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Dietary folic acid requirement of juvenile abalone *Haliotis discus hannai* Ino

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ABSTRACT

A 16-week growth study was conducted to determine the dietary folic acid requirement of juvenile abalone (initial mean weight 482.2 ± 2.2 mg). Six semi-purified diets were formulated to contain graded levels of folic acid (0.06, 1.44, 2.86, 5.88, 14.27 and 35.71 mg/kg). Each diet was fed to three replicate tanks of abalone in a re-circulated water system. The results showed that the weight gain rate (WGR) was significantly increased when dietary folic acid contents increasing from 0.06 to 2.86 mg/kg ($P < 0.05$). Higher dietary folic acid (2.86–35.71 mg/kg) did not result in further increase of WGR. When dietary folic acid increased from 0.06 to 5.88 mg/kg, the viscera folic acid concentration (VFAC) significantly increased ($P < 0.05$). And then, there were no significant differences in VFAC among treatments as dietary folic acid contents increased from 5.88 to 35.71 mg/kg. Dietary folic acid did not significantly influence survival and the soft body compositions of abalone. Based on WGR and VFAC, broken-line analysis showed that dietary folic acid requirements of abalone were 2.62 and 5.29 mg/kg, respectively.

1. Introduction

Folate is the generic name for all derivatives of the B-vitamins with biological activity of pteroylmonoglutamic acid. Folic acid is the fully oxidized monoglutamate form of the vitamin. After being converted to active tetrahydrofolate coenzymes, it functions as a one-carbon donor or acceptor in a variety of reactions involved in amino acid and nucleotide metabolism (Stokstad and Koch, 1967). In terrestrial animals, folic acid deficiency is consistently characterized by megaloblastic anemia, together with anorexia and associated with low weight gain. Quantitative requirements of folic acid in chicks (0.55 mg/kg diet), rats (1.0 mg/kg diet), piglets (0.3 mg/kg diet) and several other species have been established (McDowell, 1989).

Folic acid is also an essential nutrient for aquatic animals. In vertebrates, some fish species have been demonstrated to need dietary folic acid for normal growth and hematopoiesis. They are yellowtail *Seriold quinqueradiata* (Shimeno, 1991), rainbow trout *Oncorhynchus mykiss* (Cowey and Woodward, 1993), channel catfish *Ictalurus punctatus* (Duncan et al., 1993), hybrid tilapia *Oreochromis aureus* × *O. aureus* (Shiau and Huang, 2001a), grouper *Epinephelus malabaricus* (Lin et al., 2011), etc. Duncan et al. (1993) described the symptoms of folic acid deficiency in channel catfish as a reduction in growth, and macrocytic normochromic anemia characterized by pale livers, spleens, gills and kidneys. Dietary folic acid deficiency led to growth retardation and abnormal hematopoiesis in rainbow trout and Nile tilapia *Oreochromis niloticus* (Cowey and Woodward, 1993; Lim and Klesius, 2001). In aquatic invertebrates, published data on dietary folic acid requirement has been only found in a crustacean species grass shrimp *Penaeus monodon*. And it was estimated to be 1.9–2.1 mg/kg (Shiau and Huang, 2001b). However, up to now, there is no available data on the nutrition of folic acid in mollusk.

Abalone *Haliotis discus hannai*, a large algivorous marine mollusk, is one of the most commercially important gastropods in aquaculture. Much interest has been focused on developing nutritionally balanced and effective artificial feeds for abalone farming. The requirements of dietary protein, lipid, carbohydrate, essential fatty acids and minerals for *H. discus hannai* have been well established (Mai and Tan, 2000; Mai et al., 1995a, 1995b, 1996; Tan and Mai, 2001a; Tan et al., 2001). Meanwhile, requirements of many vitamins were also studied in this species, such as fat-soluble vitamins (A, D, E, K) (Tan and Mai, 2001b; Zhang et al., 2003; Zhou and Mai, 2000; Zhou et al., 2001) and water-soluble vitamins (vitamin C, thiamin, myo-inositol, biotin and pyridoxine) (Mai, 1998; Mai et al., 2001, 2007Wu et al., 2002; Zhu et al., 2002). The purpose of this study is to quantitatively estimate the requirement of dietary folic acid for juvenile abalone *H. discus hannai*.

2. Materials and methods

2.1. Diet preparation

The formulation and proximate composition of the experimental diets are shown in Table 1. Vitamin-free casein and gelatin were used as the dietary protein sources to provide 31.71% crude protein,

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Table 1
Ingredients and proximate analysis of the experimental diets (% on dry weight basis).

Ingredients	Content (g/100 g diet)
Casein, vitamin-free (Sigma, St. Louis, MO, USA)	25.0
Gelatin (Sigma)	6.0
Dextrin (Shanghai Chemical, Shanghai, China)	34.0
CM-cellulose (Shanghai Chemical)	5.0
Sodium alginate (Shanghai Chemical)	20.0
Vitamin mixture [a], folic acid-free	2.0
Mineral mixture [b]	4.0
Choline chloride (Shanghai Chemical)	0.5
SO/MFO[c](1:1)	3.5
Proximate analysis (means of triplicate) (%)	
Protein	31.71
Lipid	3.73
Ash	11.11

[a] Vitamin mixture: each 1000 g of diet contained thiamin-HCL, 120 mg; riboflavin, 100 mg; para-aminobenzoic acid, 400 mg; pyridoxine HCl, 40 mg; niacin, 200 mg; Ca pantothenate, 200 mg; inositol, 4000 mg; biotin, 12 mg; vitamin E, 450 mg; menadione, 890 mg; VB_{12}, 0.18 mg; ascorbic acid, 4000 mg; retinol acetate, 100,000 IU; cholecalciferol, 2000 IU; ethoxyquin, 400 mg.
[b] Mineral mixture: each 1000 g of diet contained NaCl, 0.4 g; $MgSO_4 \cdot 7H_2O$, 6.0 g; $NaH_2PO_4 \cdot 2H_2O$, 10 g; KH_2PO_4, 12.8 g; $Ca(H_2PO4)_2 \cdot H_2O$, 8 g; Fe-citrate, 1.0 g; Ca-lactate, 1.4 g; $ZnSO_4 \cdot 7H_2O$, 141.4 mg; $MnSO_4 \cdot 4H_2O$, 64 mg; $CuSO_4 \cdot 5H_2O$, 12.4 mg; $CoCl_2 \cdot 6H_2O$, 0.4 mg; KIO_3, 1.2 mg; Na_2SeO_3, 0.4 mg.
[c] Soybean oil and menhaden fish oil (1:1) with 0.001% ethoxyquin.

which was considered to be sufficient to maintain optimum growth for juvenile abalone (Mai et al., 1995a). Soybean oil and menhaden fish oil (1:1) were used as the lipid sources. Dietary lipid level was 3.73%. It was sufficient to support optimum growth and provide enough essential fatty acids for abalone (Mai et al., 1995b). The vitamin mixture (except for folic acid) and mineral mixture were the same as described by Zhang et al. (2009). Six experimental diets were prepared from the purified ingredients to provide graded levels (0.0, 1.4, 2.8, 5.6, 14.0 and 35.0 mg/kg) of folic acid (Sigma Chemical Co., St. Louis, MO, USA). Final contents of folic acid in diets were 0.06, 1.44, 2.86, 5.88, 14.27 and 35.71 mg/kg, respectively, as determined by a microbiological assay procedure (AOAC, 1995).

Prior to supplementation into the experimental diets, folic acid was microencapsulated with sodium alginate by an emulsion coacervation process (Mai et al., 2001).

2.2. Leaching

A leaching test for dietary folic acid was carried out according to the method described by Mai et al. (2001). Diet (15 g) was put in a nylon mesh bag (100 μm) and allowed it to the bottom of an experimental tank without abalone. The water temperature was adjusted to 19 ± 1 °C. At the end of the allotted time (2, 6 and 12 h, respectively), the remaining diet was removed from the bags and lyophilized for 24 h. Then, the folic acid concentration was measured (AOAC, 1995). The leaching of dietary folic acid was expressed as retention efficiency (RE):

$$RE(\%) = 100N_t/N_0$$

where N_t and N_0 are final and initial dietary folic acid concentration, respectively.

2.3. Animal rearing

Abalone juveniles were obtained from a commercial hatchery at Maidao Fisheries Co., Shandong, China. Before initiation of the experiment, abalone juveniles were acclimated to the culture system and the basal diet for 2 weeks. After that, healthy juveniles with similar size (initial mean weight: 482.2 ± 2.2 mg) were assigned to the 150-L tanks. A completely randomized design with six triplicated treatments was used. Each tank was a replicate, and was stocked with 30 abalone juveniles in a re-circulated water system. Animals were hand-fed with the test diets at a rate equaling 5–10% of wet body weight once daily at 17:00. Every morning, feces and uneaten feed were removed to maintain the water quality. During the 16-week feeding trial, the water temperature was 17.8–20.0 °C, salinity 30–34‰, pH 7.6–7.9. Dissolved oxygen was not less than 7.0 mg/L, and there were negligible levels of free ammonia and nitrite.

2.4. Sample collection and analysis

At the termination of the feeding trial, animals were not fed for 3 days. All abalone were removed from the tanks, counted, weighed and measured. Then, abalone from each replicate were immediately frozen (−70 °C) for subsequent analyses. Growth performances were expressed as the weight gain rate (WGR, %). The calculation formula is as follows:

$$WGR(\%) = [(W_t - W_i)/W_i] \times 100$$

where W_t, W_i are final and initial mean weight (mg), respectively.

Ten abalone juveniles from one tank were sampled and pooled as a replicate to determine the contents of protein, lipid and moisture in the soft body, and the concentration of folic acid in viscera by the standard methods (AOAC, 1995). There were three replicates/tanks in each treatment. The method of enzyme preparation to liberate folic acid in viscera was according to Ryu et al. (1994).

2.5. Statistical analysis

Statistical analysis was performed using SPSS 15.0 package (SPSS Inc., Chicago, USA). All percentage data were square root arcsine transformed before analysis. Data were subjected to one way ANOVA test. When overall differences were significant at less than 5% level, Tukey's test was used to compare the mean values between individual treatments. Dietary folic acid requirement of juvenile abalone was estimated by the broken-line regression analysis (Robbins et al., 1979).

3. Results

3.1. Leaching

The results of the leaching test for dietary folic acid are shown in Table 2. Generally, dietary folic acid content decreased with the increasing of immersion time. The RE ranged from 95.2% to 98.5% after 2-h immersion in seawater. After 6-h immersion, RE ranged from 80.0 % to 85.8%. Furthermore, RE decreased to 55.4%–60.0% after 12-h immersion. There were no significant differences in RE among all dietary treatments regardless of the immersion time ($P > 0.05$).

Table 2
The retention efficiency (RE) of folic acid in the experimental diets at different intervals (2, 6 and 12 h) of immersion in seawater[1].

Dietary folic acid (mg/kg)	Retention efficiency (%)		
	2 h	6 h	12 h
0.06	ND[a]	ND	ND
1.44	98.5 ± 1.2	85.8 ± 2.5	58.9 ± 4.0
2.68	98.3 ± 0.7	84.9 ± 1.8	60.0 ± 2.1
5.88	96.5 ± 1.6	81.8 ± 1.3	55.4 ± 2.0
14.27	96.8 ± 1.6	81.1 ± 1.7	56.0 ± 3.0
35.71	95.2 ± 1.9	80.0 ± 2.5	56.0 ± 2.2
ANOVA			
F value	1.673	3.709	3.273
P value	0.232	0.062	0.058

[1]Values are means ± SD, n = 3.
[a] ND: not detected.

3.2. Survival and growth

The results of survival and growth performance are presented in Table 3. There was no significant difference in survival of abalone fed with the different experimental diets ($P > 0.05$). The survival rate ranged from 84.2% to 92.5%.

When dietary folic acid increased from 0.06 to 2.86 mg/kg, the weight gain rate (WGR) significantly increased from 136.6% to 174.6% ($P < 0.05$). And then, there were no significant differences in WGR among treatments as dietary folic acid contents increased from 2.86 to 35.71 mg/kg. Broken line analysis indicated that the dietary folic acid requirement for juvenile abalone was estimated to be 2.62 mg/kg (Fig. 1). The regression equation was as follows: $Y = 168.73 - 13.613\ (2.62 - X)$, $R^2 = 0.944$, where, X is the dietary folic acid levels and Y is the WGR.

3.3. Soft body composition and viscera folic acid concentration

Results of the soft body compositions and folic acid concentrations in viscera are shown in Table 4. Contents of protein, lipid and moisture in the soft body of abalone were not significant influenced by dietary folic acid contents ($P > 0.05$). The protein contents ranged from 53.49% to 55.14%. Those for soft body lipid contents were 6.87% to 7.01% and those for moisture were 77.70% to 78.67%.

When dietary folic acid increased from 0.06 to 5.88 mg/kg, the viscera folic acid concentration (VFAC) significantly increased from 0.28 to 1.61 μg/g ($P < 0.05$). And then, there were no significant differences in VFAC among treatments as dietary folic acid contents increased from 5.88 to 35.71 mg/kg. Based on the data of VFAC, broken line analysis indicated that the dietary folic acid requirement for juvenile abalone was estimated to be 5.29 mg/kg (Fig. 2). The regression equation was as follows: $Y = 1.5373 - 0.2452\ (5.29 - X)$, $R^2 = 0.9053$; where, X is the dietary folic acid levels and Y is the VFAC.

4. Discussions

Abalone is known to be slow feeder or nibbler. Previous study indicated that abalone usually reached satiation in 2 h after feeding (Mai et al., 1998). Therefore, it is necessary to take measures to reduce the leaching of water-soluble vitamins in diets for abalone. Microencapsulation is one of the potential methods to reduce leaching by sheltering encapsulated materials from outer environment (Mai et al., 2001; Marchetti et al., 1999; Petitjean and Csengeri, 1995; Yúfera et al., 2003). According to the results from Mai et al. (2001), the RE values of lipid-encapsulated myo-inositol in pelleted feed were 89.5% and 82.5% for 1- and 2-h immersion in seawater, respectively. In the present study, the RE of folic acid was up to 93.4%–99.8% after 2-h immersion. Furthermore, no significant difference can be observed in RE of experimental diets when all diets were immersed in seawater for 12 h. It was suggested that using of microencapsulation in the present study was good to quantify dietary folic acid requirement of abalone precisely.

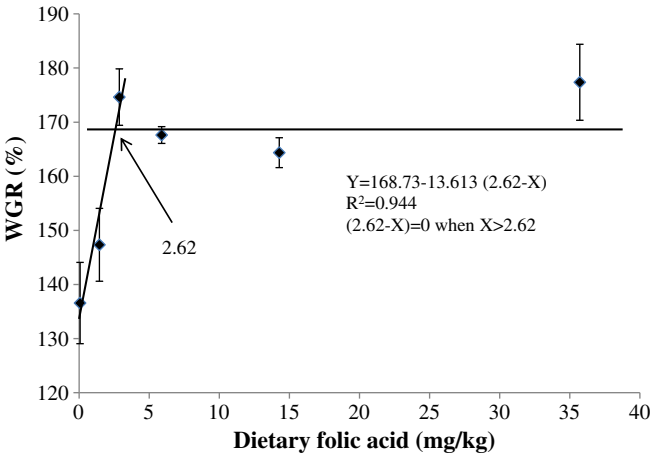

Fig. 1. Broken-line analysis of the relationship between dietary folic acid and the weight gain rate (WGR) of abalone *Haliotis discus hannai* indicates that the dietary folic acid requirement for abalone was 2.62 mg/kg. Each point represents the mean of three replicates.

No deficiency signs were observed in rainbow trout *O. mykiss* after feeding low folic acid (0.3–0.6 mg/kg) diet for 16 weeks. It was possibly because of synthesis of folic acid by intestinal bacteria (Cowey and Woodward, 1993). When sulfonamide (an antagonist of folic acid) was included in diet, reductions in growth and survival, serious impaired hematopoiesis were found in channel catfish *I. punctatus*. However, these could not be found when folic acid free diet was used without sulfonamide (Duncan et al., 1993). So, Duncan et al. (1993) pointed out that intestinal microorganisms were a great source of folic acid for channel catfish. It has been generally believed in fish that intestinal microorganisms may contribute a considerable quantity of folic acid to the host (Shimeno, 1991). However, there is little published data on the capacities of vitamins synthesis by gut micro-organism in abalone, even in mollusk. In the present study, abalone fed diets containing folic acid higher than 2.8 mg/kg had significantly higher WGR than those in folic acid deficient groups. It was suggested that juvenile abalone required dietary folic acid for better growth. This result was different to that in some fish mentioned above. A question is rising. Are there differences in abilities of folic acid synthesis by intestinal micro-organism between abalone and fish, even between mollusk and aquatic vertebrates? Further study is needed to answer this question.

In previous studies, growth performance, tissue folic acid concentration and hematology were usually used as indices to estimate the requirement of dietary folic acid. For example, Cowey and Woodward

Table 3
Effects of dietary folic acid on survival and growth of abalone *Haliotis discus hannai* [1].

Dietary folic acid (mg/kg)	Initial weight (mg)	Final weight (mg)	WGR[2] (%)	Survival (%)
0.06	486.7 ± 2.6	1151.7 ± 41.3^{a}	136.6 ± 7.5^{a}	86.7 ± 0.8
1.44	490.3 ± 3.0	1212.2 ± 26.1^{a}	147.3 ± 6.7^{b}	92.5 ± 1.4
2.86	473.2 ± 3.3	1299.5 ± 27.0^{a}	174.6 ± 5.2^{c}	85.0 ± 2.9
5.88	477.9 ± 6.9	1279.2 ± 25.9^{a}	167.6 ± 1.6^{bc}	88.3 ± 2.2
14.27	479.3 ± 6.9	1267.2 ± 22.7^{a}	164.4 ± 2.8^{bc}	84.2 ± 0.8
35.71	486.0 ± 3.6	1348.5 ± 43.6^{b}	177.4 ± 7.0^{c}	91.7 ± 2.2
ANOVA				
F value	1.859	4.611	8.238	3.335
P value	0.176	0.014	0.001	0.075

[1]Values are means ± SD, n = 3; values in the same column sharing a common superscript letter are not significantly different ($P > 0.05$, Tukey's test).
[2]WGR: weight gain rate.

Table 4
Effects of dietary folic acid on proximate compositions of the soft body and folic acid concentrations in viscera of abalone *Haliotis discus hannai* (dry matter basis)[1].

Dietary folic acid (mg/kg)	Protein (%)	Lipid (%)	Moisture (%)	VFAC[2] (μg/g)
0.06	53.49 ± 0.73	6.87 ± 0.14	77.70 ± 0.41	0.28 ± 0.06^{a}
1.44	54.31 ± 0.23	6.99 ± 0.21	78.17 ± 0.72	0.38 ± 0.05^{a}
2.86	53.90 ± 0.49	6.90 ± 0.34	78.18 ± 0.57	1.20 ± 0.06^{b}
5.88	54.24 ± 0.52	6.91 ± 0.24	77.95 ± 0.79	1.61 ± 0.03^{c}
14.27	53.71 ± 0.24	7.01 ± 0.27	78.67 ± 0.25	1.43 ± 0.02^{bc}
35.71	55.14 ± 0.20	7.00 ± 0.41	78.17 ± 0.60	1.56 ± 0.14^{c}
ANOVA				
F value	1.696	0.255	0.298	284.784
P value	0.210	0.929	0.905	0.000

[1]Values are means ± SD, n = 3; values in the same column sharing a common superscript letter are not significant different ($P > 0.05$, Tukey's test).
[2]VFAC: viscera folic acid concentration.

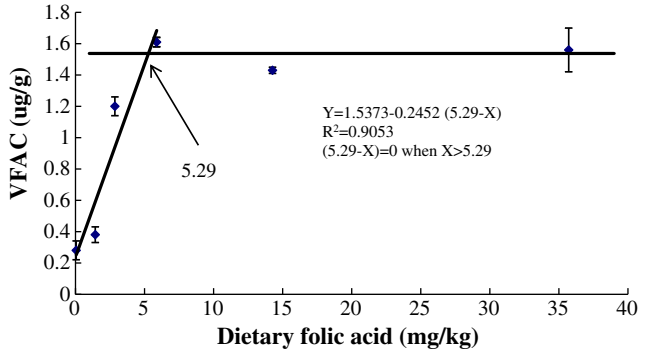

Fig. 2. Broken-line analysis of the relationship between dietary folic acid and the viscera folic acid concentration (VFAC) of abalone *Haliotis discus hannai* indicates that the dietary folic acid requirement for abalone was 5.29 mg/kg. Each point represents the mean of three replicates.

(1993) suggested that 0.3–0.6 mg folic acid/kg diet was optimum for young rainbow trout *O. mykiss* based on survival and growth, and that was 1.1 mg folic acid/kg diet based on tissue folic acid saturation. Lim and Klesius (2001) pointed out that 0.5–1.0 mg folic acid/kg diet was optimum for Nile tilapia *O. niloticus* based on growth response and hematology. Lin et al. (2011) concluded that the optimal dietary folic acid level for juvenile grouper *E. malabaricus* was about 0.8 mg/kg based on the analyses of weight gain, hepatosomatic index, hepatic folic acid concentration and hepatic thiobarbituric acid-reactive substance value. In aquatic invertebrates, Shiau and Huang (2001b) suggested that 1.9–2.1 mg folic acid/kg diet was optimum for grass shrimp *P. monodon* based on weight gain percentage, hepatopancreatic folic acid concentration and hepatosomatic index. In the present study, dietary folic acid requirement of abalone was estimated to be 2.62 mg/kg based on WGR. According to the published data in fish, shrimp and abalone above, it was suggested that fish (0.3–1.1 mg/kg) had less requirement of dietary folic acid than shrimp (1.9–2.1 mg/kg) or abalone (2.62 mg/kg). One of the reasons could be the species difference, especially between vertebrates and invertebrates. Further study is needed to find the exact reasons. Meanwhile, in the present study, dietary folic acid requirement could be estimated as 5.29 mg/kg based on the visceral folic acid concentration. It was much higher than that (2.62 mg/kg) based on WGR. Usually, requirements derived from relevant physiological parameters are higher than those from growth performance, and tend to vary when different parameters are examined (Cowey and Woodward, 1993; Lim and Klesius, 2001).

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