was assayed by the method cited by Hodge and Hofreiter (1962).

All data obtained were analyzed using one-way analysis of variance (ANOVA). If there was a significant difference (P<0.05) among the treatments, we further analyzed significant differences (P<0.05) among means of variables by Tukey’s multiple test comparison using the SPSS Advanced Models 12.0 package (SPSS Inc. Tokyo, Japan)

Results

The spawning performance of striped knifejaw broodfish fed test diets with graded levels of APM was presented in Table 2. Overall egg fecundity was higher in groups fed diets 2-5 with APM than the group fed the control diet 1 without APM. The highest egg fecundity was observed in group fed diet 2 with 52-spawning

<table>
<thead>
<tr>
<th></th>
<th>Valuable</th>
<th>Diet</th>
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<tr>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Egg production (×10⁷)</td>
<td>2.27 ± 1.61*¹</td>
</tr>
<tr>
<td></td>
<td>Number of spawning</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Egg diameter (mm) (n=1000)</td>
<td>0.97 ± 0.002</td>
</tr>
<tr>
<td></td>
<td>Egg oil globule (mm) (n=1000)</td>
<td>0.22 ± 0.001</td>
</tr>
<tr>
<td></td>
<td>Egg weight (mg) (n=105–154)</td>
<td>0.39 ± 0.006</td>
</tr>
<tr>
<td></td>
<td>Buoyancy rate (%) (n=105–154)</td>
<td>62.34 ± 7.20</td>
</tr>
<tr>
<td></td>
<td>Hatching rate (%) (n=105–154)</td>
<td>54.80 ± 9.68</td>
</tr>
<tr>
<td></td>
<td>Larval SAI (n=105–154)</td>
<td>12.25 ± 1.80</td>
</tr>
<tr>
<td></td>
<td>Larval deformity (%) (n=105–154)</td>
<td>6.18 ± 2.09</td>
</tr>
</tbody>
</table>

*¹ Values are mean ± SEM.
*² Number of buoyant egg × 100 / total number of egg.
*³ Number of hatched larvae × 100 / total number of egg.
*⁴ SAI = 1/N ∑(N - ki) / i where N: total number of larvae, ki: cumulated mortality by i-th day and k: number of the days elapsed until total larval mortality. SAI was conducted on unfed larvae.
*⁵ Number of deformed larvae × 100 / number of hatched larvae.
during a 10-week spawning period. The lowest egg fecundity was found in group fed diet 1 with only 35 spawning during the same spawning period. Egg diameter, weight and oil globule size were not differed among the test diet groups. Although no significant difference, higher egg buoyancy and hatching rates were observed in groups fed diets 2-5 than the group fed diet 1. In larval survival activity index (SAI), the highest value was obtained in group fed diet 2 however, without remarkable difference among the test diet groups. Lower SAI was also obtained in the group fed diet 1. Diets 4 and 5, high dietary APM levels at 2,640 mg A₃₅/kg diet and above, induced a tendency of high incidence of abnormal hatched larvae.

At the end of the spawning period, that is the end of the feeding trial, body weight of broodfish was lower than the start of the feeding trial (Table 3). No significant difference was found in feed intake among the dietary groups, thus APM intake was increased with an increasing dietary APM levels. No significant difference of haematocrit and haemoglobin was observed among the broodfish.

Egg AsA level of each test diet group was presented in Fig. 1. Egg AsA level in group fed diet 1 decreased significantly while those groups fed APM fortified diets increased significantly towards the end of the spawning season. The egg AsA level in diets 2 and 5 increased remarkably from 14 days after initial spawning (DAIS) onwards; while 21 and 35 DAIS onwards was observed in diets 4 and 3, respectively.

Table 3. Body weight (BW), daily feed intake, total AsA intake, haematocrit and haemoglobin of female broodfish fed test diets 1-5

<table>
<thead>
<tr>
<th>Diet</th>
<th>Initial BW (g)</th>
<th>Final BW (g)</th>
<th>Gain (%)</th>
<th>Daily feed intake/ind. (g)</th>
<th>Calculated total AsA intake/ind. (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>589</td>
<td>530</td>
<td>-10.02</td>
<td>8.6</td>
<td>0.33</td>
</tr>
<tr>
<td>2</td>
<td>587</td>
<td>524</td>
<td>-10.73</td>
<td>8.8</td>
<td>372.1</td>
</tr>
<tr>
<td>3</td>
<td>598</td>
<td>505</td>
<td>-15.55</td>
<td>7.3</td>
<td>616.6</td>
</tr>
<tr>
<td>4</td>
<td>595</td>
<td>508</td>
<td>-14.62</td>
<td>7.6</td>
<td>1411.2</td>
</tr>
<tr>
<td>5</td>
<td>573</td>
<td>514</td>
<td>-10.30</td>
<td>8.8</td>
<td>3491.9</td>
</tr>
</tbody>
</table>

*1 Values are mean of n=8.  
*2 Values are mean ± SEM, n=8.

Fig. 1. Egg AsA levels of broodfish groups fed the test diets. Changes in egg AsA levels at one week interval across the spawning period. DAIS is day after initial spawning. Within the same diet, histogram bars with different superscript alphabet indicate significant difference (P<0.05).
Discussion

In the present study, dietary APM was valid to improve the egg fecundity and quality, and significant egg AsA accumulation of striped knifejaw. Ishibashi (1994) showed that striped knifejaw can utilize AsA-Na and AsA-Ca as AsA source and present results revealed that APM is a highly bioavailable AsA source for striped knifejaw broodfish, as shown in juvenile hybrid tilapia, Oreochromis niloticus × O. aureus (Shiau and Hsu 1999) and grass shrimp, Penaeus monodon (Hsu and Shiau 1998). Shiau and Hsu (1999) indicated that APM was ca. 85% as effective as AsA-2-monophosphate-Na in meeting the AsA requirement for the hybrid tilapia. Reversely, Hsu and Shiau (1998) reported AsA-2-monophosphate-Na was ca. 84% as effective in meeting the AsA requirement for the grass shrimp. These differences may be attributed to the enzymatic specificity of phosphatases in digestive and other organs, the metal element of AsA derivatives and species specificity.

Dietary AsA fortification improved not only growth performance (Ishibashi 1994; Lee et al. 1998; Ai et al. 2004) and immune activity (Lin and Shiau 2005) but reproductive performance of fish (Sandnes et al. 1984; Waagybo et al. 1989; Blom and Dabrowski 1995; Dabrowski and Ciereszko 2001). In the present study, diets with AsA induced higher egg fecundity than diet without AsA same as those reported in rainbow trout (Sandnes et al. 1984; Blom and Dabrowski 1995), even though other studies on dietary AsA fortification showed negligible effect on egg production in cod Gadus morhua (Mangor-Jensen et al. 1994) and rainbow trout (Gavrilidou et al. 2003). Likewise, in striped knifejaw broodfish fed white fish meal based diet fortified with 3,000 mg AsA/kg diet equivalent of AsA-Ca tended to increase egg fecundity than diet containing 250 mg AsA/kg diet (Ishibashi 1994). In the present study, APM fortified diet also promoted early onset spawning. Ishibashi et al. (1994) reported that 1-year-old striped knifejaw fed diets with 300, 1,000 and 3,000 mg AsA/kg diet equivalent of AsA-Ca salt in an 8-month-feeding trial had clearly promoted the gonadal development and maturation as compared with the fish fed diet without AsA. Similar to most of the teleost marine fish, AsA is an essential water soluble vitamin for striped knifejaw broodfish and/or juveniles (Ikeda et al. 1988; Ishibashi et al. 1994) which may be can biosynthesize AsA less than its requirement or not at all. The presence of adequate or high amount of AsA in the early ovary development may have promoted the oocytes development as reflects by its roles as cofactor in steroidogenesis in follicle and adrenal cells as proposed by Levine and Morita (1985); besides other roles in biosynthesis of peptide hormone, prevent molecules oxidation or hydroxylation of proline and lysine in collagen formation in the gonad (Blom and Dabrowski 1995; Luck et al. 1995; Dabrowski and Ciereszko, 2001). However, its complex mechanism still remain unclear.

In the present study, broodfish fed control and test diets spawned eggs with similar size, oil globule diameter and weight. Similar egg size had also been demonstrated in other broodfish fed diet with or without AsA such as rainbow trout and cod (Sandnes et al. 1984; Mangor-Jensen et al. 1994; Blom and Dabrowski 1995; Gavrilidou et al. 2003). However, higher egg buoyancy rate, hatching rate, larval SAI and egg AsA level were obtained from broodfish fed test diets with APM than those fed control diet without APM in the present study. The beneficial effect of dietary AsA on improving hatchability had also been reported in rainbow trout (Gavrilidou et al. 2003) and O. mossambicus tilapia (Soliman et al. 1986). Furthermore, the diet 2 contained equivalent of 441 mg AsA/kg diet induced higher larval SAI. On the contrary, the larval deformity from diets 4 and 5 at AsA level 2,429 mg/kg diet and above were reversely higher than diet 2. Gavrilidou et al. (2003) reported that in rainbow trout, broodfish fed diet with 2,400 mg AsA/kg diet produced significant lower hatching rate and number of fry than those fed diet with 1,200 AsA/kg diet. We could not find reports relating the hypervitaminosis of AsA in fishes. The high percentage of the deform larvae occurred in diets with high APM levels.
might originated from high APM or AsA doses. The magnesium (Mg\(^{2+}\)) deficiency has not been demonstrated in fish in a seawater environment, where they obtain Mg\(^{2+}\) by drinking the water (NRC 1998). Dabrowska et al. (1989) demonstrated that excess Mg in a low-protein diet produced toxicity signs in tilapia. High body Mg\(^{2+}\) phosphate or Mg\(^{2+}\) contents originated in excess APM ingestion has possibility to offer toxicities for egg and sperm during maturation processes and/or egg development. However, there is no gross overdose sign observed among the broodfish at the end of the feeding trial, and the data (Table 3) suggests that all broodfish were in a healthy condition. Wang et al. (2002) in a study examined the effect of reactive oxygen species on blastocyst development with or without AsA and vitamin E supplementation on mouse embryo reported that a higher AsA concentration of 200 and 400 \(\mu\)mol resulted in embryo toxicity. This result although not directly comparable to the present study, however, high APM fortification in the present study may have caused undesirable side effect on the egg and larval quality, hence requires further verification and investigation. The present study proposes that the suitable dietary AsA level for 3-year-old primiparous striped knifejaw broodfish is 441 mg/kg diet or lower in an equivalent basis of APM under this experiment conditions.

### Acknowledgement

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ビタミン C 源であるアスコルビン酸-2-モノリン酸 Mg\(^{2+}\)がイシダイの産卵に及ぼす効果

A.S.K. Yong・瀬岡 学・大川有紀・高岡 治・滝井健二・熊井英水

イシダイの産卵に及ぼす飼料アスコルビン酸 (AsA) の効果を、アスコルビン酸-2-モノリン酸 Mg\(^{2+}\) (APM) を用いて検討した。魚粉主体の飼料へ APM を AsA 换算で 0、500、1000、3000および 6000 mg/kg になるよう配合し、各水槽に収容した親魚を 5 尾（平均体重 567 g）および 2 尾（平均体重 595 g）に、1 日1 匹飼育給与して2006年4月から25週間飼育した。なお、飼育試験は各飼料に付与する 2 反復区を設けた。APM 配合は産卵を早め、卵質の向上に有効であることが示され、500 mg 飼料区では産卵数、浮上卵率、仔魚の無給食生残指数などが高い傾向にあった。一方、APM 配合量と卵の AsA 含量に正の相関関係が得られ、有意な区間差がなかったが、3000および6000 mg 飼料区のふ化仔魚に形態異常が多かった。これらの結果から、イシダイ親魚は AsA 源として APM を有効に利用して産卵成績を向上させるが、多量の APM 配合は反則に産卵成績や卵質に悪影響を及ぼすことが示唆された。


