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SHORT COMMUNICATION

Effects of dietary vitamin K on growth performances, blood coagulation time and menaquinone-4 (MK-4) concentration in tissues of juvenile large yellow croaker *Pseudosciaena crocea*

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Introduction

Vitamin K is essential for the synthesis of blood clotting factors of animals. So it is also called anti-haemorrhage vitamin. All types of compounds of vitamin K have the phylloquinone biological activity of 2-methyl-1,4-naphthoquinone. Phylloquinone (VK_1), menaquinones (VK_2) and menadione (VK_3) exhibit vitamin activity (Udagawa, Nakazoe & Murai 1993). Phylloquinone is synthesized by plants and algae, whereas the menaquinone family (MK-n) is the products of bacterial biosynthesis. Menaquinones include a range of vitamin K forms, which was named according to the number (*n*) of prenyl groups in the unsaturated side chain, thus designated as MK-n, with *n* ranging from 2 to 14 (Lambert & De Leenher 1992). Menaquinone-4 and MK-7 are the most relevant nutritional menaquinones (Fodor, Albu, Poantã & Porojan 2010). Water-soluble salts of menadione are usually used in animal diets. And menadione sodium bisulfate (MSB) is widely supplemented in fish diets (Grahl-Madsen & Lie 1997). It was confirmed that dietary MSB could be converted to MK-4 in fish, such as cod *Gadus morhua* (Grahl-Madsen & Lie 1997) and sardine *Sardinops melanostictus* (Udagawa *et al.* 1993).

In fish, typical signs of vitamin K deficiency include increased blood coagulation time, reduced growth, anaemia, haemorrhage, loss of fin tissue, weak bones, and occurrence of spinal curvature, short tails and increased mortality (Taveekijakarn, Miyazaki, Matsumoto & Arai 1996; Udagawa 2004; Lall & Lewis-McCrea 2007). However, the minimum requirement of vitamin K is difficult to estimate owing to natural occurrence in feed ingredients, feed processing and storage stability of inherent and added vitamin K, vitamin leaching, variable feed intakes and variable bioavailability of the different K vitamins (Krossoy, Waagbo & Ornsrud 2011).

Large yellow croaker *Pseudosciaena crocea* is a widely cultured species in China because of its delicious meat and commercial importance (Mai, Zhang, Ai, Duan, Xu, Zhang, Liufu & Tan 2006). At present, no report has been published as to the essentiality or quantitative requirements of vitamin K for this species. The aim of this study was to investigate the effect of dietary vitamin K on survival, growth, blood coagulation time and MK-4 concentration in tissues of juvenile large yellow croaker.

Materials and methods

Experimental diets

Casein, gelatin and fish muscle protein were used as the dietary protein sources (Table 1). Fish oil

Table 1 Formulation and proximate composition of the basal diet

Ingredient	%
Casein,vitamin-free*	31
Gelatin	8
Fish muscle protein	7
Dextrin	28
Fish oil	8
Lecithin	2
Attractant†	0.8
Mold inhibitor‡	0.1
Ethoxyquin	0.05
Vitamin mix, vitamin K-free§	2
Mineral mix¶	0.5
Calcium phosphate	1
Cellulose	11.55
Proximate composition (% dry matter)	
Crude protein	45.21
Crude lipid	12.37
Ash	2.65

*Casein, vitamin-free: crude protein 96.39%, crude lipid 0.47% (Sigma Chemical, St. Louis, MO, USA).
†Attractant: L-Glycine and betaine.
‡Mold inhibitor: 50% of calcium propionate acid and 50% of fumaric acid.
§Vitamin mix, vitamin K-free (mg kg^{-1} diet dry matter): retinol acetate, 32; cholecalciferol l,5; α-tocopherol, 240; thiamin-HCl, 25; riboflavin, 45; pyridoxine-HCl, 20; vitamin B_{12}, 10; D-pantothenic acid calcium, 60; niacin acid, 200; folic acid, 20; biotin, 60; inositol, 800; ascorbic acid, 2000; choline chloride, 4000; cellulose, 12463.
¶Mineral mix: kindly provided by the Qingdao Master Biotechnology, China.

was used as the main dietary lipid source. Isonitrogenous (45.21% crude protein) and isolipidic (12.37% crude lipid) semi-purified diets were formulated to contain 6 graded levels of MSB (0, 3, 6, 12, 24 and 48 mg kg^{-1} dry basis respectively). And the 7th diet contained 11 mg kg^{-1} of antibiotic (sulfanilamide) without MSB supplementation. The reagents of MSB and sulfanilamide were purchased from Sigma. The final contents of MSB in diets were 0.06, 3.45, 7.15, 12.82, 26.00, 47.54 and 0.04 mg kg^{-1}, respectively, as analyzed by the UV spectrophotometer (UV-2401PC; Shimadzu, Kyoto, Japan).

Experimental procedure

The large yellow croakers were obtained from a commercial farm in Ningbo, Zhejiang, China. Before the initiation of the feeding trial, animals were reared in floating sea cages (3.0 × 3.0 × 3.0 m) for 2 weeks to adapt to the experimental diet and culturing environment.

At the start of the feeding trial, animals were not fed 24 h. Fish of similar sizes (10.71 ± 0.10 g) were randomly distributed into 21 seawater cages (1.5 × 1.5 × 2.0 m), and each cage was stocked with 60 fish. There were seven triplicated treatments, and each cage was a replicate. Seven groups of animals were hand-fed one of the experimental diets twice daily at 05:00 and 17:00 hours, respectively, for 8 weeks. During the experimental period, the water temperature ranged from 19.5 to 25.5°C, salinity 25–28‰ and dissolved oxygen concentration was about 7 mg L^{-1}.

Sample collection and analyses

At the termination of the feeding trial, animals were not fed 24 h. Number and body weights of fish were measured. The growth and survival of large yellow croaker were calculated as follows:

$$\text{Specific growth rate (SGR, \% day}^{-1}) = (\text{Ln}W_t - \text{Ln}W_0) \times 100/t$$

$$\text{Survival rate (SR, \%)} = N_t \times 100/N_0$$

where W_t and W_0 were final and initial body weight, respectively; N_t and N_0 were final and initial numbers of fish, respectively; t is the feeding trial period (56 days).

Five fish from each cage were sampled for the whole body composition analysis. Determinations of moisture, crude protein, crude lipid and ash were conducted using the standard procedures (AOAC 1995).

Three animals in each cage were used for determination of the blood coagulation time, which was measured by the method of Shen (2000).

Five fish in each cage were used for analysis of MK-4 concentrations in muscle and liver. A high-performance liquid chromatography (HPLC) method was used (Udagawa *et al.* 1993). Standard MK-4 was purchased from Sigma.

Statistical analysis

All the data were subject to one-way ANOVA. When overall differences were significant at less than 0.05, Tukey's test was used to compare the mean values between individual treatments. Statistical analysis was performed using the SPSS 17.0. Based

on MK-4 concentrations in muscle and liver, the broken-line model (Robbins, Norton & Baker 1979) was used to estimate the minimum dietary contents of vitamin K for the maximum accumulation of tissue MK-4 in large yellow croaker.

Results

Growth and survival

The growth and survival data are presented in Table 2.

During the 8 weeks experiment, no apparent vitamin K deficiency signs were observed in any of the fish. The SGR varied from 1.84% to 2.17% day^{-1}, but no significant differences were observed among the all treatments ($P > 0.05$). Survival of large yellow croaker fed dietary sulfanilamide was significantly lower than the other all treatments ($P < 0.05$). There were no significant differences in survival rate among the all treatments without sulfanilamide supplementation.

Whole body composition

Data on the whole body compositions are shown in Table 3. There were no significant differences in contents of moisture, crude protein, crude lipid or ash among the all treatments ($P > 0.05$). The whole body moisture ranged from 75.11% to 75.89%. The body protein, lipid and ash ranged from 67.52% to 69.66%, 3.91% to 4.68% and 3.79% to 4.08% respectively.

Blood coagulation time

The results of blood coagulation time are presented in Table 4. There was significant difference in blood coagulation time between treatments with

Table 2 Survival and growth of juvenile large yellow croaker fed the experimental diets with different level of menadione sodium bisulfate (MSB) (means ± SEM, $n = 3$)*

Dietary MSB contents (mg kg^{-1})	Initial weight (g)	Final weight (g)	SGR† (% d^{-1})	Survival (%)
0.06	10.59 ± 0.10	29.87 ± 0.70^{ab}	2.04 ± 0.07	100 ± 0.00^{b}
3.45	10.81 ± 0.04	33.65 ± 1.04^{bc}	2.04 ± 0.15	100 ± 0.00^{b}
7.15	10.63 ± 0.05	27.78 ± 0.28^{a}	1.91 ± 0.06	100 ± 0.00^{b}
12.82	10.77 ± 0.05	35.07 ± 0.86^{c}	2.17 ± 0.10	100 ± 0.00^{b}
26.00	10.71 ± 0.01	29.79 ± 0.20^{ab}	1.97 ± 0.11	100 ± 0.00^{b}
47.54	10.73 ± 0.10	31.27 ± 0.88^{abc}	1.96 ± 0.11	100 ± 0.00^{b}
0.04 (sulfanilamide)	10.70 ± 0.17	29.65 ± 1.35^{ab}	1.84 ± 0.13	91.11 ± 3.89^{a}
ANOVA				
F value	1.471	10.306	1.136	5.224
P value	0.258	0.002	0.392	0.005

*Means in the same column sharing a same superscript letter are not significantly different determined by Tukey's test ($P > 0.05$).
†SGR: specific growth rate.

Table 3 The whole body compositions of juvenile large yellow croaker fed the experimental diets with different level of menadione sodium bisulfate (MSB) (means ± SEM, $n = 3$)*

Dietary MSB contents (mg kg^{-1})	Moisture (%)	Crude protein (%)	Crude lipid (%)	Ash (%)
0.06	75.69 ± 0.27	69.39 ± 0.41	3.91 ± 0.13	4.01 ± 0.04
3.45	75.63 ± 0.18	68.23 ± 0.46	4.14 ± 0.26	4.05 ± 0.04
7.15	75.87 ± 0.23	69.64 ± 0.63	3.91 ± 0.23	4.08 ± 0.04
12.82	75.11 ± 0.05	67.52 ± 0.87	4.68 ± 0.09	4.01 ± 0.07
26.00	75.89 ± 0.32	69.00 ± 0.44	3.98 ± 0.22	4.05 ± 0.01
47.54	75.80 ± 0.71	69.14 ± 1.37	4.08 ± 0.54	4.03 ± 0.02
0.04 (sulfanilamide)	75.73 ± 0.48	69.66 ± 0.38	4.19 ± 0.10	3.79 ± 0.12
ANOVA				
F value	0.501	1.179	1.005	2.743
P value	0.798	0.371	0.460	0.056

*Means in the same column sharing a same superscript letter are not significantly different determined by Tukey's test ($P > 0.05$).

Table 4 Blood coagulation time and concentrations of menaquinone-4 (MK-4) in muscle and liver of large yellow croaker fed the experimental diets with different level of menadione sodium bisulfate (MSB) (means ± SEM, $n = 3$)*

Dietary MSB contents (mg kg^{-1})	Coagulation time (s)	MK-4 concentrations (ng g^{-1} wet weight)	
		Muscle	Liver
0.06	68.33 ± 0.73^{a}	103.41 ± 0.92^{a}	501.03 ± 1.10^{a}
3.45	59.25 ± 1.25^{b}	109.18 ± 0.35^{b}	506.22 ± 0.27^{a}
7.15	54.75 ± 0.75^{bc}	114.06 ± 0.36^{b}	513.70 ± 1.04^{b}
12.82	55.25 ± 1.25^{bc}	122.23 ± 1.30^{c}	523.57 ± 0.11^{c}
26.00	55.25 ± 0.75^{bc}	122.10 ± 0.27^{c}	522.44 ± 1.41^{c}
47.54	52.75 ± 0.75^{c}	121.87 ± 1.46^{c}	522.40 ± 1.47^{c}
0.04 (sulfanilamide)	68.50 ± 0.50^{a}	102.14 ± 1.06^{a}	504.61 ± 0.82^{a}
ANOVA			
F value	60.55	89.81	86.12
P value	0.000	0.00	0.00

*Means in the same column sharing a same superscript letter are not significantly different determined by Tukey's test ($P > 0.05$).

0.06 mg kg^{-1} and 3.45 mg kg^{-1} dietary vitamin K ($P < 0.05$). Moreover, further increment of dietary vitamin K up to 26.00 mg kg^{-1} did not lead to significant changes in blood coagulation time. It was suggested that large yellow croaker has a minimum dietary requirement below 3.45 mg kg^{-1} for vitamin K.

Tissue concentrations of MK-4

Concentrations of MK-4 in muscle and liver of juvenile large yellow croaker are presented in Table 4. Generally, the concentrations of MK-4 in muscle and liver increased with the increasing dietary MSB levels up to 12.82 mg kg^{-1}. Moreover, further increment of dietary vitamin K up to 47.54 mg kg^{-1} did not lead to significant changes in MK-4 concentrations in muscle and liver. Based on MK-4 concentrations in muscle and liver, broken-line analysis showed that the minimum dietary vitamin K contents were 10.42 and 10.55 mg kg^{-1}, respectively, for the maximum accumulation of tissue MK-4.

Discussion

Poston (1971) did not find any adverse effects on growth rates of brook trout *salvelinus fontinalis* fed high levels of dietary MSB (100–2400 mg kg^{-1}). Meanwhile, after an 8-week (even prolonged to 20 weeks) feeding trial, it was confirmed that dietary MSB (0.00 mg kg^{-1} and 20.8 mg kg^{-1}) had no significant effects on the growth of haddock *Melanogrammus aeglefinus* L. (Roy & Lall 2007). Besides, based on a 28-week feeding trial, no significant effects of dietary menadione nicotinamide bisulphite (MNB) on the growth of Atlantic salmon *Salmo salar* were found (Krossøy, Waagbø, Fjelldal, Wargelius, Lock, Graff & Ornsrud 2009). However, 30 mg kg^{-1} of dietary MSB reduced the growth of Atlantic salmon after 20 weeks of feeding (Grisdale-Helland, Helland & Asgard 1991). Meanwhile, 20 mg kg^{-1} of dietary MSB decreased the growth of Atlantic cod after 23 weeks of feeding (Grahl-Madsen & Lie 1997). In the present study, no significant effect of dietary MSB on the SGR of juvenile large yellow croaker was observed. At the same time, no external signs of vitamin K deficiency or toxicity were found. Up to now, information on the physiological role of vitamin K in aquatic animals is limited, and its necessity for survival and growth has not yet been well established (Krossoy *et al.* 2011). At the present, it is hard to draw a conclusion that vitamin K is not essential to the growth of large yellow croaker due to the 56-day feeding trial period. A longer experimental duration could be necessary for dietary vitamin K to completely exert its effects on the growth of this fish species. Further study is needed to confirm it.

In the present study, the blood coagulation time generally decreased with the increasing of dietary MSB levels. It was suggested that vitamin K had the function of promoting blood clotting in large yellow croaker. Some previous studies had the same results. For example, the vitamin K-free diet or the antibiotics-supplemented diet can prolonged the blood clotting time of brook trout (Phillips, Podoliak & Livingston 1963). Dietary vitamin K

deficiency could also cause the prolongation of blood clotting time and tissue bleeding in amago salmon *Oncorhynchus rhodurus* (Taveekijakarn *et al.* 1996). At the same time vitamin K-deficient diets or antibiotics-supplemented diets resulted in the signs of increased blood clotting time, anaemia, haemorrhages in gills, eyes and vascular tissues in salmonids (Kitamura, Suwa, Ohara & Nakagawa 1967; Halver 1989; Graff, Waagbo & Fivelstad 2002). However, studies on cod (0–20 mg kg^{-1} MSB) (Grahl-Madsen & Lie 1997) suggested that the blood coagulation time was not the results of dietary vitamin K deficiency. The reason could be that cod had a high tolerance of vitamin K deficiency. Longer time was needed to exhibit the vitamin K deficiency symptoms (Grahl-Madsen & Lie 1997). However, there could be some other reasons for variation in the results on effects of dietary vitamin K on fish blood coagulation time, such as different experimental diets (purified, semi-purified or practical) and animal species.

In the present study, MK-4 was detected in muscle and liver of large yellow croaker fed the experimental diets. It was indicated that dietary MSB could convert to MK-4 in the body. This was the same with those in mammals (Billeter, Bollinger & Martius 1964) and abalone *Haliotis discus hannai* (Tan & Mai 2001). Menadione must be alkylated enzymatically to MK-4 in animal tissues to become biologically active (Udagawa 2000). Although micro flora in the intestinal track may produce menaquinones in terrestrial animals, typical intestinal vitamin K synthesizing micro flora has not been isolated from fish (Lall & Lewis-McCrea 2007). Moreover, no significant difference in tissue MK-4 concentration was found between the dietary MSB-free treatment and the dietary sulfanilamide supplemented treatment. It was suggested that micro flora in large yellow croaker could not synthesize MK-4, or the synthesis ability was very limited. However, the exact role of the micro flora on the MK-4 synthesis in large yellow croaker needs further studies.

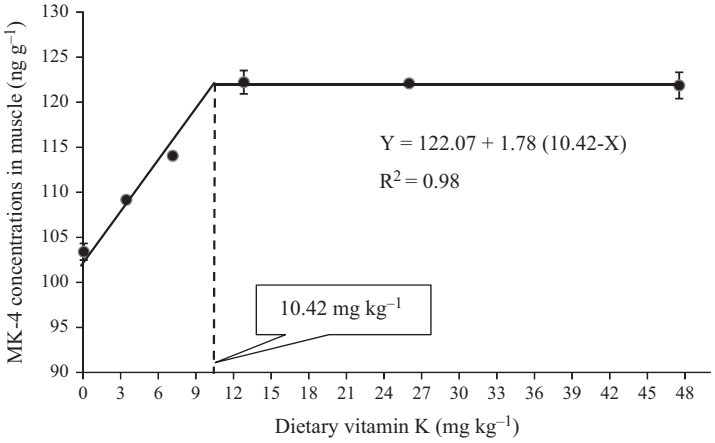

Figure 1 Broken-line analysis of the relationship between dietary menadione sodium bisulfate (MSB) and concentrations of menaquinone-4 (MK-4) in muscle indicates that the minimum dietary vitamin K content was 10.42 mg kg^{-1} for the maximum accumulation of MK-4. Each point represents the mean of three replicates.

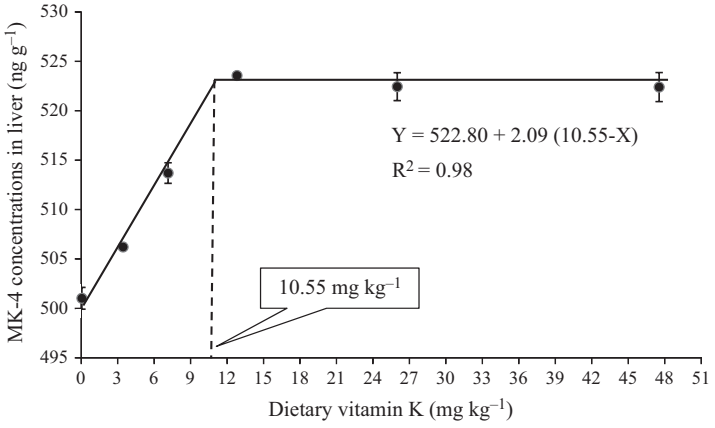

Figure 2 Broken-line analysis of the relationship between dietary menadione sodium bisulfate (MSB) and concentrations of menaquinone-4 (MK-4) in liver indicates that the minimum dietary vitamin K content was 10.55 mg kg^{-1} for the maximum accumulation of MK-4. Each point represents the mean of three replicates.

Based on growth data, the requirements of vitamin K for both chinook salmon *Oncorhynchus tschawytscha* and European seabass *Dicentrachus labrax* were determined as 1.5 mg kg^{-1} (Kaushik, Gouillou-Coustans & Cho 1998). Those for salmonids (Halver 2002) and haddock (Roy & Lall 2007) were 10 mg kg^{-1} and 20 mg kg^{-1} respectively. However, as discussed above in the first paragraph of Discussion, there were also reports that found no significant effects of dietary vitamin K on the growth of some fish species. In those studies, the blood coagulation time was used to determine the minimum requirement of dietary vitamin K, such as 0.5–1.0 mg kg^{-1} for lake trout *Salvelinus namaycush* (Poston 1976), 0.2 mg kg^{-1} for cod (Grahl-Madsen & Lie 1997), and 0.1 mg kg^{-1} for Atlantic salmon (Krossøy *et al.* 2009). In the present study, the growth was not significantly affected by the dietary vitamin K levels yet. Based on the blood coagulation time, the minimum requirement of vitamin K for large yellow croaker was determined as less than 3.45 mg kg^{-1}. At the same time, in this study, the concentrations of MK-4 in muscle and liver increased with the increasing dietary MSB levels up to 12.82 mg kg^{-1}. Further increases of dietary MSB levels did not significantly influence the MK-4 concentrations (Table 4). According to the MK-4 concentrations in muscle and liver, broken-line analysis showed that the minimum dietary vitamin K contents were 10.42 and 10.55 mg kg^{-1}, respectively, for the maximum accumulation of tissue MK-4 (Fig. 1 and Fig. 2). There is a distinction between minimum requirement and requirement for optimal growth or optimal health, which could lead to the definition of higher requirement or recommendation levels adapted to a specific function or to certain conditions (Krossoy *et al.* 2011). To clearly understand the relationship between the dietary vitamin K doses and the growth as well as the health of large yellow croaker, further work is needed.

In conclusion, under the present study conditions, it was suggested that supplementation of MSB in diet is not necessary to the survival and growth of juvenile large yellow croaker in a short period (56 days) culture. However, with regard to the blood coagulation time, a minimum requirement of dietary MSB (<3.45 mg kg^{-1}) was needed. Furthermore, for the maximum accumulation of MK-4 in muscle and liver, the minimum dietary vitamin K contents were 10.42 and 10.55 mg kg^{-1} respectively.

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