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# Studies on the Distribution and Physiological Function of Vitamin K in Fish [*1]

Miho Udagawa [*2]

**Abstract:** Basic information on the distribution and role of vitamin K in fish was studied in relation to dietary intake.

Chapter 1 describes the concentrations of phylloquinone (PK) and menaquinones (MKs) in various tissues of wild and cultured fishes as the results of analysis based on the HPLC method. The major vitamin K in wild pelagic fish was PK, and the stomach and intestinal contents of pelagic fish showed much higher levels of PK than MK-4. Demersal fish contained relatively large amounts of MK-4 and PK but only small amounts of long chained MKs. In demersal fish, the contents of the gastrointestinal tracts were rich in MK-long chains. However, a discrepancy between the composition pattern of vitamin K in the tissues and contents of the gastrointestinal tract was observed. Cultured fish fed a menadione (MD) supplemented feed were rich in MK-4, which indicates that MD was converted to MK-4 in the body. From these results, it was concluded that either PK or MK-4 deposited in the body originates from their food and stored mainly in the liver, and the different forms of vitamin K are absorbed and/or accumulated in the tissues based on different physiological pathways.

In Chapter 2, the contents of vitamin K in the plasma and tissues (kidney, liver and gonad) of fish fed diets supplemented with different vitamin K groups were determined. The PK rich diet raised the PK concentration in the plasma and the tissues much higher than the diets supplemented with short and/or long chain MKs. This indicates that PK is more easily accumulated into the body of fish than the MK homologues.

Chapter 3 describes the effect of different dosages of vitamin K, either as PK or menadione sodium bisulfite (MSB), on mortality and vertebral formation. Rearing during the spawning season with a vitamin K free diet for 11 weeks, led to most of the male fish dying. In the kidney tissue of the fish fed with a vitamin K free diet, a large number of immature erythrocytes was observed. This indicates that the female fish had been affected by hematoporia and/or hematopoiesis brought about by a deficiency in vitamin K. Diets without vitamin K caused a significantly higher incidence of bone deformity in larvae than diets supplemented with vitamin K. The maternal effect of PK deficiency on bone structures was examined in the larvae. The vitamin K deficient larvae had an abnormal vertebral formation, while PK rich larvae showed a low rate of abnormality. When fed the vitamin K deficient diet, larvae which had been fed the vitamin K rich and deficient diets had a both high rate of abnormality after 30 days, with only larvae which wrere hatched from vitamin K rich eggs and then fed on a vitamin K rich diet showed a low rate of abnormality. These results indicate that vitamin K deficiency affects bone structure both in early development and during growth.

In Chapter 4, the effect of PK on the bone structure in fish was observed histochemically. The bone structure of vitamin K deficiency larvae was thin and rough after the feeding experiment for 30 days. It seemed to be connected after fine fracture of a bone surface. In

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[*2] 中央水産研究所　〒236-8648　横浜市金沢区福浦2-12-4（National Research Institute of Fisheries Science, 2-12-4, Fukuura, kanazawa, Yokohama 236-8648, Japan）

contrast, in larvae fed a PK rich diet this phenomenon was not observed. These results imply that vitamin K deficiency induced bone abnormality with thin and weak structure. A vitamin K rich diet increased the osteoblast-like cells. These results imply that PK is necessary to activate the osteoblasts. It is indicated that at least part of the effects of PK on bone resorption may regulate the osteoblasts activity through an unidentified mechanism.

In Chapter 5, a comprehensive discussion is given of the all results and conclusions so far described.

**Key Words:** phylloquinone, menaquinone, tissue distribution, vitamin K deficiency,,

## Contents



## Introduction

Vitamin K was first discovered by Dam (1935) as a substance playing an important role in the synthesis of blood-clotting factors. Vitamin K is a group name for a series of related compounds that act as a cofactor for the microsomal enzyme γ-glutamylcarboxylase. This enzyme is involved in the post-translational conversion of peptide-bound glutamate residues into γ-carboxyglutamate (Gla), which occurs in a number of blood coagulation factors and bone proteins (Furie B. and Furie B. C. 1988; Hauschka *et al.* 1989; Vermeer 1990). Furthermore, a variety of diseases due to vitamin K deficiency has been reported in mammals. In particular, a few week old human infants are prone to hemorrhage due to vitamin K deficiency. Attention has been increasingly focused on the importance of vitamin K for normal bone development. However, the role of vitamin K in fish has not been well documented. In this study, the physiology, distribution and absorption of vitamin K in relation to the intake of vitamin K in fish is described.

There are several derivatives of vitamin K as follows: 1) phylloquinone (PK) called vitamin K1, is produced by plants; 2) menaquinone (MK) which is synthesized by microorganisms is called vitamin $K_2$ and has long side-chains arranged in MK-n series. MK-4 consists of short isoprene units and MK-9 is one of a typical long-chain MK produced

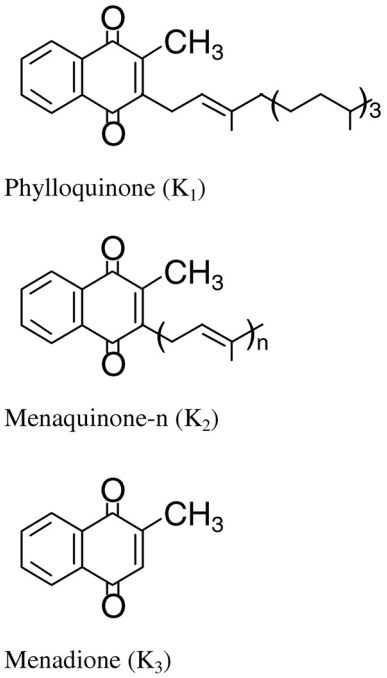



**Fig. 1.** Chemical structure of vitamin K.

by bacteria; 3) Synthetic analogues of menadione (MD) designated as vitamin $K_3$ have no side-chains, and are utilized for animal and fish commercial diets (Fig. 1). Water-soluble salt of synthetic menadione, menadione sodium bisulfite (MSB) is generally supplemented in commercial fish diets. Parts of PK, MK and MD are metabolized to MK-4 in the tissues (Billeter *et al.* 1964). Therefore, as basic information on the utilization of vitamin K, the distribution of the various forms of vitamin K in tissues and origins were determined.

There are many reports on vitamin K requirements for mammals except for fish, and vitamin K is generally considered to be an essential nutrient for mammals. Woodward (1994) gave quantitative estimates of the dietary requirement of vitamin K for salmonids. Poston (1964) reported that in the brook trout blood coagulation time was delayed and the microhematocrit value decreased by vitamin K deficiency, but that the growth rate was not appreciably affected. Kitamura *et al.* (1967) also reported that in the rainbow trout, vitamin K deficiency was associated only with anemia. As for the catfish, no symptoms of vitamin K deficiency were observed (Murai and Andrews 1977). On the other hand, Taveekijakarn *et al.* (1996) reported that the mortality level in vitamin K deficient amago salmon reached 50% and the histopathological changes were detected.

The physiological function of vitamin K was originally thought to be limited to the role of cofactor in blood coagulation. Recently, the important role of vitamin K in normal bone development has been revealed in mammals (Vermeer *et al.* 1996; Shearer 1996). Residue-specific carboxylation of proteins is one of the functions of vitamin K and calcium-binding proteins such as osteocalcin and matrix-Gla protein also require vitamin K for their carboxylation. Thus, the importance of vitamin K in mammalian bone development has attracted the interest of many researchers (Kohlmeier *et al.* 1996), while the participation of vitamin K in fish bone development has not been investigated.

In this study the effect of vitamin K deficiency on fish mortality and bone development are described.

## Chapter 1. Distribution of vitamin K in fish

Vitamin K is essential for the synthesis of clotting factors in the liver of mammals. Its metabolic role also has been more clearly defined than that of the other three fat-soluble vitamins, even though it was the last of the fat-soluble vitamins to be discovered (Suttie 1980; Korman and Weiser 1983). Many types of compounds such as PK, MKs and MD exhibit vitamin K activity. In terrestrial mammals, long chain MKs (MK-5 through MK-13) are usually synthesized by the intestinal bacteria and PK comes from food being consumed (Will *et al.* 1992). On the other hand, information on the physiological roll of vitamin K is limited in fish, and even its essentiality for fish is not well established yet among fishes (Murai and Andrews 1977; Kawatsu and Ikeda 1988; Kawatsu *et al.* 1989). In this chapter, distribution of vitamin K especially difference by food habitat in fish is described.

### 1.1. Tissue distribution of phylloquinone and menaquinone-4 in fish

More basic information, such as the distribution of various forms of vitamin K in tissues and those origins are necessary as the first step to elucidate the physiological roles of vitamin K in fish. In

this section, the author attempted to measure the concentrations of PK and MK-4 in various tissues of fish with a HPLC method modifying the sample preparations. Using these techniques, the author determined distribution of PK and MK-4 in various tissues of wild sardine *Sardinops melanostictus* feeding on PK-rich phytoplanktons such as diatoms and cultured sardine fed MD-supplemented feed to determine the origin of these compounds.

## Materials and Methods

### Materials

Cultured sardine was raised in a $20m^3$ round tank at National Research Institute of Aquaculture. A commercial trout feed supplemented with MSB was fed to the fish at a daily rate of about 2% of biomass for one year. Five fish were randomly sampled and immediately frozen after the blood sample was collected from the caudal vein. The first lot of wild sardine (wild sardine 1) were purchased from the Tukiji fish market on Nov. 1991. They were kept in ice after captured at unknown area and were still extremely fresh condition. Five fish having a similar body length to the cultured fish were selected but the blood sample was not available. The second lot of wild sardine (wild sardine 2) were caught in the Pacific Ocean approximately 90km off Shionomisaki, Japan, by angling from the research vessel Soyo Maru on Feb. 23 1992. They were kept at −80℃ immediately after being caught but the blood was not sampled. The stomach contents and intestinal contents were separately collected from wild sardine 2. Planktons were collected with 100 $\mu$m mesh NORPAC net by vertical tow (0-150m) simultaneously at the same area where wild sardine 2 were angled. The gonad somatic index (GSI) was calculated as follows: GSI=GW×100/BW, where GW and BW represent gonad weight (g) and body weight (g).

### Chemicals

Standard PK and MK-4 were purchased from Sigma Chemical Co. (St Louis, MO). The hexane, methanol and ethanol were of HPLC grade and purchased from Wako Pure Chemical Industries, LTD. Frolisil (100-200mesh) was also product of Wako Pure Chemical Industries, LTD.

### Analysis of vitamin K

In this section, only PK and MK-4 were measured because no rich source of typical vitamin K synthesizing microorganisms has been described in the intestine of cultured fishes (Margolis, 1953) and also Will *et al.* (1992) have reported that long chain MKs (MK-5 through MK-13) of gut origin make a relatively minor contribution to the hepatic stores of vitamin K in rats and chicks.

The samples of heart, kidney, muscle and serum were prepared with Sep-Pak as has been described by Nagaoka *et al.* (1989). If enough quantity was available, about 1 g of the tissue was used but otherwise used whole tissue. A sample was homogenized with 1 ml of distilled water and 4 ml of ethanol. Then 6 ml of *n*-hexane was added. And the mixture was shaken for 5min, followed by centrifugation at 800*g* for 5min. A 5ml portion of the upper layer was transferred into a centrifuge tube and evaporated. The residue was dissolved in 2 ml of *n*-hexane. A silica Sep-Pak cartridge (Waters) was washed with 10ml of *n*-hexane/ether (93/7) and 10ml of *n*-hexane. The sample was loaded, washed with 10ml of *n*-hexane, and eluted with 5ml of *n*-hexane/ether (93/7). This eluate was evaporated and dissolved in 0.2ml of ethanol.

The liver sample was applied to frolisil column prior to the Sep-Pak treatment in order to remove various compounds which was found only in the liver and affected the reading. The frolisil was washed with *n*-hexane, packed into a glass column (5mm (i.d.)×60mm), and washed with 10ml of *n*-hexane. The residue from the silica Sep-Pak cartridge was dissolved in 0.2ml *n*-hexane and loaded, washed with 10ml of *n*-hexane, eluted with 10ml of *n*-hexane/chroloform (60/40). This eluate was evaporated and dissolved in 0.2 ml of ethanol.

The total sample completed these procedures was injected into the HPLC system. MSB was measured by an ordinary method (Chiba, 1985). All chamical analysis was conducted under the absence of daylight by using brown glassware.

### HPLC system

The HPLC system consisted of a Hitachi L-6000

pump, Lichrospher RP-8 (e) column (4×250mm, 5m), platinum oxide column (4×20mm, EICOM Co.) for the reduction column, Hitachi F-1050 fluorescence spectrophotometer and Hitachi D-2500 chromato-integrator. The mobile phase was methanol containing 2% sodium perchlorate and deaerated by bubbling argon gas. Flow rate was 0.5ml/min. Detection was made fluorometrically at an excitation wavelength of 254 nm and an emission wavelength of 430 nm as described by Shino (1988).

### Statistics

Data on the size of fish and gonad somatic index (GSI) were analyzed by Duncan's multiple range and multiple *F*-test (Duncan, 1955). Man-Whitney *U*-test and *t*-test for paired samples were used for the contents of PK and MK-4 in each tissue. The statistical differences between the treatment groups were assessed at a 5% level of probability. The values shown in the text are means ±SEM.

## Results and discussion

The physical characteristics of the sardine used for this study were shown in Table 1. Although all fish used in this study had a similar body length, there were significant differences in body weight, liver weight and maturation stage. The GSI clearly indicates, wild sardine 1 were still immature while wild sardine 2 were fully matured and cultured sardine were intermediate. The liver of wild sardine 1 were significantly smaller than those of cultured sardine and wild sardine 2.

As clearly shown in HPLC profile (Fig. 2), identifications of PK and MK-4 peaks became possible by the previous treatment with the frolisil column. Thus, the HPLC method employed can be applicable for determination of PK and MK-4 even for the liver sample if various other compounds were removed in advance by passing through the frolisil column.

The concentrations of PK in the various tissues examined were shown in Fig. 3. PK contents in all tissues of wild sardine 2 were much higher than those of cultured sardine and wild sardine 1, especially in the liver. PK content in the liver of cultured sardine was only 1.65±1.11ng/g of wet tissue which was less than 1/9 of those of wild sardine 1 (14.75±8.23) and wild sardine 2 (46.48 ±10.86). In wild sardine 2, the gonad contained significantly higher level of PK than the kidney and heart (*t*-test for paired sample) but it was about 1/3 in the liver. While appreciable quantity of PK was found only in the liver of wild sardine 1. On the other hands, only fractional quantity of PK was detected among the tissues of cultured sardine including the serum. PK contents in both ordinary and dark muscle were trace amounts in all fish tested.

The concentrations of MK-4 in the various tissues were presented in Fig. 4. MK-4 contents in cultured sardine were significantly higher (at least 2-fold) than those of wild sardine 1 and 2 in all tissues examined except the muscles (Mann-Whitney *U*-test). The highest value (14.20±3.6) was detected from the heart but it was not significantly different from those in the gonad and liver (*t*-test for paired samples). More than 5ng of MK-4 was also found in the kidney and serum. MK-4 concentrations in these tissues of wild sardine 2

**Table 1.** Physical characteristics of sardine used for determination of vitamin K

| | Body length (mm) | Body weight (g) | Liver weight (g) | Condition factor | GSI* |
|---|---|---|---|---|---|
| Cultured sardine | 200±3.9[a] | 129.1±10.8[a] | 2.20±0.30[a] | 1.62±0.11[a] | 2.78±1.85[a] |
| Wild Sardine 1 | 192±2.7[a] | 100.3±4.7[b] | 0.91±0.12[b] | 1.41±0.08[b] | 0.31±0.16[b] |
| Wild Sardine 2 | 296±0.6[a] | 101.7±6.1[b] | 1.67±0.10[c] | 1.36±0.05[b] | 8.69±1.57[c] |

*Gonad somatic index.
Values are means ± SEM of five fish.
Values in the same column with different superscripts are significantly different ($P$ <0.05).

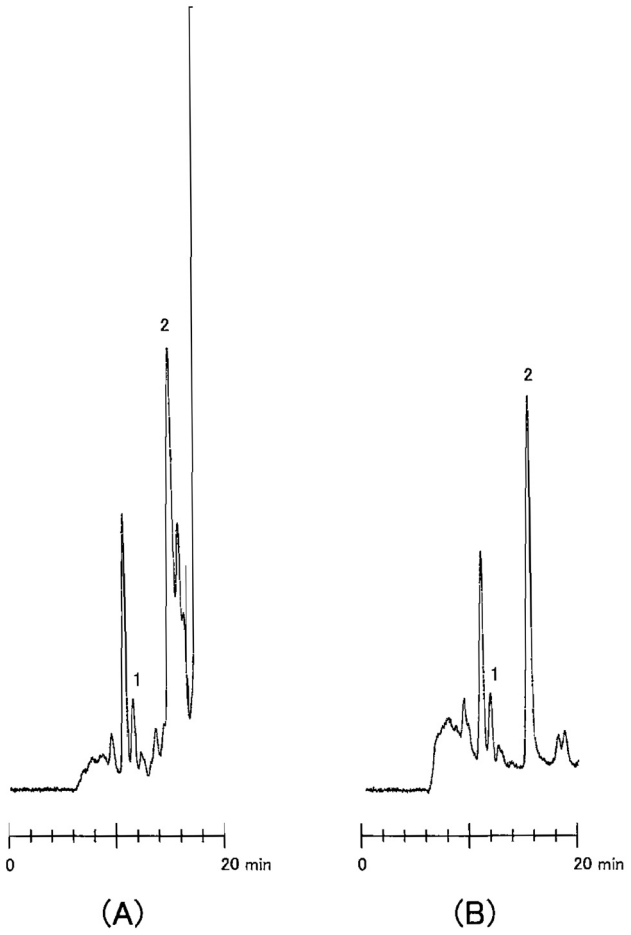



**Fig. 2.** HPLC separation of vitamin K in liver tissue of sardine. Peak 1 is MK-4, peak 2 is PK. (A): only Sep-Pak was used; (B): Frolisil column was used after Sep-Pak.

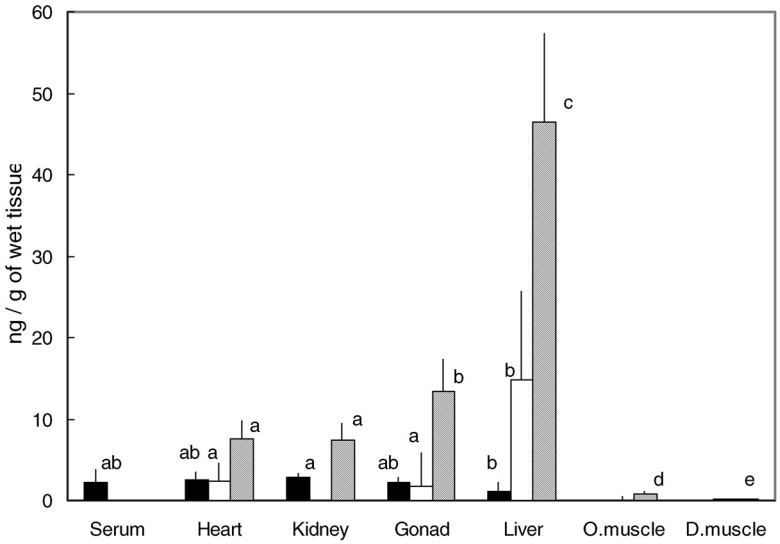



**Fig. 3.** Phylloquinone contents in various tissues of sardine. Groups of sardine were as follows: solid bars, cultured sardine; open bars, wild sardine 1; hatched bars, wild sardine 2. O. muscle is ordinary muscle and d. muscle is dark muscle. Values in each group with different superscripts are significantly different ($P<0.05$). Values are means ± SEM of five fish.

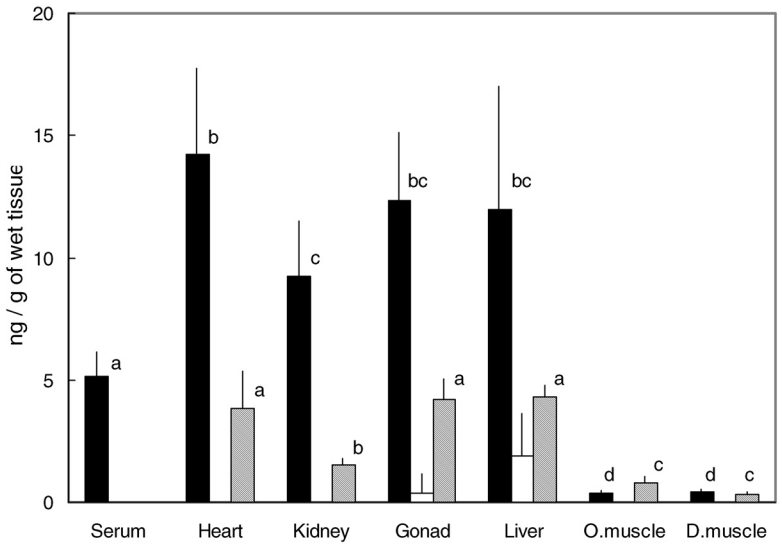



**Fig. 4.** Menaquinone-4 contents in various tissues of sardine. Groups of sardine were as follows: solid bars, cultured sardine; open bars, wild sardine 1; hatched bars, wild sardine 2. O. muscle is ordinary muscle and d. muscle is dark muscle. Values in each group with different superscripts are significantly different ($P<0.05$) . Values are means ± SEM of five fish.

**Table 2.** Phylloquinone, menaquinone-4 and menadione contents in food consumed by sardine

| | Phylloquinone (ng /g of wet) | Menaquinone-4 (ng /g of wet) | MSB[1] (ng /g of wet) |
|---|---|---|---|
| Plankton | 61 | 2 | -[3] |
| Stomach contents* | 120±60 | 12±12 | - |
| Intestinal contents* | 160±150 | 6±5 | - |
| Commercial trout feed | ND[2] | ND | 2.0 |

*Values are means ± SEM of five specimens.
[1]MSB: menadione sodium bisulfite.
[2]ND: not detected.
[3]-: not measured.

were significantly higher than those of wild sardine 1 but were only comparable values to PK levels in the respective tissues of cultured sardine. Again, the muscle samples contained only trace quantity of MK-4 in all groups.

It is possible that the significant differences in PK and MK-4 contents between wild sardine 1 and 2 were due to the difference in nutritional condition and maturation stage between these two group because the liver weight and GSI were much higher in the latter. Also unknown period of storage in ice can be another factor attributable for the low values in wild sardine 1. Even though these factors were taken into consideration, it can be concluded at least that major vitamin K deposited in the body was PK in wild sardine and MK-4 in cultured sardine.

Analytical results of PK, MK-4 and MSB in the stomach and intestinal contents, planktons which were supposed to be consumed by wild sardine and commercial trout feed which was fed for cultured sardine were shown in Table 2. The planktons contained almost 30 times higher level of PK than MK-4. The stomach and intestinal contents of wild sardine 2 also had at least 10 times higher level of

PK than MK-4. However, no significant difference in both PK and MK-4 concentrations were detected between the stomach and intestinal contents (*t*-test for paired samples). On the other hand, only MSB was found in the commercial feed. These results strongly indicate that vitamin K found in the body of sardine came from food being consumed. Also the lack of any significant difference in MK-4 concentrations between the stomach and intestinal contents suggests that contribution of MK-4 synthesized by intestinal microorganism is minor in sardine. MSB seemed to be converted to MK-4 in the body of sardine as has been well known to take place in the liver of mammals (Billeter *et al*., 1964) since the commercial trout feed fed for cultured sardine contained only MSB.

More studies are needed to elucidate physiological role of vitamin K in fish. However, the difference in distribution pattern in the tissues between cultured and wild sardine, and its accumulation in the gonad of matured sardine and extremely low concentrations in the muscle could be useful information for this purpose.

## 1.2. Difference in the distribution of vitamin K with feed habitat in fish

### 1.2.1. Content of phylloquinone and menaquinone derivatives in the organs of pelagic and demersal fishes

Food habits differ greatly between pelagic fish and demersal fish. Pelagic fish feeds on plankton and small fishes, while demersal fish feeds chiefly on benthos. Benthos are organism which lives in benthic of the sea. Generally, phytoplankton contains a high concentration of PK. On the other hand, it can be assumed that benthos are rich in long chain MKs produced by microorganisms growing on them. This section describes the study to elucidate the relationship between type of vitamin and/or their K levels in various tissues of fish which have the different food habits. The fish species tested were mackerel, *Scomber japonicus* and saury, *Cololabis saira* as pelagic fish examples, and marbled sole, *Limanda yokohamae* and sillago, *Sillago japonica* as demersal fish examples.

#### Materials and methods

##### Materials

Mackerel were caught by angling in the Sagami bay, Japan, on 11 February 1993. Saury were also angled in the sea off the Pacific coast of Hokkaido, Japan on 10 September 1993. Marbled sole and sillago were purchased from Kosiba Fishing Port Market in Kanagawa prefecture shortly after being caught at Tokyo bay on 6 November 1993. All the fish samples were kept at −40℃ until used. Five fish of each species were used for analysis. The tissues of heart, kidney, liver, gonad and muscle were separated and the vitamin K content in each specimen was measured, except for the mackerel gonad, because it was not mature. In the cases of mackerel and saury, the muscle tissue was separated further into the ordinary and dark muscles for separate analysis.

The stomach and intestinal contents were collected separately from individual fish. Frozen Pacific krill *Euphausia pacifica* was purchased from Ishinomaki Fishing Port Market in Miyagi prefecture. *Polychaeta spp.* was collected at Kosiba Fishing Port, in Kanagawa prefecture. These materials were also subjected to analysis of vitamin K.

##### Chemicals

Authentic PK was purchased from Sigma Chemical Co. (St Louis, MO). Authentic MK-4 was kindly provided by Eizai Co. (Tokyo, Japan), and MK-6, 7, 8 and 9 by Nippon Roche Tokyo (Tokyo, Japan). Ethanol, methanol and *n*-hexane, HPLC grade, were purchased from Wako Pure Chemical Industries, (Osaka, japan).

##### Analysis of vitamin K

PK and MK were measured by HPLC. The tissue specimens were homogenized with a mixture of *n*-hexane, $H_2O$ and ethanol (6:1:4) and shaken for 5min. The solvent extracts were then pre-treated by passing through a Sep-Pak silica cartridge (Waters, Milford, MA) by elution with *n*-hexane and ether mixture. Each eluate was evaporated to dryness under reduced pressure and the residue

was dissolved in 200 μl ethanol (Nagaoka *et al.*, 1989). This solution was used as a sample solution for HPLC analysis. An aliquot of the sample solution was injected into the HPLC system.

The HPLC system consisted of same composition described in section 1.1. The column of Merck Lichrospher 100 RP-8 (e) 4×250mm was used for the analysis of vitamin K in mackerel and saury, and the Cosmosil 5C18-AR column 4.6×100mm (Nacalai Tesque, Inc. Kyoto, Japan) for marbled sole and sillago. A platinum oxide column, 4×20mm (EICOM Co., Kyoto Japan) was used as a reduction column. The mobile phase, flow rate and detection of vitamin K were same manner as section 1.1. The peaks were identified by comparison of retention times with those of the standards and disappearance of peaks without reduction.

## Results

The body measurements of the fishes used for this study were shown in Table 3. The size of fish was not greatly different from each other in samples of the same species. Except for marbled sole, sillago and saury were not mature. The gonad of mackerel was too small to determine GSI.

The PK and MKs contents in the fish tissues were illustrated in Fig. 5. None of the samples showed even trace of MK-9. In mackerel, though MK-6 was detected, no other long chain Mks were detected. Similarly, in the case of sardine, the content of PK was much higher than those of MKs. This was especially so in the liver where the PK content was as high as 3-4 times when compared to those in heart and kidney. However, only a trace amount of vitamin K was detected in the ordinary and dark muscle tissues. Thus the vitamin K distribution pattern in mackerel was very similar to that of sardine, although the amount in the former was two times higher than that of the latter.

In striking contrast to mackerel, MK-4 appeared to be the major vitamin K in the heart and gonad of saury with very low level of PK. Furthermore, little vitamin K was present in the liver. The total vitamin K content was, however, much lower than that of mackerel.

In the case of marbled sole, significant amounts of long chain MKs (MK-6 and 7) were detected together with MK-4 in the heart and kidney. Furthermore, MK-8 was also found in the kidney. On the other hand, in sillago, PK and MK-4 were commonly distributed in the various organs, but MK-6, 7 and 8 were found only in the liver. In both marbled sole and sillago, the PK level was higher than that of saury on the whole but very much lower than that of mackerel.

As shown in Table 4, the contents of PK and MKs in the contents of gastrointestinal (GI) tract contents differed greatly among species. In the GI tract contents of marbled sole and sillago, large amounts of the long chain MKs were detected. However, in the mackerel and saury, the contents of long chain MKs was less than 1/100 of those of marbled sole and sillago. The GI tract content of mackerel was

**Table 3.** Body length, body weight, sampling locality and date of fish used for determination of vitamin K

| | Body length(cm) | Body weight(g) | GSI[1] | Sampling locality | Sampling date |
|---|---|---|---|---|---|
| Mackerel | 24.6±0.5 | 197±21 | -[2] | Sagami Bay | 2 Nov. 1993 |
| Saury | 29.0±0.6 | 156±14 | 0.20±0.17 | East of Hokkaido | 2 Nov. 1993 |
| Marbled sole | 16.7±0.4 | 113±9.0 | 4.07±2.59 | Tokyo Bay | 2 Nov. 1993 |
| Sillago | 15.0±0.5 | 44.1±4.5 | 0.62±0.20 | Tokyo Bay | 2 Nov. 1993 |

[1] Gonad somatic index (Gonad weight × 100 /Body weight).

[2] Not measured.

Values are means ± SEM of 5 fish.

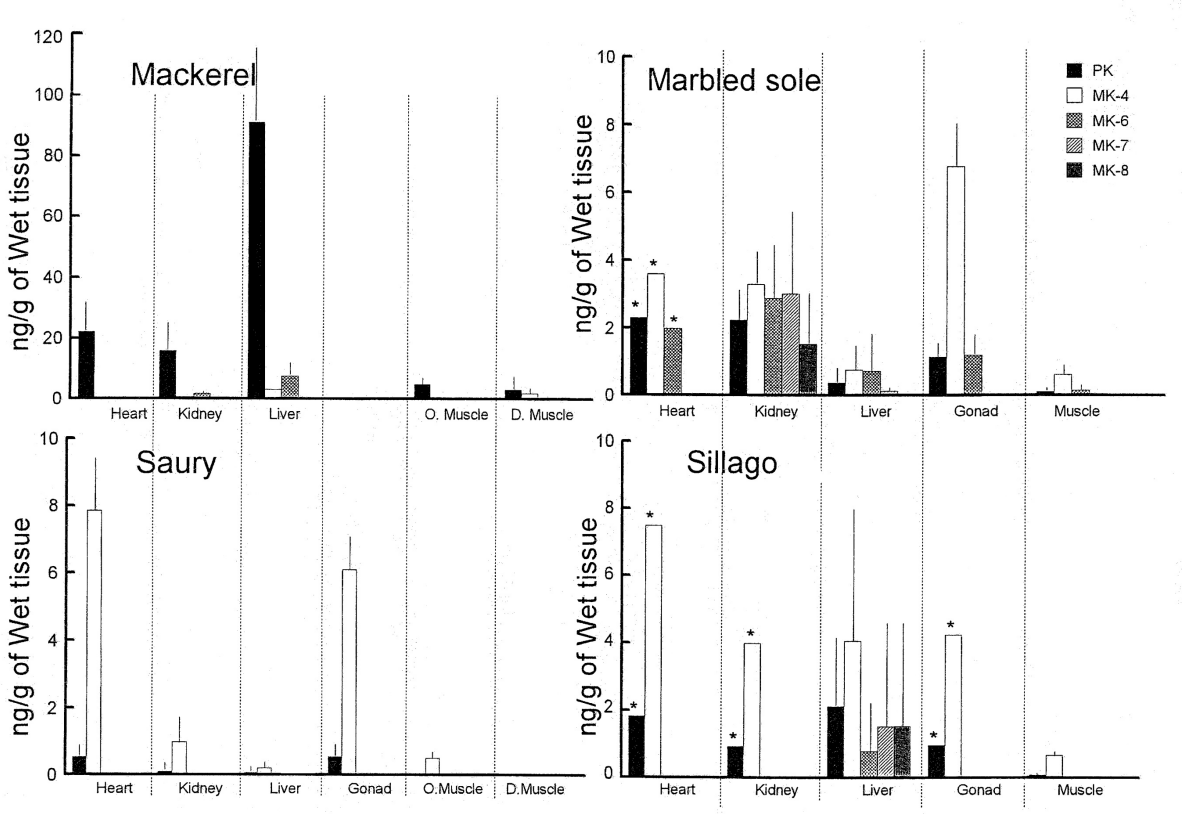



**Fig. 5.** Contents of phylloquinone (PK) and menaquinone (MK)-4, 6, 7, 8, 9 in various tissues of mackerel, saury, marbled sole and sillago. O muscle: Ordinary muscle, D muscle: Dark muscle. Values are means ± SEM of 5 fish. * Indicates that the values were measured by pooling specimens from 5 fish. MK-9 was also analyzed, but not detected in all the tissues examined.

**Table 4.** Contents of phylloquinone and menaquinone in gastrointestinal tract contents of fish
(ng/g of wet substance)

| | PK (%) | MK-4 (%) | MK-6 (%) | MK-7 (%) | MK-8 (%) | MK-9 (%) |
|---|---|---|---|---|---|---|
| Mackerel | | | | | | |
| Stomach | 3.97±2.04(32) | 1.29±0.26(10) | 3.83±0.87(31) | 3.28±2.31(27) | N.D. | N.D. |
| Intestine | 2.01±0.98(34) | 0.24±0.03 (4) | 2.37±1.05(40) | 1.07±0.24(18) | 0.18±0.28 (3) | N.D. |
| Saury | | | | | | |
| Stomach | 0.74±0.65(10) | 2.62±1.49(35) | 2.11±2.04(28) | 1.96±1.53(26) | N.D. | N.D. |
| Intestine | 2.48±0.69(13) | 5.79±1.03(29) | 5.61±2.05(28) | 5.98±4.23(30) | N.D. | N.D. |
| Marbled sole | | | | | | |
| Stomach | 54.8±50.8 (6) | 20.3±15.1 (2) | 228±343 (24) | 408±582 (44) | 160±143 (17) | 62.7±67.0(7) |
| Intestine | 12.6±11.7 (4) | 4.76±2.72 (2) | 95.1±164(30) | 159±279 (49) | 39.1±54.0(12) | 13.7±23.8(4) |
| Sillago | | | | | | |
| Stomach | 9.82±11.8 (6) | 6.34±3.66 (4) | 67.5±103(43) | 53.5±66.7(34) | 14.6±14.6 (9) | 3.98±5.97(3) |
| Intestine | 19.9±21.9 (8) | 10.0±5.37 (4) | 75.1±89.0(28) | 94.1±54.4(36) | 47.2±48.4(18) | 18.4±25.8(7) |

N.D.:not detected.

Values means ±SEM of five fish.

(%):percentage of each vitamin K to total vitamin K volume

**Table 5.** Phylloquinone and menaquinone contents in food consumed by fish
(ng /g of wet substance)

| | PK (%) | MK-4 (%) | MK-6 (%) | MK-7 (%) | MK-8 (%) | MK-9 (%) |
|---|---|---|---|---|---|---|
| *Euphausia pacifica* | 0.35(6) | 1.76(28) | 2.63(41) | 1.61(25) | N.D. | N.D. |
| *Polychaeta spp.* | 11.6(4) | 28.8(11) | 48.9(18) | 139 (51) | 32.4(12) | 14.2(5) |

N.D.: not detected.

rich in PK, MK-6 and MK-7 and that of saury was rich in MK-4, 6 and 7. However, in the GI tract contents of marbled sole and sillago, MK-6 and 7 were the major components and the proportions of PK and MK-4 were very low. The composition of vitamin K was very similar between the stomach and intestinal contents of the same species of fish.

The PK and MK contents in food organisms are shown in Table 5. In *Euphausia pacifica*, the PK and MK levels were low, and its composition of vitamin K was similar to those of the GI tract contents of mackerel and saury with an exception of the low PK. On the other hand, in *Polychaeta spp.* MK-7 was the major vitamin K followed by MK-6 and MK-8, with a trace or negligible amount of PK. This composition resembled those of the GI tract contents of marbled sole and sillago. From these results it can be concluded that the compositions of vitamin K in food organisms and that of GI tract contents are similar to each other.

## Discussion

Mackerel and saury feed on plankton, small fish and copepoda (Usami, 1968). The vitamin K composition of the GI tract contents of saury was similar to that of, Pacific krill *Euphausia pacifica*, suggesting that vitamin K in the GI tract of saury came from their food. While in mackerel, the vitamin K composition of GI tract seems to be different from that of *Euphausia pacifica*. However, it can be regarded that significant difference in vitamin K composition is only PK between content of the GI tract and that of *Euphausia pacifica*, so that there may be another supplementary source for PK in the case of mackerel. On the other hand, marbled sole and sillago feed on benthos such as *Polychaeta spp.* (Maeda 1955; Maeda 1955; Omori 1974). The composition of vitamin K of the GI tract contents of these specimen were similar to that of *Polychaeta spp.* This also suggests that the vitamin K in the GI tract contents depends on their food. Therefore, it is possible that the vitamin K in the tissue of the fishes studied originated from their GI tract, because all forms of vitamin K present in GI tract were also detected in the tissue of a certain organ, though a slight difference was detected between the vitamin K composition of stomach and that of intestinal contents. It implies that fish cannot produce long chain MKs in the intestine on its own.

In the GI tract contents, MK-7 was one of the major vitamin K components in mackerel and sillago, however there was little MK-7 in the other tissues of these fishes. In the cases of mackerel and saury, their GI tract contents were rich in long chain MKs, but, in the tissue, only a little long chain Mks were detected. This suggests that all of vitamin K in the food was not transferred into the tissues of fish and there must be a selected absorption mechanism. It is known that MK-4 is conversion from PK at chick and rat tissues (Thijssen and Drittij-reijnders 1994; Will *et. al.* 1992). It is possible that Mk-4 in these fish tissues comes from PK which depends on their food.

In the liver of mackerel, the PK contents was outstanding. There was, however, not such a high level in the tissue of other fishes examined. There must be a possibility that vitamin K in different forms might be selectively absorbed and deposited in the tissue. Furthermore, the selection and degree of deposition may differ among the species of fish and/or different organs. Though the contents of

long chain MKs in the tissue of fish were not so high as those in the GI tract, it is characteristic of the kidney and liver to contain various forms of long chain MKs. These data suggest that vitamin K in fish was used in various forms, but the absorption rates may differ in the form of vitamin K.

### 1.2.2. Comparison of the composition of vitamin K in different forms between cultured and wild fish

Long chain MKs such as MK-7 and MK-9 are major forms of the utilizable vitamin K in mammals (Usui *et al.* 1989), but for fish the major utilizable forms are PK and MK-4. However, the utilizable form of vitamin K differs with feeding habits and fish species. In this section, ayu *Plecoglossus altivelis* was studied because it is an abundant, economically import and valuable freshwater fish. Wild ayu inhabit rivers and feed on diatoms. Ayu is also extensively cultured in Japan. The contents of PK and long chain MKs in the tissues were measured and the tissue distribution pattern between the wild and cultured fish was compared.

#### Materials and Methods

Wild ayu caught in the Chikuma river in Nagano Prefecture on 15 September 1993 were used for this study (body length: 17.5cm; body weight: 69.5g). Ayu at the larvae stage caught at Biwa Lake in Siga Prefecture were reared in an outdoor tank at the Ueda Station of National Research Institute of Fisheries Science in Nagano Prefecture, were used as the cultured fish sample. They were fed with a commercial diet containing MSB for 5 months. They were 14.5cm in length and 45.0g in weight. All of the fish were frozen-stored at −40℃ until use. Diatoms were collected from the surfaces of stones at the bottom of river where the wild ayu were caught. The PK and MKs contents were measured by the HPLC method in the same manner as section 1.2.1. MSB was analyzed according to the same method as section 1.1.1.

#### Result and Discussion

The PK and MKs contents in various tissues are illustrated in Fig. 6. In the tissues of wild ayu, PK appeared to be the major form of vitamin K, and MKs was present at very low levels. Long chain

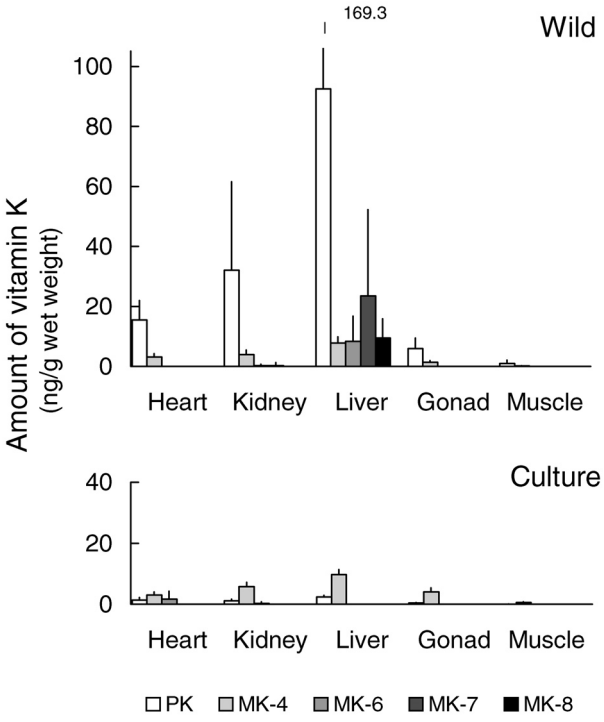



**Fig. 6.** Amount of phylloquinone and menaquinones in different tissues of wild and cultured ayu. Values are means ± SEM of five fish (cultured fish) and ten fish (wild fish). MK-9 was not detected in all tissues examined.

**Table 6.** Phylloquinone and menaquinones contents in feed consumed by ayu (ng/g)

| | PK | MK-4 | MK-6 | MK-7 | MK-8 | MK-9 | MSB |
|---|---|---|---|---|---|---|---|
| Diatom | 164 (38) | 31.8 (7) | 30.8 (7) | 137 (32) | 71.5 (16) | - | |
| Commercial diet | 38.6 | 10.2 | - | - | - | - | $1.2\times10^3$ |

MSB=menadion sodium bisulfite (Figures in parentheses are expressed as percentage.)

**Table 7.** Amount of different forms of vitamin K in the stomach and intestine of wild and cultured ayu

(ng/g wet weight)

| | PK | MK-4 | MK-6 | MK-7 | MK-8 | MK-9 |
|---|---|---|---|---|---|---|
| Wild | | | | | | |
| Stomach(0.22)* | 107.7 (62.1) | 8.2 (4.7) | 4.8 ( 2.8) | 32.7 (18.9) | 20.1 (11.6) | - |
| Intestine(0.34)* | 440.9 (79.3) | 6.9 (1.2) | 8.6 ( 1.6) | 74.9 (13.5) | 24.3 ( 4.4) | - |
| Cultured | | | | | | |
| Stomach(0.24)* | 80.1 (30.6) | 24.7 (9.5) | 98.7 (37.7) | 51.8 (19.8) | 6.4 ( 2.4) | - |
| Intestine(0.37)* | 22.6 ( 9.5) | 5.8 (2.4) | 120.8 (50.8) | 65.5 (27.5) | 23.2 ( 9.8) | - |

(Figures in parentheses are expressed as percentage of total vitamin K in each specimen.)
*= weight/body weight (percentage).

MKs in the different forms were present in the liver, but their quantities were lesser than that of PK. Only MK-6 was detected in tissues other than the liver. On the other hand, in the tissues of cultured ayu, the major form of vitamin K was MK-4, while MK-6 was detected only in the heart and kidney. Unlike the wild ayu, no long chain MKs were observed in the liver.

The PK and MKs contents in the feed given to ayu are shown in Table 6. PK is abundant in diatoms. In the gastrointestinal (GI) tract content of wild ayu fed with diatoms, the PK content accounted for 60-80% of total vitamin K (Table 7). It is, therefore, certain that the PK in the GI tract originated from diatoms in the diet. As for cultured ayu, long chain MKs was the major form of vitamin K in the GI tract. However, MSB could not be detected in the GI tract. This is because MSB which was added to commercial feed, a water soluble form, cannot be detected by this HPLC method. It was reported that the alkylation of MD to MK-4 in the liver differs between chick and rat liver (Dialameh *et al.* 1970, 1971). In the ayu, this conversion process is thought to be active resulting in high levels of MK-4 in the tissues.

In each group of fish, large quantities of long chain MKs, which were considered to be of bacterial origin, were detected in the contents of GI tract (Table 7). However, only small amounts were found in the tissues (Fig. 6). This suggests negligible absorption of long chain MKs into the tissues of the fish. Further studies are required to prove the existence of MK-producing bacteria in the GI tract and the low utilization of their by-products. From this observation, it is probable that MK-producing bacteria could not be a major source of vitamin K in the ayu. These findings also suggest that the extent of absorption and/or conversion of vitamin K in the tissues of the ayu varies with the different forms of vitamin K.

In the liver, most of the vitamin K present was in the form of PK in the wild ayu and MK-4 in the cultured ayu (Fig. 6). The quantity of PK in the wild ayu was about 10 times higher than that of MK-4 in the cultured ayu. This observation is similar to those results for sardine and mackerel. The reason why PK is accumulated at a much higher concentration as compared with MK-4 has yet to be elucidated. However, since evidence has been reported that mice and chickens have the ability to convert PK into MK-4 (Thijssen and Drittij-Rejinders 1994; Thijssen *et al.* 1996; Will *et al.* 1992), the fact, the much higher concentration of PK and low concentration of MK-4 in wild ayu, may be attributed to the weak conversion ability in ayu.

### Chapter 2. Absorption of vitamin K in fish

The dietary necessity and physiological roles of vitamin K are fairly well established for mammals (Dan 1935) and its importance in bone health has been recently a focus for research (Vermeer *et al.* 1996). However, it is still not clear whether dietary supplementation of this vitamin is indispensable for the growth of fish because of contradictory results have been reported between fish species (Poston

1964; Kitamura *et al.* 1967; Murai and Andrews 1977; Taveekijakarn *et al.* 1996).

PK, MK and MD are known to be converted partly into MK-4 in the tissues of calves (Nestor and Conrad 1990). Similarly, PK has been shown to be converted into MK-4 in mice and chickens (Will *et al.* 1992), and MD into MK-4 in cod (Grahl-madsen and Lie 1997). The distribution pattern of vitamin K in the tissues of fish has been reported to differ with the feeding habit among fish species and the PK content is usually much higher than MK in wild fish. The PK content in the gastrointestinal tract is high, then always high PK level is detected in the liver, while even though relatively large amounts of MKs are found in the gastrointestinal level, not all forms of MKs are detected in fish. However, the mechanism and rate of absorption of the various vitamin K groups have not been clarified yet in fishes.

This chapter describes the concentrations of the vitamin K derivatives in the tissues of males and females mummichog after feeding diets supplemented with different forms of vitamin K as a first step to elucidate the physiological role of vitamin K in fish.

## Materials and Methods

Mummichog *Fundulus heteroclitus* were fed a

**Table 8.** Composition of the experimental diet

| | Diet | | | | | | |
|---|---|---|---|---|---|---|---|
| Experimental group | No. 1 Group 1 | No. 2 Group 2 | No. 3 Group 3 | No. 4 Group 4 | No. 5 Group 5 | No. 6 Group 6 | No. 7 Group 7 |
| Ingredient (g / 100g) | | | | | | | |
| Vitamin-free casein*[1] | 30 | 30 | 30 | 30 | 30 | 30 | 30 |
| Gelatin*[2] | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| Dextrin | 30 | 30 | 30 | 30 | 30 | 30 | 30 |
| Feed oil*[3] | 8 | 8 | 8 | 8 | 8 | 8 | 8 |
| Mineral mix*[4] | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Vitamin mix*[5] | 4 | 4 | 4 | 4 | 4 | 4 | 4 |
| Calcium lactate | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 | 0.08 |
| Sodium phosphate, monobasic | 0.48 | 0.48 | 0.48 | 0.48 | 0.48 | 0.48 | 0.48 |
| CMC*[6] | 5 | 5 | 5 | 5 | 5 | 5 | 5 |
| Cellulose | 13.44 | 13.44 | 13.44 | 13.44 | 13.44 | 13.44 | 13.44 |
| Vitamin K (mg / kg) | | | | | | | |
| Phylloquinone | - | 1 | - | - | - | - | - |
| Menaquinone-4 | - | - | 1 | - | - | - | - |
| Menaquinone-6 | - | - | - | 1 | - | - | - |
| Menaquinone-7 | - | - | - | - | 1 | - | - |
| Menaquinone-8 | - | - | - | - | - | 1 | - |
| Menaquinone-9 | - | - | - | - | - | - | 1 |

*[1]: (Lot no. ECF7046) Wako Pure Chemical Industries (Osaka, Japan).

*[2]: DIFCO Laboratories (Detroit, USA).

*[3]: (Lot no. SNo. J-450) Riken Vitamin Company, Ltd. (Tokyo, Japan).

*[4]: Mc.Collum Salt 820517 Iwai Chemical Co., Ltd. (Tokyo, Japan).

*[5]: The premix reported by the National Research Council was partly modified as follows (mg / 4 g premix): thiamin HCl 5, riboflavin 20, pyridoxine HCl 5, choline chloride 500, nicotinic acid 75, ca-pantothenate 50, inositol 200, biotin 0.5, folic acid 1.5, ascorbic acid 100, alpha-tocopherol 40, vitamin $B_{12}$ 0.01, activated 7-dehydro-cholesterol 200IU, retinol 3200IU.

*[6]: Carboxymethylcellulose-Na.

commercial diet for common carp in an outdoor tank of 4.5m$^3$ (sea water depth: 1.5m). All the experimental fish were acclimated to the experimental conditions described below by feeding the vitamin K deficient diet for three weeks prior to the start of the experiment. The experimental fish (average body weight of 18.1g) were divided into seven groups of 3 pairs each. Each group was transferred to respective 20L glass aquariums placed in the laboratory and supplied with running natural seawater. The experimental fish were reared in a 3mm mesh screen cage placed in the aquarium so that they were unable to eat their own feces and eggs. The water temperature was kept at 20℃ and the laboratory was lit with fluorescent lights for 13 hours from 7: 00 throughout the experimental period.

The basal diet was designed according to Murai *et al.* (1984). Fish in each group were fed an experimental diet respectively supplemented with different vitamin K derivatives (Table 8). No. 1: a vitamin K deficient diet; Nos. 2 to 7: supplemented with PK, MK-4, -6, -7, -8 and -9 at a ratio of 1mg/kg, respectively. Experimental diets were given at 2% of the body weight per day for 12 days.

Approximately 3 hours after the final feeding, samples of blood, kidney, liver, gonad and gastrointestinal tract were collected from individual fish to analyze the content of vitamin K derivatives in each specimen.

The PK and MKs contents were measured by the HPLC method in the same manner as section 1.2.1.

## Results

The contents of PK and MKs in the gastrointesti-

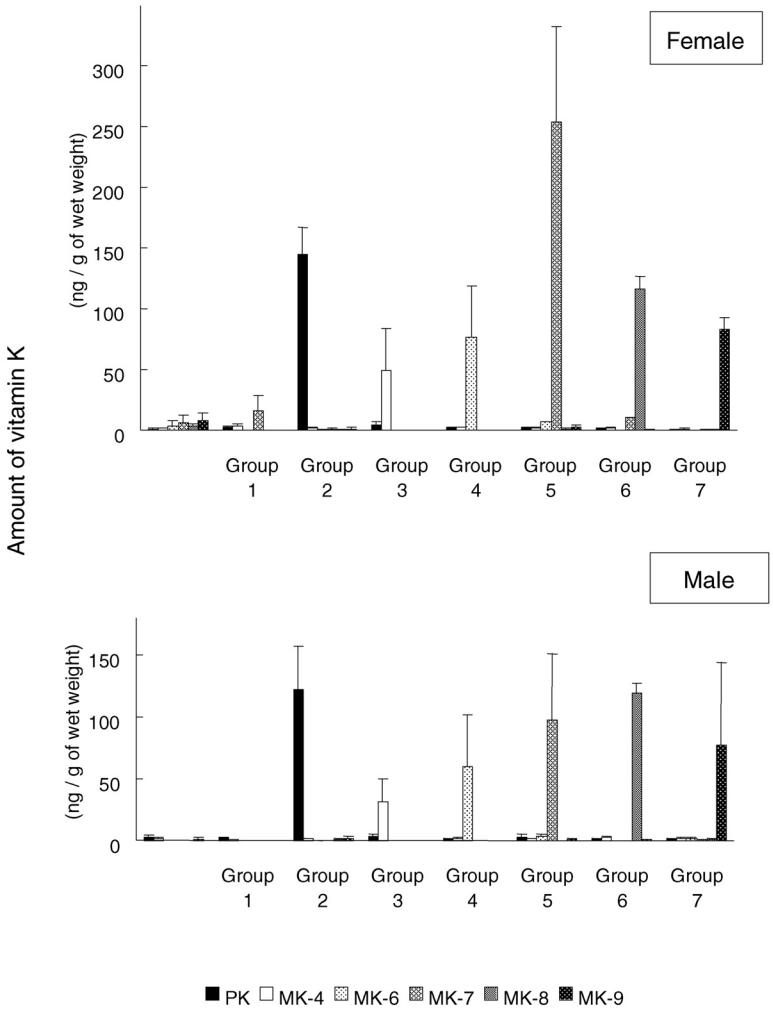



**Fig. 7.** Phylloquinone and menaquinone contents in the gastrointestinal tract contents of the each group of fish. Values are means ± SEM of 3 fish.

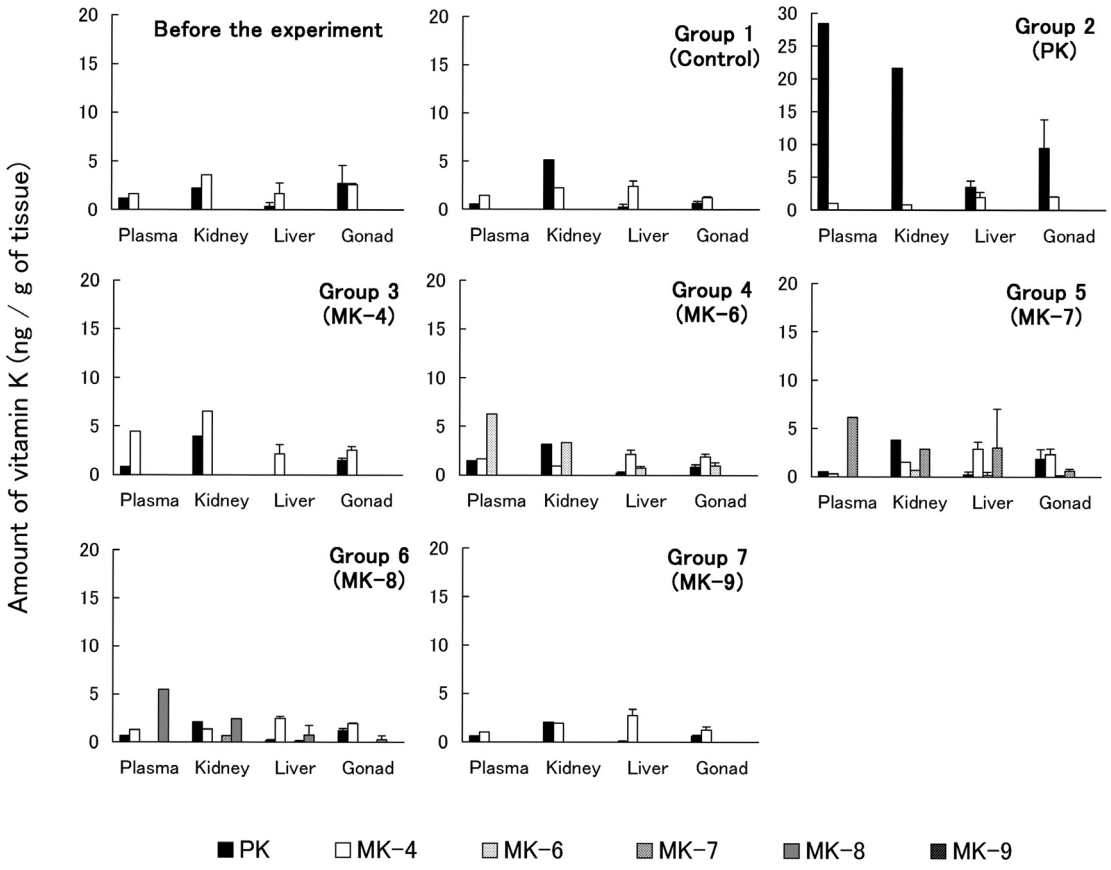



**Fig. 8.** Phylloquinone and menaquinone contents in four different tissues of female mummichog of each group of fish. Control fish (no vitamin K added) and each vitamin K added to the experimental diet. Values are means ± SEM of 3 fish.

nal tract are shown in Fig. 7, which were collected at about 3 hours after the final feeding of the respective diets. The contents of the various vitamin K derivatives reflected almost exclusively the dietary supplements even though there were some difference in their levels. These levels were somewhat higher in females than in males, especially for Group 5. Also, a small but significant quantity of MK-7 was detected in the females only of Group 1 although both females and males of this group were fed the vitamin K free diet for 33 days including the acclimatization period.

The concentrations of PK and MKs in the plasma, kidney, liver and gonad are shown in Fig. 8 for females and Fig. 9 for males. In both females and males, feeding of the vitamin K free diet for an additional 12 days (control group) made no change in the concentrations of vitamin K in the plasma and tissues which consisted only of PK and MK-4. The plasma levels of the various vitamin K derivatives which are a certain indication of absorption from the intestine, differed among the vitamin K fed, and between females and males. The plasma level of PK in Group 2 fed the PK supplemented diet was 30ng/ml in females but only about 1/3 of that value in males. Values of the MK derivatives were much lower than the PK value not only in males but also in females. On the whole, the values for females were much higher than the corresponding values for males. MK-9 having the longest side chain was not detected at all in the plasma.

PK was accumulated in the kidney, liver and gonad of fish fed the PK-supplemented diet in both females and males. Reflecting the plasma level, the PK concentration in the kidney of females was much higher than males, but this was not true for the liver and gonad. In case of males, contents of MK-4 in the liver were highest even in the groups fed the other

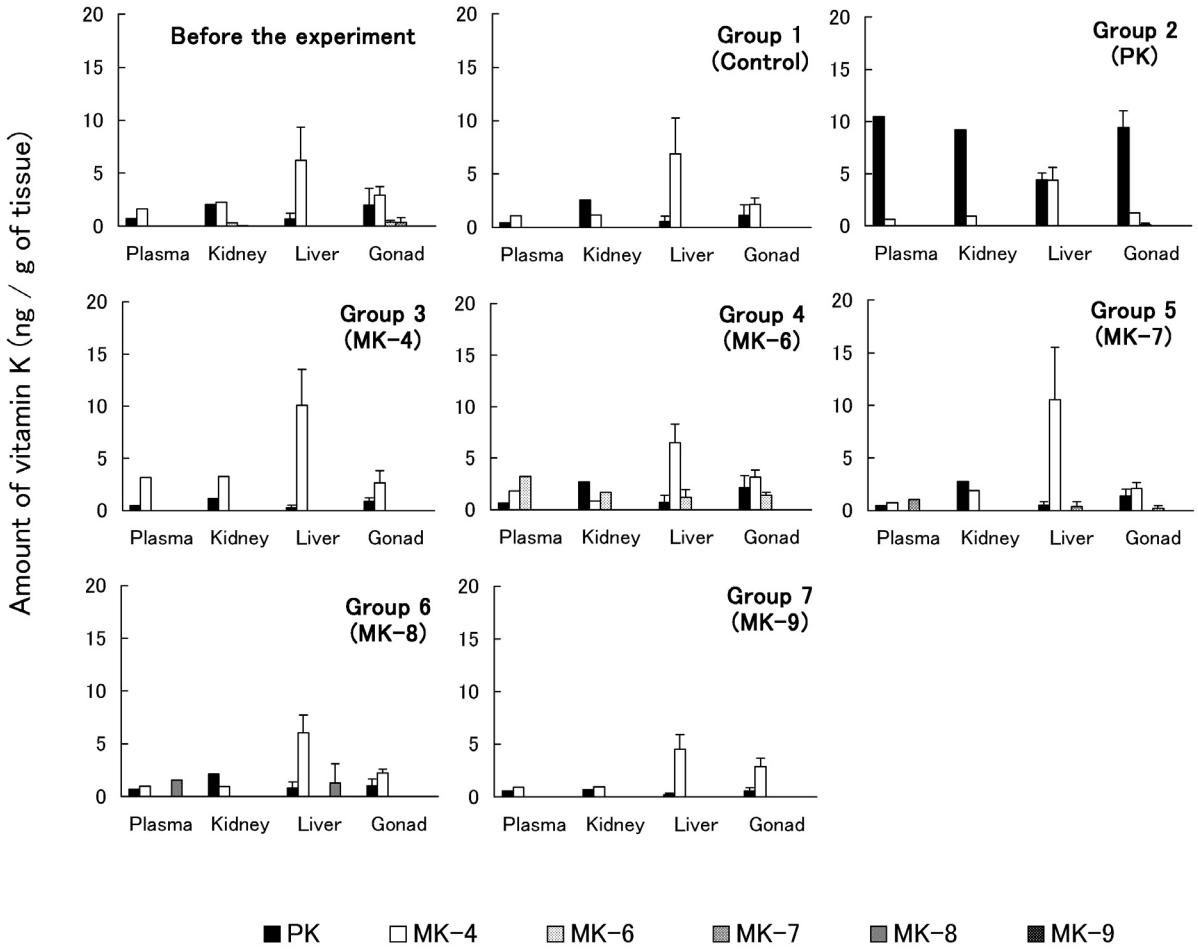



**Fig. 9.** Phylloquinone and menaquinone contents in four different tissues of male mummichog of each group of fish. Control fish (no vitamin K added) and each vitamin K added to the experimental diet. Values are means ± SEM of 3 fish.

derivatives. While, MK-6, MK-7, and MK-8 were more clearly detected in the tissues of females than in males. However, MK-9 was not detected at all in any tissues examined.

PK and MKs which were not supplemented in the diet were detected in the tissues of almost every dietary groups at similar levels as prior to the experiment and of the control group (Group 1).

## Discussion

As shown in Fig. 7, the major vitamin K in the gastrointestinal tracts of each experimental group was that administered in the feed to the respective group and only minute amounts of the other homologues were recognized in each group. This clearly indicates that the major vitamin K source is almost entirely of dietary origin.

The fish were found to contain very small amounts of PK and MK-4 in the plasma at the beginning of the experiment. Because the experimental fish were fed a commercial diet with MSB until the start of feeding the experimental diets, the small amount of MK-4 detected in all groups should be taken into account. The plasma levels of vitamin K derivatives shown Figs. 8 and 9 imply that PK is the most efficiently absorbed into the blood in both females and males because its level was much higher than those of the MK derivatives. This result agrees well with Birgit and Suttie (1992) who reported that the PK level in the plasma of rats fed a PK-supplemented feed was about three times higher than rats fed a MK-9 supplemented feed. Ichihashi *et al.* (1992) described that the absorption

rates of MKs decrease markedly with an increase in the number of isoprenoid units in rats. Akiyama *et al.* pointed out that the number of isoprene units of MK is an important factor in its absorption and incorporation into the liver in rats (1995). No clear relationship between the number of isoprene units and absorption was detected in this study. However, the side chain of MK-9 may be too long for mummichog to absorb it into the blood, because MK-9 was not detected at all in the plasma of both females and males.

As to the difference in the effectiveness in vitamin K activity, Groenen-van Dooren *et al.* observed that MK-9 was definitely more active than PK and MK-4 in rats (1995). While Thijssen reported that MK-4 rather than PK may be the functional vitamin in rats (1996). From the viewpoint of the effective accumulation of vitamin K in the tissues, PK can be considered to be a more available vitamin K in fish than any of the MK homologues and MD, differing from the case of rats. This view may be supported by the fact that the major vitamin K detected in the body of fish is generally PK. Therefore, further studies on the differences in the mechanism of absorption of PK and MKs are needed with respect to the supplementation of commercial feed for fish culture with vitamin K.

Judging from the vitamin K derivatives in the tissues, PK and MK-4 may play a major role in males as has been described at Chapter 1 in wild fishes. However, not only PK but also other MKs except MK-9 may play a certain important role in females. There were also apparent differences in the absorption of PK and MK-4, and in the accumulation of MKs in the tissues, especially MK-4 in the liver between females and males.

## Chapter 3. Physiological function of vitamin K in fish

Vitamin K is well known for its participation in blood coagulation. Further, a variety of vitamin K deficiency diseases have been reported for mammals. In particular, human infants of a few weeks old are said to be exposed to a risk of hemorrhage due to vitamin K deficiency (Greason and Kerr 1989). Recently, the importance of vitamin K to bone health has also been emphasized (Vermeer *et al.* 1996).

There are some reports on vitamin K requirements for fish. As for rainbow trout and catfish, no perceivable syndrome of vitamin K deficiency was observed (Kitamura *et al.* 1967; Murai and Andrews 1977). On the other hand, Taveekijakarn *et al.* (1996) reported that mortality in vitamin K deficient fish, amago salmon, reached to about 50%.

From these researches on the role of vitamin K in fish it is difficult to conclude categorically whether vitamin K is essential for fish. In this chapter, experiment was undertaken to determine the vitamin K requirements of fish and to examine if there is any change in the distribution and/or the content of vitamin K in the organs, including eggs of fish fed with a vitamin K deficient diet for a long term spawning stage.

### 3.1. Physiological abnormality and the tissue vitamin K level in the fish fed with a vitamin K free diet

Mummichog *Fundulus heteroclitus* which is widely distributed along the eastern coast of North America, were chosen as the experimental fish, as this species is relatively easy to rear and the spawning season is long lasting. This section describes the experimental results of the change in vitamin K content in different organs of fish fed with a vitamin K supplemented or deficient diet for 11 weeks. Microscopic observations on the tissues of the fish are also described.

Since MSB, which has been proven to be converted into MK-4 after intake, is generally supplemented in the commercial diet of cultured fish, MSB was used for a control diet in this study.

#### Materials and Methods

##### Experimental fish and diet

Mummichog were fed a commercial diet in an outdoor-tank of 4.5m$^3$ (sea water depth: 1.5m). All fish were acclimated to the experimental conditions as follows for three weeks. The experimental fish were divided into three groups of 18 pairs each. Each group was transferred to respective 60L glass aquariums filled with running natural sea water

**Table 9.** Percentage composition of experimental diet

| Ingredient | Diet | | |
|---|---|---|---|
| | No. 1 | No. 2 | No. 3 |
| Vitamin-free casein*[1] | 30 | 30 | 30 |
| Gelatin*[2] | 5 | 5 | 5 |
| Dextrin | 30 | 30 | 30 |
| Feed oil*[3] | 8 | 8 | 8 |
| Mineral mix*[4] | 4 | 4 | 4 |
| Vitamin mix*[5] | 4 | 4 | 4 |
| Calcium lactate | 0.08 | 0.08 | 0.08 |
| Sodium phosphate, monobasic | 0.48 | 0.48 | 0.48 |
| CMC*[6] | 5 | 5 | 5 |
| Cellulose | 13.44 | 13.44 | 13.44 |
| MSB*[7] | 0.0025 | 0 | 0 |
| Sulfaguanidine | 0 | 0 | 0.1 |

*[1]: (Lot no. ECF7046) Wako Pure Chemical Industries (Osaka, Japan).
*[2]: DIFCO Laboratories (Detroit, USA).
*[3]: (Lot no. SNo.J-450) Riken Vitamin Company, Ltd. (Tokyo, Japan).
*[4]: Mc.Collum Salt 820517 Iwai Chemical Co., Ltd. (Tokyo, Japan).
*[5]: The premix reported by National Research Council was partly modified as follows (mg / 4.5 g premix): thiamin HCl 5, riboflavin 20, pyridoxine HCl 5, choline chloride 500, nicotinic acid 75, ca-pantothenate 50, inositol 200, biotin 0.5, folic acid 1.5, ascorbic acid 100, alpha-tocopherol 40, vitamin $B_{12}$ 0.01, activated 7-dehydro-cholesterol 200IU, retinol 3200IU.
*[6]: Carboxymethylcellulose-Na.
*[7]: Menadione sodium bisulfite.

in the laboratory. The fish in respective groups were referred to as Groups 1, 2 and 3. The basic diet was designed according to Murai *et al.* (1984). The composition of the diet is given in Table 9. Group 1 was fed with the control diet (No. 1) which was supplemented with MSB. Group 2 was fed with the vitamin K free diet (No. 2). Group 3 was given another vitamin K free diet (No. 3) to which sulfaguanidine (SG) was added to eliminate the possibility of any effect of vitamin K production by intestinal bacteria. All of the fish were fed five times a day on 2.5% of body weight with an automatic feeder for 11 weeks. The fish were kept at 22℃ with 14 h of light per day (6:00 to 20:00). Experimental fish were reared in a 3-mm mesh screen cage placed in the aquarium so that they did not eat their own feces and eggs. The inner wall of each aquarium was carefully cleaned three times a week. On all such occasions, fish were transferred within the screen cage to another aquarium, and naturally spawned fertilized eggs that sank through the screen to the aquarium bottom were collected once a week with a fine mesh net. The experiment started on June 6, 1995. After 11 weeks of feeding, five fish from each group were killed to measure the vitamin K levels in the heart, kidney, gonad, liver, muscle, gill and plasma, and for another five fish kidneys, livers, spleens were histologically examined. The eggs were transferred in a fine net to a separate tank (area: 60×40cm, depth: 10cm) with running sea water. Larvae (about 200) were fed with the same diet as that given to their parent fish beginning immediately after hatching. They were reared in a 5L aquarium with running natural sea water. The mortality of experimental fish after 4 weeks of feeding was examined. Triplicated

**Table 10.** Effect of dietary MSB on the growth, condition factor and mortality of mummichog

| Group | Sex | Body weight (g) Initial*2 | Body weight (g) 11 weeks | Length (mm) 11 weeks | Condition factor | Growth*1 rate (%) | Mortality (%) |
|---|---|---|---|---|---|---|---|
| 1 | female | 11.47 | 13.28[a] | 81.41[ab] | 2.44[a] | 1.16[a] | 16[a] |
| | male | 11.47 | 10.94[b] | 78.81[b] | 2.21[b] | 0.95[b] | 11[a] |
| 2 | female | 10.50 | 11.51[ab] | 77.94[b] | 2.44[a] | 1.10[a] | 16[a] |
| | male | 10.50 | - | - | - | - | 100[b] |
| 3 | female | 11.47 | 13.25[a] | 83.72[a] | 2.23[ab] | 1.16[a] | 26[a] |
| | male | 11.47 | - | - | - | - | 94[b] |

Values in the same column with different superscripts are significantly different ($p<0.05$).
*1: body weight (11 weeks) / Initial body weight.
*2: The fish were pooled and weighed.

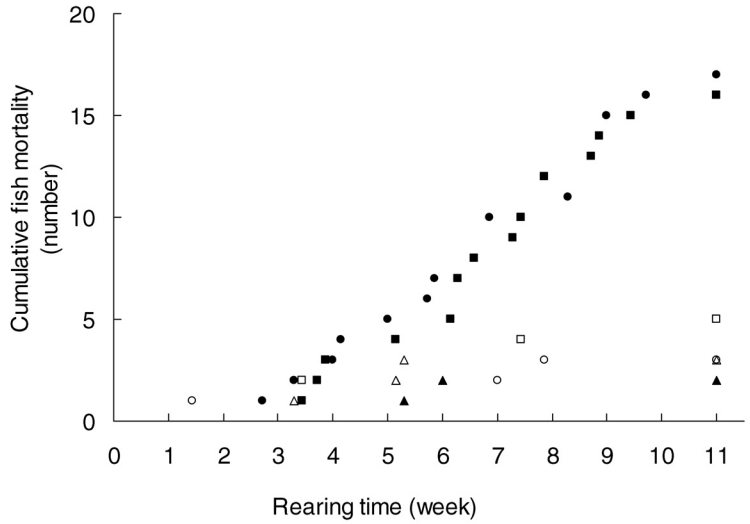



**Fig. 10.** Cumulative fish mortality of the three groups.
Open and solid symbols represent female and male fish, respectively. Triangle (△, ▲), control fish fed with the No. 1 diet which was supplemented with MSB; Circle (○, ●), experimental fish fed with No. 2 diet which is MSB free; Square (□, ■), experimental fish fed with No. 3 diet which is MSB free containing sulfaguanidine.

sampling was carried out on eggs and larvae to examine the hatching rate and mortality of the larvae.

### Vitamin K Analysis

PK and MK were measured by the same method described in Section 1.2.1. In the case of spawned egg and larvae specimens, 100 individuals of each were pooled and homogenized.

### Histological Observations

The kidney, liver and spleen excised from the experimental fish were fixed with Bouin's fluid and embedded in paraffin. Serial sections 5 $\mu$m thick were stained with hematoxylin and eosin.

### Statistical Analysis

Data for the gonads of female fish at the beginning of the experiment and those in Group 1 were analyzed by the Wilcoxon rank sum test, the other

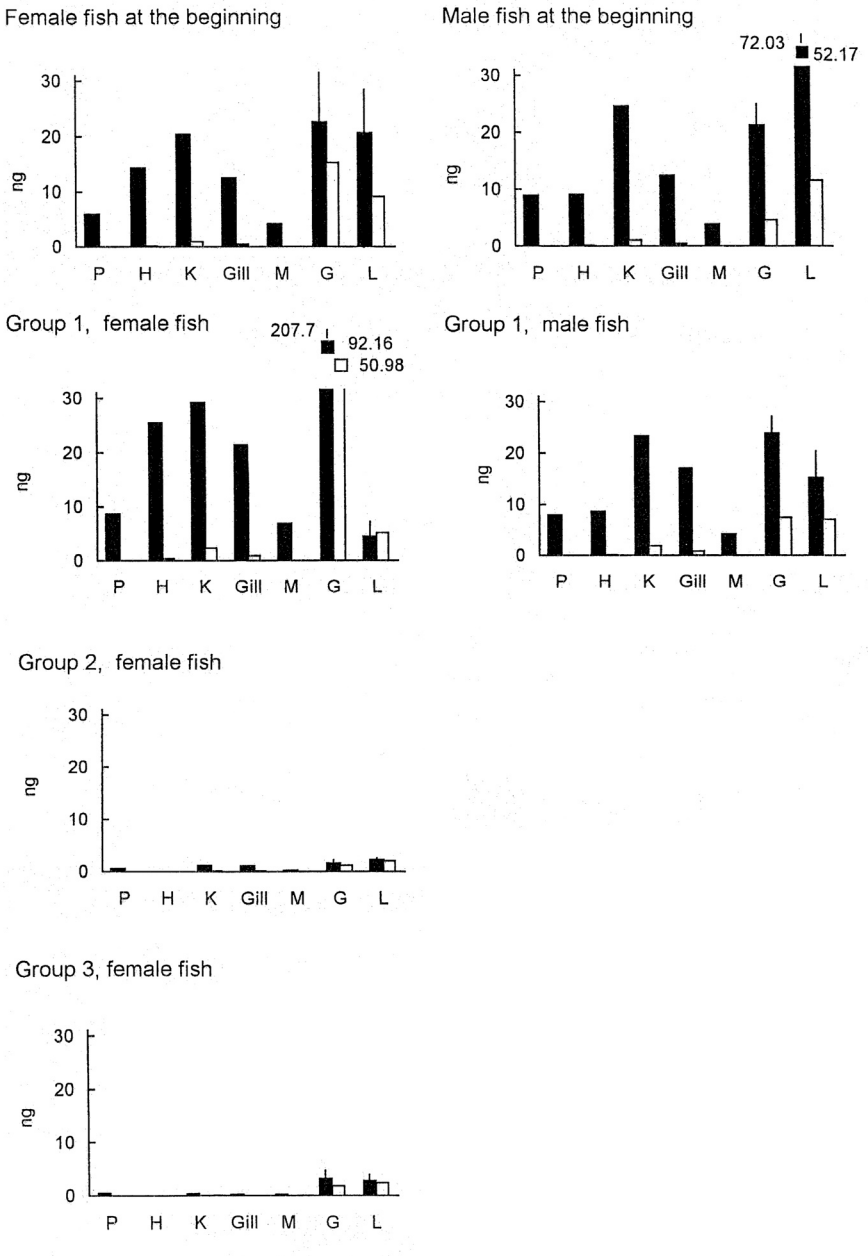



**Fig. 11.** Menaquinone-4 (MK-4) contents in various tissues of mummichog.
P, plasma; H, heart; K, kidney; M, muscle; G, gonad; L, liver. Values for the gonad and liver are means ± SEM of 5 individuals. Other tissues were measured using pooled samples of the 5 fish. Solid column, ng per g of tissue; open column, ng per whole body.

data by Duncan's multiple range, multiple *F*-test (Duncan 1955).

## Results

There was a striking contrast in mortality between the control (Group 1) and experimental fish (Groups 2 and 3) as shown in Table 10. In Groups 2 and 3, most male fish died. There was no such great difference in the female fish mortality. No significant difference was observed in the growth rate of female fish among the 3 groups. Figure 10 shows the cumulative number of fish that died during the experimental period. In Group 2, the first male fish died on day 20, and by the 11th week all the male fish had died. For the fish fed with the

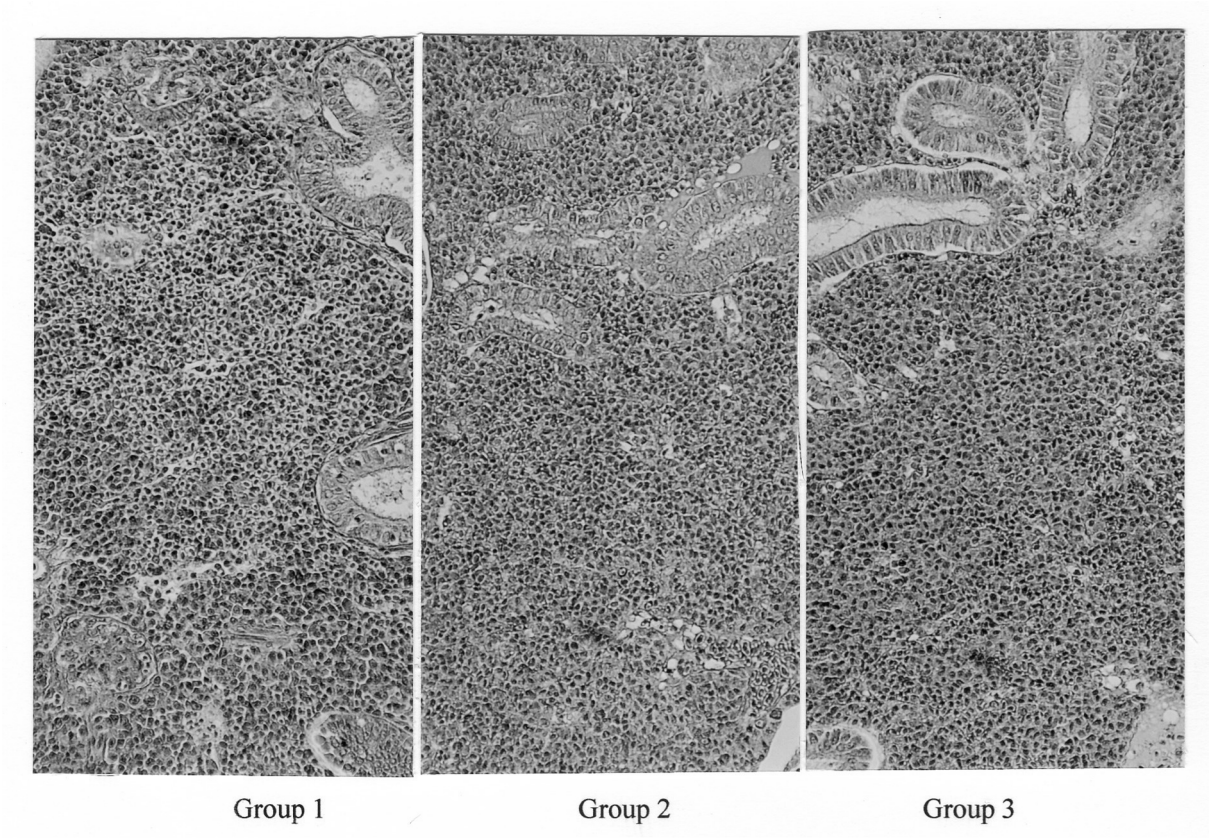



**Fig. 12.** Effects of vitamin K deficient diets on the kidney of a female mummichog (hematoxylin and eosin), showing a large number of erythrocytes including immature cells.
Group 1, fed with control diet; Group 2, fed with vitamin K deficient diet; Group 3, fed with vitamin K deficient diet supplemented with sulfaguanidine. Magnification: ×66.

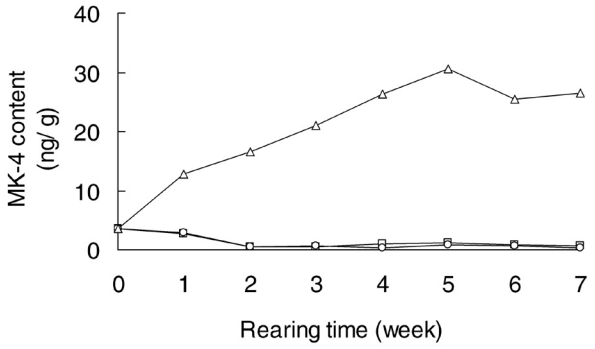



**Fig. 13.** Changes in menaquinone-4 (MK-4) contents in the eggs.
Triangle (△), egg spawned by the fish fed with a MSB supplemented diet; circle (○), eggs spawned by the fish fed with vitamin K free diet; square (□), eggs spawned by the fish fed with a sulfaguanidine supplemented diet. All eggs were pooled for analysis.

No. 3 feed (Group 3), male fish also died frequently as in the case of fish fed with the No. 2 feed (Group 2), and only one male survived after the 11 weeks of experimental feeding with the vitamin K free diet. However, in the case of female fish, there was no significant difference in the mortality between the control (Group 1) and the experimental fish (Groups 2 and 3).

The MK-4 content in various tissues of the fish at the beginning of the experiment and for female fish after 11 weeks of feeding are summarized in Fig. 11. The MK-4 contents were markedly higher in the gonad and liver of the fish at the beginning of the experiment. In Group 1, the MK-4 content increased to some extent in most parts of the body, and markedly in the gonad, within the 11 weeks of feeding. However, in the liver, the content decreased significantly. The MK-4 content in the fish in Group 2 or 3 decreased to about one tenth

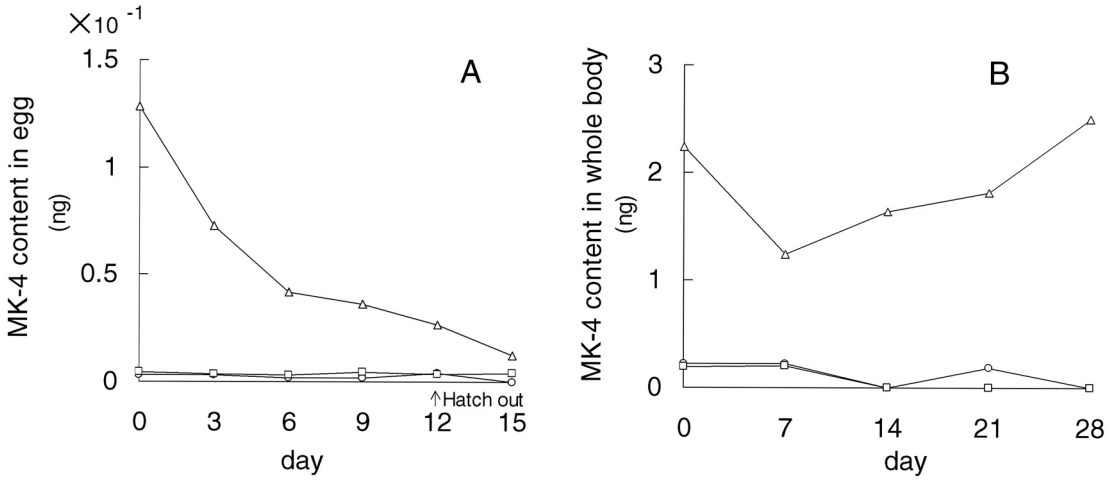



**Fig. 14.** A: Changes in the content of menaquinone-4 (MK-4) in the eggs of the three groups of fish during development. B: Changes in the content of MK-4 in the larvae from the three groups of fish.
The larvae were fed with the same feed as that for their parents. Triangle (△), egg or larvae from the fish fed with a MSB supplemented diet (No. 1); circle (○), egg or larvae from the fish fed with a vitamin K free diet (No. 2); square (□), egg or larvae from the fish fed with a sulfaguanidine supplemented diet (No. 3)

**Table 11.** Hatching rate and mortality of the three groups of mummichog larvae*

| Diet | No.1 | No.2 | No.3 |
|---|---|---|---|
| Hatching rate | 59.7% | 61.1% | 72.2% |
| Mortality | 27.8% | 30.3% | 20.6% |

*All figures are the average value of triplicate experiments.

or less of the initial level. There was, however, no essential difference in the levels between these two groups of fish. Analysis of MK-4 for male fish in Groups 2 and 3 was not possible, because almost all of the male fish has died during the 11 week experiment. The reason for the extremely high content in the liver of the male fish at the beginning of the experiment can not be presently explained. PK was not detectable in every tissue.

According to microscopic observation of the tissues, abnormality was observed only in the kidney of fish in Groups 2 and 3 fed with the vitamin K free diets. A number of erythroblasts existed in the kidney of the fish in Groups 2 and 3. Particularly, many more erythroblasts were observed in Group 3 fish (Fig. 12).

The effect of vitamin K deficiency of the mother fish on the eggs and larvae, i.e. the hatching rate and the mortality of larvae, was investigated. Firstly, the relationship between egg MK-4 content and the duration of feeding of the mother fish fed with the experimental diet (No. 2 or No. 3) is shown in Fig. 13. The MK-4 content in eggs produced by the experimental fish (Groups 2 and 3) decreased to almost zero within the first 2 weeks, while the content in the eggs of Group 1 (control fish) rose steadily from the beginning of feeding, and reached a maximum level after 5 weeks.

Secondly, using eggs collected between the 6th and 7th week in the feeding experiment, the effects of MK-4 content of the eggs on the hatching rate and mortality of the larvae were examined. The content change of MK-4 in eggs and larvae are shown in Fig. 14 and B, respectively. MK-4 contents in both eggs and larvae from Groups 2 and 3 were very low throughout the egg development period and subsequent feeding period, respectively. On the other hand, MK-4 content in eggs spawned by

Group 1 fish decreased markedly from the beginning of development (Fig. 14). As for the larvae of Group 1 fish, the content became high after the first feeding (Fig. 14). However, there was no significant difference in the hatching rate and mortality of larvae among the three experimental groups (Table 11).

## Discussion

The MK-4 content in fish fed with a vitamin K free diet decreased markedly during the 11 weeks of feeding (Fig. 11). This indicates that MK-4 in the control fish (Group 1) is derived from MSB in their feed. There was no significant difference in the MK content or mortality between the experimental group of fish fed with the SG added diet and the non SG diet, indicating that MK-long chain, if produced by intestinal bacteria in the fish in Group 2, does not influence the tissue levels of vitamin K.

In Group 1, the MK-4 content decreased significantly ($p<0.05$) in the liver of male and female fish but increased significantly in the gonad of female fish ($p<0.05$). However, no such apparent change in the MK-4 content was observed in other tissues. The reason for the marked decrease in the liver and marked increase in the gonad of the fish in Group 1 can not be presently explained. The fact that the MK-4 content in eggs decreased to a low level soon after the fish were fed with a vitamin K free diet (Fig. 13) may be related to the marked decrease in the gonadal levels (Fig. 11). Male fish began to die about 20 days after the start of the feeding experiment, seemingly coincident with the decrease of MK-4 levels in the tissue.

That a large number of immature cells were observed in the kidney of the fish fed with a vitamin K free diet suggests a high incidence of hematoporia and/or hematopoiesis in the renal tissue. Since hematopoiesis in fish is considered to occur mainly in the spleen and kidney (Branson 1993), we suggest that the vitamin K free diet brought about some changes in the level of vitamin K in the blood, and resulted in the hematopoiesis in the kidney.

The reason why only male fish died when fed with the vitamin K free diet is a matter for question. Since it is known that the hepatosomatic index of female fish is generally two to three times higher than that of male fish, and that the liver becomes extremely active in the spawning season (Tamura 1977), the death of male fish might be related to such factors. On the other hand, hatchability and mortality of larvae in this study were not affected by vitamin K deficiency. Therefore, it does not seem that vitamin K deficiency affects the growth of larvae, at least that of mummichog. It may have an effect on the physiological functions of the fish only during the spawning season. These results may suggest that mummichog requires a high level of vitamin K especialy in spawning season. However, further detailed study on the cause of death in male mummichog is needed.

### 3.2. The effect of dietary vitamin K (phylloquinone and menadione) levels on the vertebral formation in fish

The participation of vitamin K in fish bone development has been investigated little. One of the purpose of this section is to elucidate the effect of vitamin K deficiency on bone development in fish, using mummichog *Fundulus heteroclitus*, as an experimental animal.

A variety of naturally occurring vitamin K compounds are known. Furthermore, synthetic vitamin K exists and there are many different derivatives. They have the same naphthoquinone structure but different aliphatic side chains. MD is generally used for fish feeds as a vitamin K supplementary agent in the form of a water-soluble sodium bisulfite derivative (MSB). MD, a synthetic product of vitamin K, is not biologically active itself. The majority of MD taken in is easily excreted in rat (Losito *et al.* 1968), and only a part becomes effective after being converted into an active form, MK-4, in the liver in rat (Taggart and Matschiner 1969) and in cod (Grahl-Madsen and Lie 1997). Hence, in practice, MSB is added to feed in excess. However, it has been reported that MD and its analogues are toxic to certain animals (mice, rats, horses and humans) and cause abnormalities in the liver, kidneys and lungs, as well as hemorrhage, hemolytic anemia and other physiological abnormalities (Shimkin 1941; Smith *et*

**Table 12.** Composition of experimental diet

| Component | Diet | | | | |
|---|---|---|---|---|---|
| | Control | MSB25 | MSB2500 | PK1 | PK100 |
| Ingredient (g / kg) | | | | | |
| Vitamin-free casein*[1] | 300 | 300 | 300 | 300 | 300 |
| Gelatin*[2] | 50 | 50 | 50 | 50 | 50 |
| Dextrin | 300 | 300 | 300 | 300 | 300 |
| Feed oil*[3] | 80 | 80 | 80 | 80 | 80 |
| Mineral mix*[4] | 40 | 40 | 40 | 40 | 40 |
| Vitamin mix*[5] | 40 | 40 | 40 | 40 | 40 |
| Calcium lactate | 0.8 | 0.8 | 0.8 | 0.8 | 0.8 |
| Sodium phosphate, monobasic | 4.8 | 4.8 | 4.8 | 4.8 | 4.8 |
| CMC*[6] | 50 | 50 | 50 | 50 | 50 |
| Cellulose | 134.4 | 134.4 | 134.4 | 134.4 | 134.4 |
| Vitamin K (mg / kg) | | | | | |
| MSB*[7] | 0 | 25 | 2500 | 0 | 0 |
| Phylloquinone | 0 | 0 | 0 | 1 | 100 |

*[1]: (Lot no. ECF7046) Wako Pure Chemical Industries (Osaka, Japan).
*[2]: DIFCO Laboratories (Detroit, USA).
*[3]: (Lot no. SNo.J-450) Riken Vitamin Company, Ltd. (Tokyo, Japan).
*[4]: Mc.Collum Salt 820517 Iwai Chemical Co., Ltd. (Tokyo, Japan).
*[5]: The premix reported by National Research Council was partly modified as follows (mg / 4 g premix): thiamin HCl 5, riboflavin 20, pyridoxine HCl 5, choline chloride 500, nicotinic acid 75, ca-pantothenate 50, inositol 200, biotin 0.5, folic acid 1.5, ascorbic acid 100, alpha-tocopherol 40, vitamin $B_{12}$ 0.01, activated 7-dehydro-cholesterol 200IU, retinol 3200IU.
*[6]: Carboxymethylcellulose-Na.
*[7]: Menadione sodium bisulfite.

*al.* 1943; Owens 1971; Rebhem *et al.* 1984). In nature, fish ingest PK and/or MK-4 as the vitamin K source and it is also known that PK and MK-4, particularly PK, are accumulated by certain fish species in the liver at considerably high concentrations. However, little investigation was made in the toxicity of PK and MK to fish. The another purpose of the present study is to estimate suitable doses of PK and MSB for mummichog.

## Materials and methods

### Experimental diets and fish

Approximately 100 mature mummichog were fed a commercial diet supplemented with MSB in a laboratory aquarium (60L) in order to produce larvae for the experiment. Spawned and fertilized eggs were collected and kept in a fine net cage which was set in flowing natural sea water in an aquarium until hatching. Some 2000 larvae which average of body weight was 2.2mg were, divided into 5 groups of 400 each. Each group was kept in an 8L indoor aquarium. Flow rate was about 0.4L/min. The water temperature was kept at 22℃ throughout the experiment. Larvae in each group were fed for 4 weeks on experimental diets differing in vitamin K source and content. Vitamin K was not supplement to Control diet. Diet MSB25 and MSB2500 are supplemented with MSB at concentrations of 25 and 2500mg/kg, respectively. Diet PK1 and PK100 are supplemented with PK at concentrations of 1 and 100mg/kg, respectively (Table 12). These diets were given every hour between 7:00 and 18:00. Illumination of the laboratory was controlled with fluorescent lamps for thirteen-hour light photoperiod from 6:30. The experiment was run in two replicate.

**Table 13.** Effect of diet on the incidence of vertebral abnormalities in the skeleton after 4 weeks feeding experiment

| Group | Average body weight(mg) | Mortality (%) | No. of fish | | | Vertebral abnormality (%) | Significance | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Normal | Abnormal | Total | | MSB25 | MSB 2500 | PK1 | PK100 |
| Experiment 1 | | | | | | | | | | |
| Control | 6.5 | 19.7 | 75 | 144 | 219 | 65.8 | ** | - | ** | ** |
| MSB25 | 5.5 | 21.9 | 98 | 113 | 211 | 53.6 | | * | - | - |
| MSB2500 | 4.7 | 21.7 | 73 | 139 | 212 | 65.6 | | | ** | ** |
| PK1 | 5.5 | 18.1 | 111 | 114 | 225 | 50.7 | | | | - |
| PK100[1)] | - | - | 39 | 35 | 74 | 47.3 | | | | |
| Experiment 2 | | | | | | | | | | |
| Control | 6.8 | 28.9 | 57 | 129 | 186 | 69.4 | ** | - | *** | ** |
| MSB25 | 7.2 | 19.2 | 100 | 121 | 221 | 54.8 | | * | - | - |
| MSB2500 | 4.8 | 40.6 | 49 | 95 | 144 | 66.0 | | | ** | * |
| PK1 | 6.3 | 29.2 | 90 | 95 | 185 | 51.4 | | | | - |
| PK100 | 4.5 | 27.5 | 86 | 105 | 191 | 55.0 | | | | |

*Significantly different ($p$<0.05), **significantly different ($p$<0.01), ***significantly different ($p$<0.001).

[1)] Some fish were lost during the experiment.

After four weeks of rearing, all fish were starved for 20h, and 50 fish (4-week-old fish) from each group were taken for vitamin K content analysis. The 40 of 2-week-old fish and 20 of 4-week-old fish were removed out from each group, and the remaining fish were checked for vertebral abnormalities.

### Observation and analysis of vertebral abnormalities

The skeletal structure was observed by double staining with alizarin red and alcian blue according to the method of Kawamura and Hosoya (1991), and examined in detail under a microscope at low magnification. All abnormal cases found were counted so as to calculate the occurrence incidence, and abnormality type was simultaneously classified. The data of abnormality were statistically analyzed by the pair-wise chi-squared tests. The abnormality type were statistically analyzed by the t-test or cochran-cox test.

### Determination of the vitamin K content in fish bodies

PK and MK were determined by HPLC using a Pt column by the same method of Section 1.2.1. Fifty test fish from each group and 100 larvae immediately after hatching of each were pooled and homogenized.

## Results

### The incidence of abnormalities in vertebrae

The incidence of abnormalities in the vertebrae and caudal skeleton of mummichog fed diets with differing vitamin K contents in the duplicated experiments are shown in Table 13. The incidence was significantly higher in control Group fish, while the incidence in Group PK1 fish (fed a diet supplemented with PK at 1mg/kg) is considered to be low level under this study conditions. The incidence in Group PK100 fish was essentially the same as that in Group PK1, suggesting that the higher dosage of PK does not effect the incidence of vertebral abnormalities. Furthermore, the incidence in Group MSB25 fish (MSB at 25mg/kg) was also much the same as that in Group PK1. This finding suggests that the content of MSB of 25mg/kg in the feed might be appropriate. This view is also supported by vitamin K activity of MSB and its effect on bone formation (to be mentioned). In contrast to fish fed higher dose of PK (100mg/kg), fish fed higher dose of MSB showed a significantly

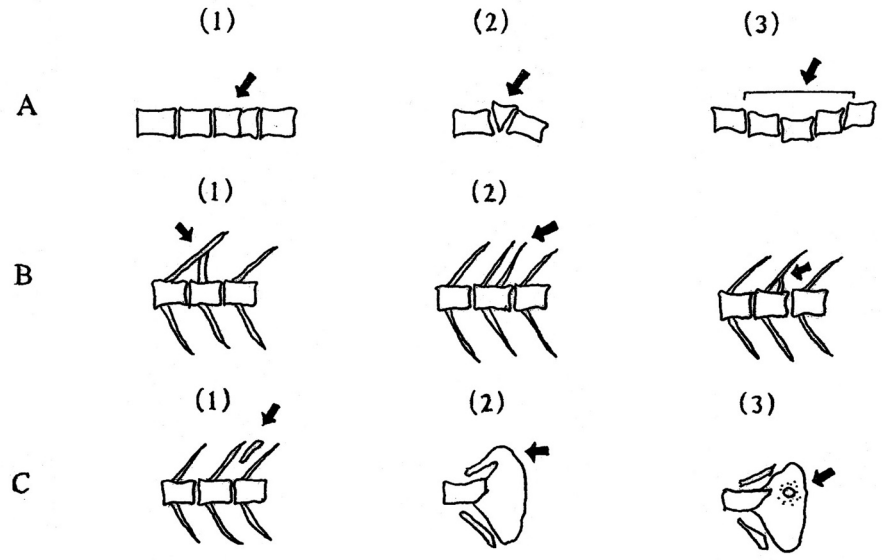
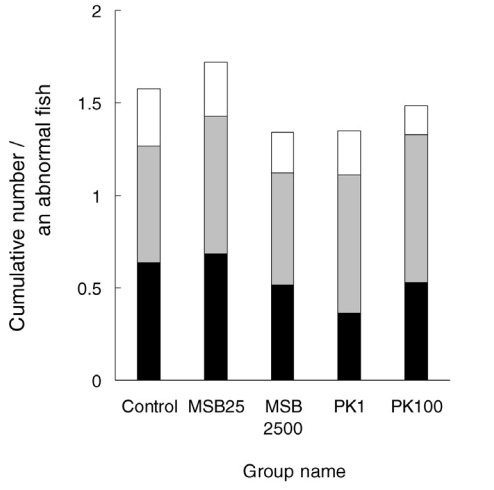



**Fig. 15.** Type of abnormalities in the skeleton. Arrows indicate the abnormal portion of the bones.

Type A: abnormalities in the vertebrae such as fusion occurred between adjacent vertebra (1), deformity of vertebra *per se* (2), and row irregularity of the vertebrae (3).

Type B: neural and haemal spines abnormalities such as fusion between adjacent spines (1), extra-ossification of a spine, and extra-ossification and fusion occurred (2).

Type C: abnormality differing from both Type A and Type B; for example, excessive ossicle (1), fusion of the epural to the hypural (2), and a deformity in the hypural (3).




Significance of Type A

| | MSB25 | MSB2500 | PK1 | PK100 |
|---|---|---|---|---|
| Control | - | - | *** | - |
| MSB25 | | - | ** | - |
| MSB2500 | | | * | - |
| PK1 | | | | - |

* = p<0.05, ** = p<0.01, *** = p<0.001

**Fig. 16.** The ratio of cases of each abnormality type to the sum of cases. Solid, stippled and open bars represent type A, B and C abnormalities, respectively.

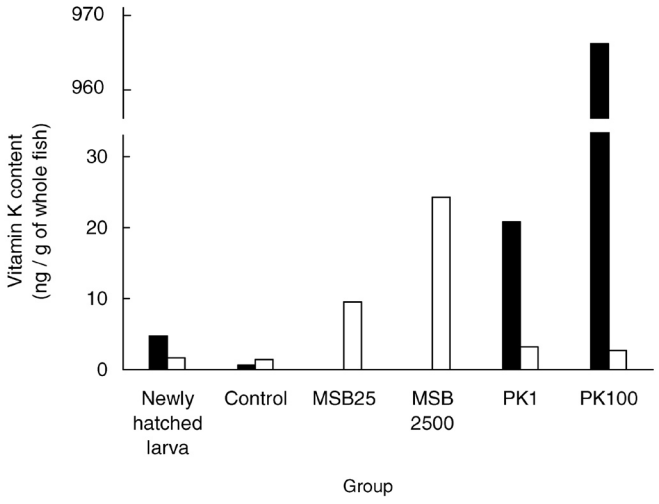



**Fig. 17.** Phylloquinone and menaquinone-4 contents in larvae. Solid bars represent phylloquinone and open bars represent menaquinone-4.

higher incidence of abnormalities. The mortality of fish during this experiment ranged from 18.1% to 40.6%, and did not differ significantly between the groups.

### Types of abnormality

Vertebral abnormalities were classified into three types based on appearance (Fig. 15). Though it was not exact morphologically, these three types were termed types A, B and C for convenience. Type A includes abnormalities such as vertebral fusion, vertebral deformity and vertebral row irregularity. Type B comprises neural and haemal spines abnormalities such as fusion, extra-ossification and a combination of the two. Cases which could not categorized into either type A or B were lumped together under type C. The incidence of the three different types of abnormality is shown graphically in Fig. 16. The incidence of type A abnormalities is apparently high significantly in Groups Control ($p<0.001$) and MSB25 ($p<0.05$) compared to that in Groups PK1, with Group PK1 having the lowest incidence among the five groups. The incidence of type C abnormalities is relatively high in Groups Control and MSB25. However, the incidence of type B abnormalities is similar across the 5 groups.

### PK and MK fish body content

Both content and form (PK or MSB) of vitamin K in experimental fish bodies were analyzed to determine differences between fish fed differing experimental diets. The results of this analysis are given in Fig. 17. The vitamin K content and form in the fish are well reflected by the experimental diets. In the newly hatched larvae analyzed for reference, a small amount of PK and a trace of MK-4 were detected. In the body of Group Control fish, only trace amounts of PK and MK-4 were found. In Groups MSB25 and MSB2500 fish, no PK and a considerable amount of MK-4 was detected. This data indicate that MSB incorporated into fish bodies was only partly, yet steadily, converted into MK-4. Even in fish exposed to a high dosage of MSB, the MK-4 level was not very high. In contrast, in Groups PK1 and PK100 fish, which were fed PK-supplemented diet, considerable amounts of PK were detected with very small amounts of MK-4. In Group PK100 fish in particular, the PK content was extremely high.

## Discussion

The incidence of bone abnormalities is apparently high in fish fed with a vitamin K free diet, compared with those fed diets supplemented with PK or MSB ($p<0.01$). This result clearly indicates that vitamin K is necessary for normal bone development in mummichog as is the case for mammals. Though there is a report that many things caused bone abnormalities (Matsusato 1986), it is supposed that vitamin K is one of them.

Wild fish ingest PK as a vitamin K source, while cultured fish are generally given MSB as a vitamin K source. The effects of PK and MSB supplemented feed on bone development of mummichog larvae differ in two ways. First, when the incidence of bone abnormalities is compared between fish given either a low dose of PK (1mg/kg of diet: Group PK1) or MSB (25mg/kg of diet: Group MSB25), there is no significant difference between the two groups. On the other hand, when fish given either a high dose of PK (100mg/kg of diet: Group PK100) or MSB (2500mg/kg of diet: Group MSB2500) are compared, the incidence is significantly higher in fish given MSB than in fish given PK ($p<0.05$). Furthermore, the incidence rate between higher and lower doses of PK was not significantly different. These findings suggest that the massive dosage of MSB is harmful to bone development.

Second, the incidence of the different types of abnormalities differed between fish given a lower dose of either PK or MSB (Fig. 16). As mentioned in terms of results, MSB mainly causes type A abnormalities, namely, fusion, deformity and row irregularity of the vertebrae from PK significantly ($p<0.01$). Although it has been reported that the growth rate of cultured Atlantic salmon Salmo salar fed MSB-supplemented feed was lower than that of PK-supplemented feed (Grisdale-Helland *et al.* 1991), effect of MSB on bone development has not determined in that study. Therefore, more extensive studies on relationship between dietary MSB supplement and bone health are necessary.

The PK and MK contents in the bodies of larvae

**Table 14.** Composition of the experimental diet

| | Diet | |
|---|---|---|
| Experimental group | No. 1<br>Group 1, 3 | No. 2<br>Group 2, 4 |
| Ingredient (g / 100g) | | |
| Vitamin-free casein*[1] | 30 | 30 |
| Gelatin*[2] | 5 | 5 |
| Dextrin | 30 | 30 |
| Feed oil*[3] | 8 | 8 |
| Mineral mix*[4] | 4 | 4 |
| Vitamin mix*[5] | 4 | 4 |
| Calcium lactate | 0.08 | 0.08 |
| Sodium phosphate, monobasic | 0.48 | 0.48 |
| CMC*[6] | 5 | 5 |
| Cellulose | 13.44 | 13.44 |
| Vitamin K (mg / kg) | | |
| Phylloquinone | - | 1 |

*[1]: (Lot no. ECF7046) Wako Pure Chemical Industries (Osaka, Japan).
*[2]: DIFCO Laboratories (Detroit, USA).
*[3]: (Lot no. SNo. J-450) Riken Vitamin Company, Ltd. (Tokyo, Japan).
*[4]: Mc.Collum Salt 820517 Iwai Chemical Co., Ltd. (Tokyo, Japan).
*[5]: The premix reported by National Research Council was partly modified as follows (mg / 4 g premix): thiamin HCl 5, riboflavin 20, pyridoxine HCl 5, choline chloride 500, nicotinic acid 75, ca-pantothenate 50, inositol 200, biotin 0.5, folic acid 1.5, ascorbic acid 100, alpha-tocopherol 40, vitamin $B_{12}$ 0.01, activated 7-dehydro-cholesterol 200IU, retinol 3200IU.
*[6]: Carboxymethylcellulose-Na.

differed depending on the diet fed. Figure 17 shows that a PK dose 100 times higher than that of lower dose caused a tissue PK concentration of about 50 times higher in fish, although an equivalently large dose of MSB caused only a 2.5 times increase in MK-4 concentration. In wild fish such as sardine *Sardinops melanostictus*, mackerel *Scomber japonicus* and ayu *Plecoglossus altivelis*, considerably large quantities of PK have been observed in the liver, while in cultured fish, fed a diet containing MSB, not so much MK-4 was observed. Although the reason why a large amount of PK, not MK, becomes accumulated in fish livers has not yet been elucidated, it is speculated that PK, rather than MK, plays a significant role in preventing vitamin K deficiency diseases of fish in nature.

### 3.3. The maternal effect of phylloquinone deficiency on bone structure in fish

The vitamin K deficient diet caused vertebral abnormality in the larvae. The effect of vitamin K deficiency of the mother fish on the eggs and larvae was not detected in term of the hatching rate and the mortality of larvae. However, the MK-4 content in eggs produced by the fish fed on the vitamin K deficient diet decreased to almost zero within the first 2 weeks. In eggs and larvae, the MK-4 contents were very low throughout the egg development period and subsequent feeding period. On the other hand, the MK-4 content in eggs spawned by fish fed on the vitamin K rich diet decreased markedly from the beginning of development and after hatching out the content became high after the first feeding.

These results indilate that vitamin K in the diet

**Table 15.** Abnormality after 5 days hatched out

| Abnormality | Normal | Abnormal | Total | |
|---|---|---|---|---|
| Group 1 (Vitamin K -) | 76 | 124 | 200 | 62.0%[a] |
| Group 2 (Vitamin K +) | 104 | 96 | 200 | 48.0%[b] |

Different superscripts are significantly different ($p<0.01$).

for maternal fish and after hatching out in the feed of the larvae are important to enhance vitamin K levels in the larval tissues, and that vitamin K seems to act on the bone structure. This section describes the maternal effect of vitamin K on the bone structure of the larvae. Secondly, the effects of vitamin K in the diet to larvae itself were studied.

## Materials and methods

Experiment 1: Table 14 shows the diet composition. Ten pairs of parental mummichog were reared after 3 weeks being fed on No. 1 diet which is the vitamin K deficient diet. Eggs were collected from these fish and obtained as vitamin K deficient eggs. Secondly, the diet for parental fish was changed for No.2 which contains PK. After 3 weeks of rearing, eggs with a vitamin K content were obtained. The eggs layered surface seawater until hatched out, and larvae were transferred to a 20L aquarium. After 5 days, 200 larvae samples were sampled for observations on the vertebral formation.

Experiment 2 : Vitamin K deficient or contained eggs which were collected same method as in Experiment 1, were reared using the different diet respectively as in Table 14. The Group 1: the vitamin K deficient eggs fed on No. 1 diet, Group 2: vitamin K contained eggs fed on No. 2 diet, Group 3: vitamin K contained eggs fed on No. 1 diet and Group 4: vitamin K deficient egg fed on No. 2 diet. They were transferred into 20L aquaria and reared on each of diet. After 10, 15, 30 days, 200 samples were collected for observations of the vertebral formation. The skeletal structure was observed by double staining with alizarin red and alcian blue according to the method in the previous Section 3.2.

The data on abnormality were statistically analyzed by the pair-wise chi-squared tests.

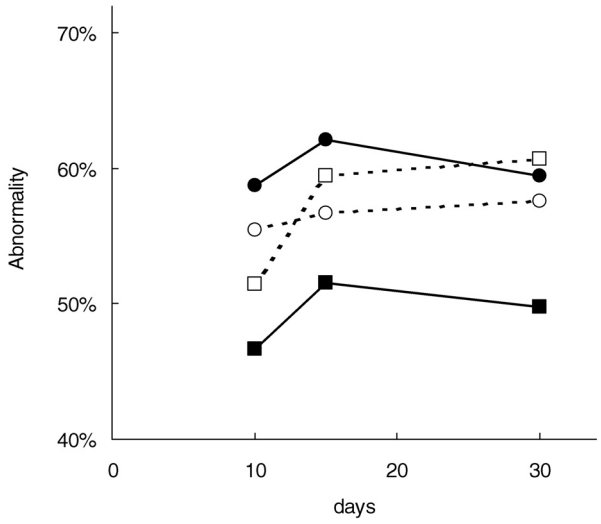



**Fig. 18.** Change of vertebral abnormalities in the skeleton of each group.
(●), Group 1; (■), Group 2; (□), Group 3; (○), Group 4.

## Results

Table 15 shows the vertebral abnormality of mummichog after 5 days from hatching out (Experiment 1). Eggs which were fertilized from fish fed the vitamin K deficient diet showed significantly more abnormality than those from fish fed vitamin K added diet.

Fig. 18 shows the change of vertebral abnormality for each group (Experiment 2). In Group 1 fed the deficient diet, vitamin K deficient eggs produced many abnormal fish through the experiment at a level the same as after 5 days at about 60%, while in the fish of Group 2 fed the vitamin K enhanced diet, vitamin K added egg produced abnormal fish significantly less than Group 1 as same as after 5

days and abnormality was about 50%. On the other hand, Group 3 vitamin K added eggs and fed the deficient diet, abnormality increased for 15 and 30 days, so that abnormality was significantly high at 30 days the as same as for the vitamin K deficient larvae. Group 4, which were fed vitamin K added feed after spontaneous from vitamin K deficient egg, abnormality increased from 10 days to 30 days and it reached about 60%.

### Discussion

Larvae which hatched out from vitamin K deficient eggs showed a significantly higher rate abnormality of the bone structure than from vitamin K rich eggs. This indicates that the maternal vitamin K content is necessary to development bone structure.

Feeding on the vitamin K deficient diet to vitamin K rich larvae led to a high rate of abnormality after 30 days. This indicates that vitamin K is necessary for bone development not only in eggs but also in the food for larvae. Vitamin K deficient eggs showed high abnormality though they were fed on vitamin K rich diet after they hatched out. This indicates that vitamin K is required in the early stage of development.

These results imply that vitamin K is necessary for the development of normal bone structure not only during production in the egg but also after hatching out.

## Chapter 4. Histochemical study of the effect of vitamin K on fish bone

The precise role of vitamin K in the skeletal system is not clear. Recently, the effects of vitamin K on bone resorption and formation have been

**Table 16.** Composition of the experimental diet

| | Diet | |
|---|---|---|
| Experimental group | No. 1<br>Group PK 0 | No. 2<br>Group PK 1 |
| Ingredient (g / 100g) | | |
| Vitamin-free casein*[1] | 30 | 30 |
| Gelatin*[2] | 5 | 5 |
| Dextrin | 30 | 30 |
| Feed oil*[3] | 8 | 8 |
| Mineral mix*[4] | 4 | 4 |
| Vitamin mix*[5] | 4 | 4 |
| Calcium lactate | 0.08 | 0.08 |
| Sodium phosphate, monobasic | 0.48 | 0.48 |
| CMC*[6] | 5 | 5 |
| Cellulose | 13.44 | 13.44 |
| Vitamin K (mg / kg) | | |
| Phylloquinone | - | 1 |

*[1]: (Lot no. ECF7046) Wako Pure Chemical Industries (Osaka, Japan).
*[2]: DIFCO Laboratories (Detroit, USA).
*[3]: (Lot no. SNo. J-450) Riken Vitamin Company, Ltd. (Tokyo, Japan).
*[4]: Mc.Collum Salt 820517 Iwai Chemical Co., Ltd. (Tokyo, Japan).
*[5]: The premix reported by National Research Council was partly modified as follows (mg / 4 g premix): thiamin HCl 5, riboflavin 20, pyridoxine HCl 5, choline chloride 500, nicotinic acid 75, ca-pantothenate 50, inositol 200, biotin 0.5, folic acid 1.5, ascorbic acid 100, alpha-tocopherol 40, vitamin $B_{12}$ 0.01, activated 7-dehydro-cholesterol 200IU, retinol 3200IU.
*[6]: Carboxymethylcellulose-Na.

reported (Hara *et al.* 1995). Vitamin K inhibits bone resorption and osteoclast-like multinucleate cell formation (Akiyama *et al.* 1994). Bone deformations have been reported to develop due to vitamin K deficiency in human and animals. Warfaren decreased bone resorption and bone formation strongly and caused remodeling abnormalities. In this chapter, effect of vitamin K on the fish bone resorption and fine structure are studied histochemically.

### 4.1. Histochemical study of the effect of vitamin K on bone development

There are reports that patients with crush fractures have very low concentrations of circulating PK (Hodges *et al.* 1993). Knapen *et al.* (1993) implied that vitamin K supplementation increases the serum markers for bone formation (including osteocalcin and bone alkaline phosphatase). These reports indicate that vitamin K functions in the bone matrix, especially the bone fine structure. The vitamin K deficient diet caused vertebral abnormality in thefish larvae. Vitamin K act as a factor in the bone mass in fish. This section describes the histochemical effect of vitamin K for the fish larvae on bone structure.

#### Materials and methods

Experiment 1: Table 16 shows the diet composition. Ten pairs of Mummichog *Fundulus heteroclitus* were reared after 3 weeks being fed on the vitamin K deficient diet (No. 1), and eggs were collected and obtained as vitamin K deficient eggs. Secondly, the diet was changed for the PK containing diet (No. 2) and after 3 weeks of rearing, eggs with a vitamin K content were obtained. The

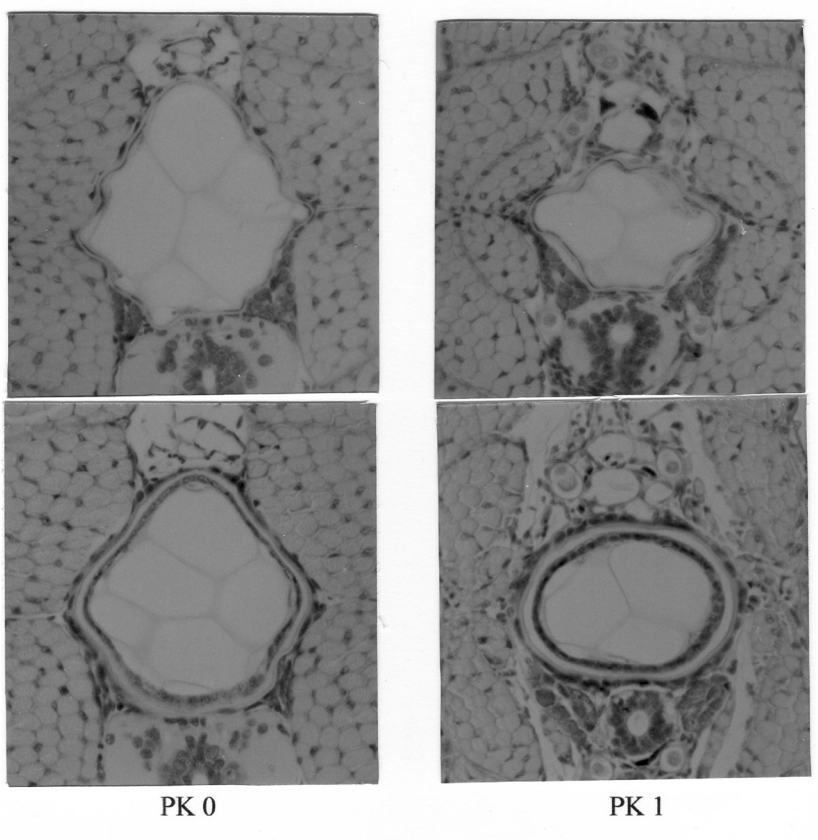



**Fig. 19.** Photomicrographs of cross section of vertebral bone in mummichog Fundulus heteroclitus, showing effects of vitamin K in eggs on the bone structure, 5 days after hatching. P0: vitamin K-deficient; P1:vitamin K supplemented.

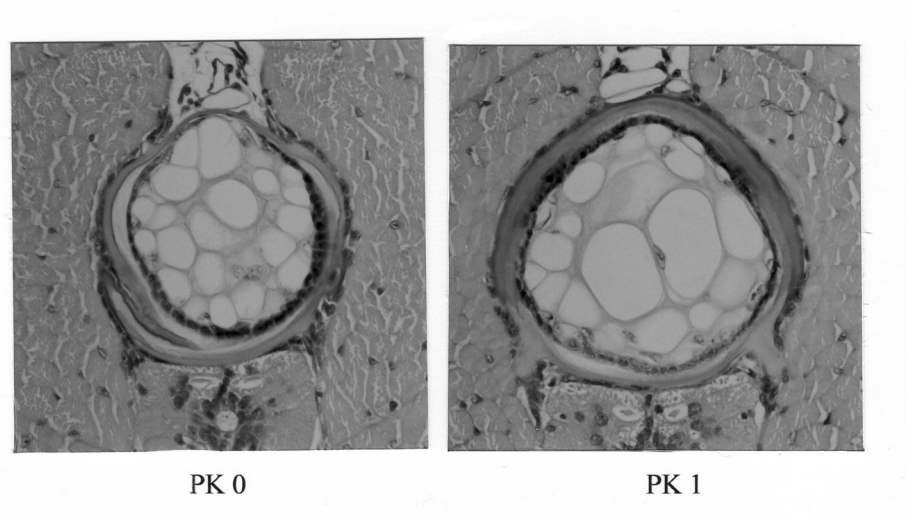



**Fig. 20.** Photomicrographs of cross section of vertebral bone in mummichog Fundulus heteroclitus, showing effects of vitamin K in eggs on the bone structure, 30 days after hatching. P0: vitamin K-deficient; P1:vitamin K supplemented.

eggs layered surface seawater until hatched out, and larvae were transferred to a 20L aquarium. After 5 days, the skeletal structure was observed by double staining according to the same method as detailed in Section 3.2.

Experiment 2 : Vitamin K deficient or contained eggs, which were same way as Experiment 1, were reared by the diet as Table 1. The larvae were transferred into 20 L aquaria and each reared on a diet of Group PK 0: vitamin K deficient eggs to No. 1 diet, Group PK 1: vitamin K content eggs to No. 2 diet. After 30 days, the skeletal structure was observed by double staining with alizarin red and alcian blue according to the same method as detailed in Section 3.2.

#### Results and discussion

Figs. 19, 20 show the observations of bone structure for the PK deficient (PK 0) and PK rich (PK 1) larvae. Fig. 19 shows the bone structure 5 days after hatching out (Experiment 1) and Fig 20 shows the bone structure 30 days after hatcheing out (Experiment 2). In the comparison of the fish bone fine structure of the Group PK 0 with that of the Group PK 1 after 5 days, the bone surface of Group PK 1 were smooth showing regular circlar but that of Group PK 0 were not constantly thick, not smooth and distorted circle. After 30 days, there was a different bone fine structure between the PK rich and the PK deficient diet larvae. The bone of the fish fed on the PK rich diet was constantly thick, while the deficient diet caused that bone was thin and rough. Thisroughness seemed that bone had been fused after being fractured finely. These results imply that PK deficient larvae had a highly decreased bone strength and formation, inducing bone structure abnormality.

### 4.2. Histochemical characteristics of the osteoblastic cells in fish fed a diet with vitamin K

Bone cells is a composition of osteoclasts, osteoblasts, osteocytes, and so on. Bone resorption have been incessantly, osteoclast clashes bone and osteoblast produce bone. Vitamin K deficiency caused thin and weak bones and decreases bone strength. It seemed that vitamin K deficiency decreased the bone cell activity on the bone development, and especially acts on the osteoblasts. In this section, the effects of vitamin K on the histochemical characteristics of osteoblasts were examined.

**Table 17.** Composition of the experimental diet

| group | Control Group | Experiment Group |
|---|---|---|
| Ingredient (g / 100g) | | |
| Vitamin-free casein*[1] | 30 | 30 |
| Gelatin*[2] | 5 | 5 |
| Dextrin | 30 | 30 |
| Feed oil*[3] | 8 | 8 |
| Mineral mix*[4] | 4 | 4 |
| Vitamin mix*[5] | 4 | 4 |
| Calcium lactate | 0.08 | 0.08 |
| Sodium phosphate, monobasic | 0.48 | 0.48 |
| CMC*[6] | 5 | 5 |
| Cellulose | 13.44 | 13.44 |
| Vitamin K (mg / kg) | | |
| Phylloquinone | - | 100 |

*[1]: (Lot no. ECF7046) Wako Pure Chemical Industries (Osaka, Japan).
*[2]: DIFCO Laboratories (Detroit, USA).
*[3]: (Lot no. SNo. J-450) Riken Vitamin Company, Ltd. (Tokyo, Japan).
*[4]: Mc.Collum Salt 820517 Iwai Chemical Co., Ltd. (Tokyo, Japan).
*[5]: The premix reported by National Research Council was partly modified as follows (mg / 4 g premix): thiamin HCl 5, riboflavin 20, pyridoxine HCl 5, choline chloride 500, nicotinic acid 75, ca-pantothenate 50, inositol 200, biotin 0.5, folic acid 1.5, ascorbic acid 100, alpha-tocopherol 40, vitamin $B_{12}$ 0.01, activated 7-dehydro-cholesterol 200IU, retinol 3200IU.
*[6]: Carboxymethylcellulose-Na.

## Materials and methods

Approximately 100 mature mummichog were fed a commercial diet supplemented with MSB in a laboratory aquarium (60L) in order to produce larvae for the experiment. Spawned and fertilized eggs were collected and kept in a fine net cage which was set in flowing natural sea water in an aquarium until hatching. Each group was kept in an 8 L indoor aquarium. Flow rate was about 0.4L/min. The water temperature was kept at 22℃ throughout the experiment. Larvae in each group were fed for 4 weeks on diets differing in vitamin K content (Table 17). Vitamin K was not supplemented to the control diet. The experimental diet was supplemented with PK at a concentration of 100mg/kg. These diets were given every hour between 7:00 and 18:00. Illumination of the laboratory was controlled with fluorescent lamps for a thirteen-hour light photoperiod from 6:30. The experiment was run in two replicates. Five of 4-week-old fish were removed from each group, and were checked for vertebral abnormalities.

The bones from the experimental fish were fixed with Bouin's fluid and embedded in paraffin. Serial sections 5 $\mu$m thick were stained with hematoxylin and eosin.

**Table 18.** The number of osteoblast-like cells per individual vertabral section at microscope

| | Diet | |
|---|---|---|
| | Experiment | Control |
| No. 1 | 110 | 48 |
| No. 2 | 62 | 52 |
| No. 3 | 60 | 52 |
| No. 4 | 64 | 52 |
| No. 5 | - | 46 |

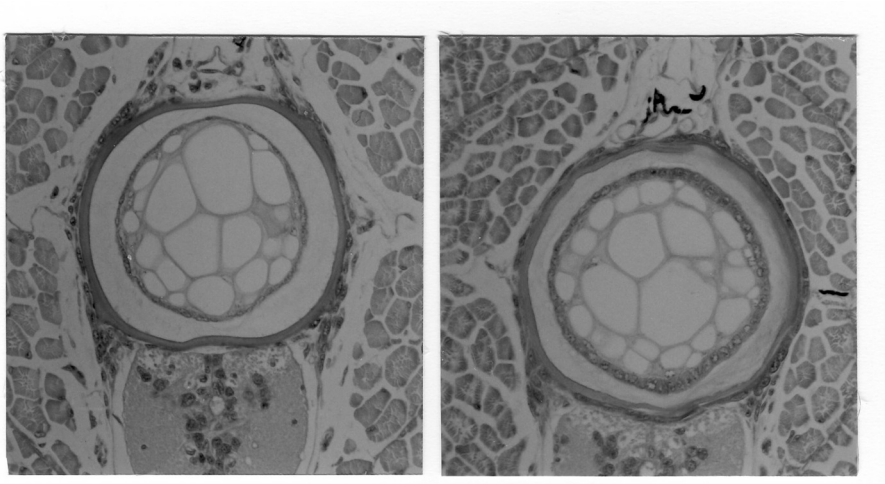


**Fig. 21.** Photomicrographs of cross section of vertebral bone in mummichog Fundulus heteroclitus, showing effects of vitamin K in diets on the osteoblast-like cells, vitamin K deficient.

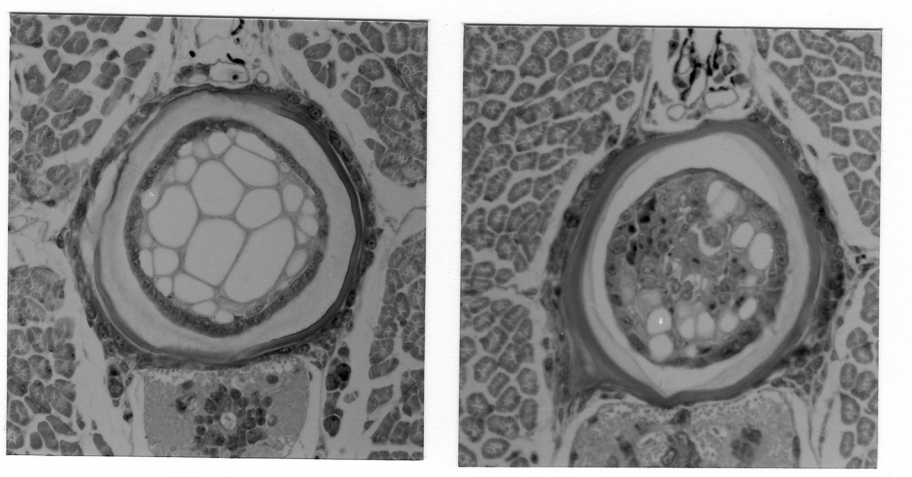


**Fig. 22.** Photomicrographs of cross section of vertebral bone in mummichog Fundulus heteroclitus, showing effects of vitamin K in diets on the osteoblast-like cells, vitamin K supplemented.

## Results and discussion

Fig. 21, 22 show observations of the bone structure of each group. In the experimental Group, osteoblast-like cells were observed more frequently than control Group.

Table 18 shows the number of osteoblast-like cells for each group. The experimental Group, more of the osteoblast-like cells were found than the control Group. These results indicate that PK is necessary for the activation of osteoblast-like cell and a large quantity of PK activates excessive osteoblast-like cell. These data indicate that at least part of the effects of PK on bone resorption may be to regulate osteoblasts activity via an as yet unidentified mechanism.

There is a report that MK inhibits *in vivo* osteoclast formation in rats (Kawata *et al.* 1999), vitamin K may effect both osteoclast and osteoblast cells, and consequently have an effect on bone metabolism. Considering that PK is more deposited in tissue than MK, there is a possibility that PK is more useful than MK in fish differing from in mammal.

## Chapter 5. Comprehensive Discussion

The contents of PK and MK-4, 6, 7, 8 and 9 in the different organs of four species of fishes were measured and compared. Mackerel, a typical pelagic fish, contained a large amount of PK but only trace amounts of MK-4 and MK-6 in the liver and kidney. Saury contained a large amount of MK-4 in the heart and gonad, and little PK. Marbled sole and sillago, demersal fish, contained relatively large amounts of MK-4 and PK but only small amounts of MK-6, 7 and 8. In marbled sole and sillago, the contents of the gastrointestinal tracts were rich in MK-long chains. While, the tissues and the digestive tracts of mackerel and saury contained low amounts of MK-long chains. In the comparison of cultured and wild ayu, the content of PK was much higher in the wild ayu, particularly in the liver, which feed on diatoms than in the cultured ayu which were fed with a commercial ayu diet. Since diatoms contain PK at a high concentration, large parts of the PK in the wild fish are considered to originate from the natural feed. Possibly, the source of vitamin K of wild ayu is considered to be the PK in the feed. On the other hand, the content of MK-4 which is known to be metabolized from menadione was high in the cultured fish. The major vitamin K in wild sardine was PK, and the stomach and intestinal contents of wild sardine as well as planktons which are consumed by the wild sardine showed a much higher level of PK than MK-4. Cultured sardine fed a MSB supplemented diet were rich in MK-4, which indicates that MD was converted to MK-4 in the body. A large quantity of long-chain MKs was found in the gastrointestinal tract content of both fish but the level was low in the tissues. These findings suggest that vitamin K in the tissues of fish originates from the food taken in and stored mainly in the liver. However, a certain discrepancy between the composition pattern of vitamin K in the tissues and contents of the gastrointestinal tract indicates that different forms of vitamin K are absorbed and/or accumulated in the tissues in a different manner. This difference suggests that the different forms of vitamin K are not equally incorporated via the metabolic system.

As feeding diets supplemented with different vitamin K groups, the vitamin K mainly detected in the gastrointestinal tract of each experimental group was the one supplemented in the respective diet, and all other forms of vitamin K were observed at low concentrations. This implies that the main vitamin K source for mummichog might be in their food, and the elevation of vitamin K concentrations in the plasma and other tissues in this experiment was brought about by the vitamin K added to the feed. The PK rich diet raised the PK concentration in the plasma and the tissues much higher than the diets supplemented with short and/or long chain MKs. This indicates that PK is more easily accumulated in the body of fish than the MK homologues. This seems to be the reason why only the PK content was higher than MKs in fish liver for sardine, mackerel and ayu. PK to MSB, a massive dose of MSB caused a high incidence of bone deformity, compared with a lower dose, while an increased dose of PK brought about no significant difference in deformity incidence. The vertebral deformity was significantly higher in fish fed a vitamin K free diet and MSB-supplemented diet compared to in fish fed a PK-supplemented diet. These results indicate that PK is more suitable than MSB as a vitamin K source in fish feed.

In mummichog reared in the spawning season on a vitamin K free diet for 11 weeks, most of the male fish died, while the female fish seldom died. In contrast, none of the male nor female fish (control fish) which were fed with a diet supplemented with MSB died. Diets without vitamin K caused a significantly higher incidence of bone deformity in fish than diets supplemented with vitamin K. These results indicate that vitamin K is essential for mummichog. The MK-4 content in the tissues markedly decreased during the experimental feeding. After 11 weeks, MK-4 was found to have accumulated in various tissues of the control fish, especially in the gonadal tissues, except for the liver. In the kidney of the female fish fed with a vitamin K free diet, a large number of immature erythrocytes were observed. This indicates that the female fish had been affected by hematoporia and/or hematopoiesis brought about by a deficiency in vitamin K. However, larval mortality was not affected by the vitamin K deficiency. These results

indicate that vitamin K intake is necessary for mummichog, particularly for the male fish during the spawning season even though they can continue to grow without any vitamin K supplement. There were apparent differences in absorption and deposition of vitamin K between females and males.

The vitamin K deficient larvae had an abnormal vertebral formation, while PK rich larvae showed a low abnormality. Vitamin K rich larvae which were fed a vitamin K rich diet after hatching had a low rate of abnormality, though one group fed vitamin K deficiency diet had high abnormality the same as larvae hatched from vitamin K deficient eggs after 30 days. On the other hand, larvae fed on a vitamin K rich diet which was hatched from vitamin K deficient egg, showed a high rate of abnormality the same as fed on vitamin K deficient diet after 30 days. These results indicate that vitamin K deficiency affects mummichog bone structure for both early development and growth.

The effect of PK on bone structure in fish were observed histochemically. The bone structure of vitamin K deficient larvae was thin and rough after 30 days feeding. It seemed to be connected after fine fracture of a bone surface. On the other hand, in larvae fed a PK rich diet this phenomenon was not observed. These results imply that vitamin K deficiency caused thin and weak bone, and it induces bone structure abnormality. Vitamin K rich diet increased the osteoblast-like cells. These results imply that PK was necessary for the activation of osteoblasts. These data indicate that at least part of the effects of PK on bone resorption may regulate osteoblasts activity via an as yet unidentified mechanism.

From these data, it can be concluded that the difference in dominant vitamin K between cultured and wild or pelagic and demersal fish is caused by differences in the food consumed by the fish. PK seemed to be absorbed more easily than MKs in the body of fish. Vitamin K deficiency causes high mortality or bone abnormality, especially PK prevents bone abnormality. It seemes to play a role in bone resorption especially on the osteoblast cells.

## Chapter 6. Summary

Basic information on the distribution and role of vitamin K in fish is described in relation to dietary intake. Vitamin K in the fish tissues is mainly concentrated in the liver and gonads, with a small amount in muscle. There was a difference in the concentration of vitamin K between demersal and pelagic fish depending on the habitat and between cultured fish and wild fish. The composition and content of vitamin K in the gastrointestinal tract which were very similar to those of the food consumed differed from the tissues, suggesting that all the vitamin K in food was not transferred to the tissues of fish. There was no clear indication that vitamin K deficiency affected the growth rate or mortality in fish, although vitamin K deficiency caused histopathological changes and bone abnormalities. The vitamin K seems to have an effect on osteoblast cells. These results imply that the role of vitamin K in fish has a variety of physiological functions.

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