Vitamin K, Much More than Blood Clotting: Roles, Metabolism and Specific Requirements. A Mini-Review.

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Abstract

Vitamin K (VK) is a fat soluble vitamin required in fish diets for normal growth and development since they are not able to synthesize it *de novo*. VK can be naturally found as phylloquinone (VK1; vegetable origin) or menaquinone (VK2; microbial or animal origin); and synthetically produced as menadione (VK3). Two major roles of VK metabolites have been described up-to-date. As a co-factor of the enzyme γ-glutamyl carboxylase which performs the conversion of Glu into Gla residues in VK-dependent proteins (VKDPs), through which VK is controlling blood clotting, representing the most widely recognized function of VK. VK also participates in transcriptional regulation, acting as ligand for the steroid xenobiotic receptor or pregnane X receptor (PXR), which is mostly known as a master regulator of xenobiotic metabolism. However, little is known about its metabolism and its specific requirements along fish development. Here, we will briefly review what is known, and identify what still remains to be unveiled about VK and fish physiology. In this sense, new findings regarding VK requirements on early fish development, metabolism and VK cycle regulation under different nutritional conditions will be enumerated. Finally, future perspectives on knowledge gaps, strategies and approaches to be applied will be discussed.

Key words: vitamin K, gamma-carboxylation, PXR, metabolism, proteomics, transcriptomics.

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Historical overview of the greatest milestones on VK physiology:

Vitamin K (VK) is a fat soluble vitamin discovered by Henrik Dam as the "*Koagulationsvitamin*" in 1935 (Dam 1935). However, its specific role in blood coagulation was only revealed in 1974, when its requirement for the conversion of glutamyl (Glu) to γ-carboxyglutamyl (Gla) residues, conferring calcium binding properties to the proteins nowadays known as VK-dependent proteins (VKDPs) was described (Magnusson *et al*. 1974; Nelsestuen *et al*. 1974; Stenflo *et al*. 1974). However, only 17 years later the enzyme catalyzing this conversion, the γ-glutamyl carboxylase (GGCX), was discovered and characterized (Wu *et al*. 1991). We still had to wait 10 years more to have a complete picture of the VK recycling process when the cloning of the enzyme vitamin K epoxide reductase complex 1 (VKORC1) was achieved (Li *et al*. 2004; Rost *et al*. 2004). Just one year before, and similarly to what has been found in the other fat soluble vitamins, the specific nuclear receptor to which VK is bind was discovered, the pregnane X receptor (PXR; Tabb *et al*. 2003). In this sense, PXR was largely known as as a master regulator of xenobiotic metabolism (Chen *et al*. 2012) and for cholesterol and bile acid metabolisms (Makishima 2005). More recently PXR was shown to have a key role in bone homeostasis, as demonstrated by the osteopenic phenotype induced in PXR knockout mouse (Azuma *et al*. 2010).

In contrast to invertebrates, vertebrate genomes include two paralogous enzymes VKORC1 and VKORC1-like 1 (VKORC1L1) likely resulting from a gene duplication of an early common VKOR ancestor. Until 2011, VKORC1 protein was considered as the only player supporting VK recycling activity. Westhofen *et al.* (2011) demonstrated that the VKORC1L1 is able to reduce VK epoxide (the VK by-product after γ-glutamyl carboxylation) to VK, although showing a low enzymatic efficiency. More recently, Hammed *et al*. (2013) reported a VK recycling activity of VKORC1L1 in extrahepatic tissues in the absence or inhibition of VKORC1 protein, completing the full picture of VK roles known up-to-date (Fig.1).

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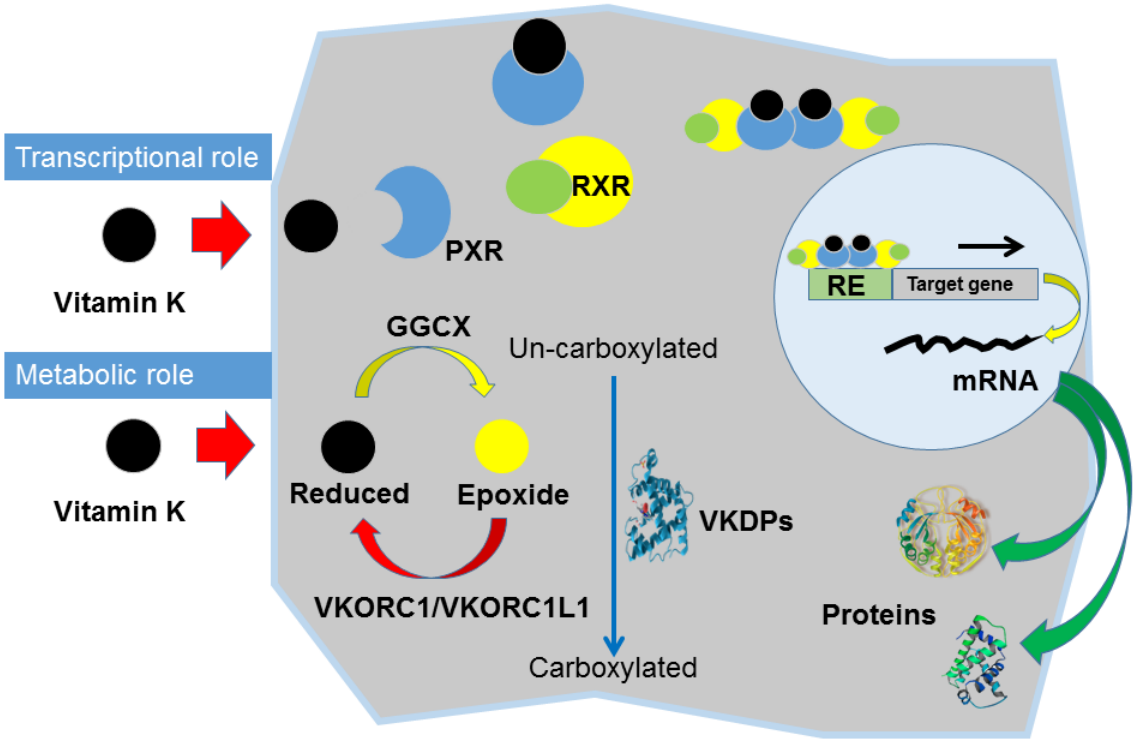

Figure 1. Vitamin K (VK) cycle showing the metabolic and transcriptional roles. VK, coming from dietary intake or glut microflora, is used by the γ-glutamyl carboxylase (GGCX) to convert glutamate into γ-carboxyl glutamate (Gla) residues in VK-dependent proteins (VKDPs), resulting in the production of a VK epoxide as a by-product. VK epoxide is then recycled to VK either by VK epoxide reductase complex 1 (VKORC1) or VKORC1-like 1 (VKORC1L1). In addition, VK is also a ligand of the pregnane X receptor (PXR). The activation of PXR by VK, promote the formation of a heterotetramer with retinoid X receptor (RXR), which finally bind to the PXR responsive elements (RE) on the promotor or enhancer regions of down-stream target genes, activating their transcription and thus the proteins synthesis.

During the last decades different members of the VKDPs have been also identified. The first ones were those involved in the blood coagulation cascade such as the different clotting factors (II, VII, IX, and X), being this the reason why VK has been largely associated with blood coagulation (reviewed in Brenner *et al*. 2009). Subsequently, other VKDPs involved different biological processes have been identified. Matrix Gla protein

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(MGP), has been identified by Price *et al*. (1983), and is known to act as an inhibitor of calcification in soft tissues such as arteries (Luo *et al*. 1997). Bone Gla protein (BGP), also known as osteocalcin, was already described in 1976 by Price and co-workers. In contrast to MGP, it is not only involved in the regulation of tissue mineralization, but it was recently shown to play a role also in glucose metabolism and spermatogenesis (Karsenty and Ferron 2012). Further, the product of growth arrest specific (GAS) gene 6, GAS6, was discovered by Manfioletti *et al*. (1993) and has been suggested to be an important regulator of vascular homeostasis and platelet signaling and being a ligand for the tyrosine protein kinase receptors AX1, TYRO3 and MER implicated in cell growth, survival, adhesion and migration (reviewed by Maree *et al*. 2007). The last member of the VKDP family to be discovered was Gla rich protein (GRP) that was found in the last decade (Viegas *et al*. 2008). It is the most densely γ-carboxylated protein and although it is expressed mainly in cartilaginous tissues (chondrocytes) and in bone cells (osteocytes) and thus, suggested to act as a modulator of calcium availability (reviewed in Cancela *et al*. 2012), however its molecular function is still to be unveiled.

The discovery of all that diverse set of VKDPs, in addition to the one of its nuclear receptor, evidenced a broader biological impact for VK than it was initially foreseen and it is expectable that it might be expanded in the nearest future.

What is known about VK in fish species?

Vitamin K metabolites

Vitamin K (VK) compile a family of compounds derived from quinone, all of them having a common 2-methyl-1,4-naphthoquinone ring, but differing in the side chain at the C3-position (Lambert and De Leenher 1992). Depending on their source, three different VK metabolites (or vitamers K) can be found. The first two can be found in nature as phylloquinone or the group of menaquinones. Phylloquinone or VK_1 is produced by photosynthetic plants serving as a cofactor for Photosystem I-mediated electron transport, with green leafy vegetables being the ones showing higher contents (Booth and Sutie

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1998). Menaquinones, VK2 or MK are a collection of isoprenologues with microbial origin that can be found in fermented products such as Nato or in foods of animal origin (Booth and Sutie 1998). Although more than 20 different menaquinoes have been described, named MK-n accordingly to the number (n) of prenyl groups in the unsaturated side chain, the most relevant from a nutritional point of view are MK-4 and MK-7 (Fodor *et al*. 2010).

Since the main sources of VK are the diet composition and the intestinal microbiota, an equilibrate intake of VK from diverse dietary sources and a healthy intestinal microbiota seems to be needed to the maintenance of proper VK levels in mammals; although contribution of colonic flora to vitamin K requirements remains controversial (Harshman *et al*. 2014). In this sense, some bacterial derived menaquinones have been found stored in the human liver (Suttie 1995). Although the use of antibiotics is known to affect intestinal microbiota (Mathers *et al*. 1990), the relevance and the impact of antibiotics on intestinal production of VK are still not known (Tan and Mai 2001). The third vitamer K is a synthetic water soluble salt, known as menadione or VK_3, majorly produced as menadione sodium bisulphite (MSB) and menadione nicotinamide bisulphite (MNB).

The complex metabolism and transformation from one vitamer K into another has been recently reviewed in detail and thus, for interested readers in this issue Shearer and Newman (2014) is recommended.

VK sources used, dietary levels recommended and biomarkers proposed in fish species

All natural vitamers K are insoluble in water, slightly soluble in alcohol and readily soluble in non-polar organic solvents. Although they are relatively thermostable compounds, vitamers K are highly sensitive to light and alkaline conditions (in Krossoy *et al*. 2011). In contrast, menadione is much more chemically unstable than the natural VK forms (Marchetti *et al*. 1999). Although it can be partly alkylated enzymatically to MK-4 in animal tissues when present in animal feeds (Graff *et al*. 2010; Krossoy *et al*. 2009), it is easily excreted and shows lower bioavailability than the naturally occurring K vitamers (reviewed in Krossoy *et al*. 2011). Furthermore, menadione cannot act directly as a co-factor for GGCX as demonstrated by Krossoy *et al*. (2010). When the different K vitamers

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are compared metabolically, it seems that conversion of menadione to MK-4 is highly rate limited, as it is its retention compared to that of phylloquinone (Graff *et al*. 2010). Importantly, meal and oil from alternative vegetable sources for the replacement of fish meal and fish oil, like soybean oil or canola oil, may contain higher levels of natural vitamin K_1 compared to marine ingredients, although its incorporation might be hampered by anti-nutritional factors present in the same vegetable resources.

Regarding the safe levels of VK metabolites, while a 50 and 100 % mortality has been observed in zebrafish (*Danio rerio*) embryos exposed from 0-5 dpf to 0.25 and 0.5 mM VK_3, respectively, the same concentrations of VK_2 and VK_1 only induced a 15 % mortality and not significantly different from the control group (Fernandez *et al*. unpublished data). Similarly, when other aquacultured species have been fed with MSB (20-30 mg Kg^{-1}) a reduced growth has been verified (Grisdale-Helland *et al*. 1991; Grahl-Madsen and Lie 1997). However, upper tolerance of 100 mg Kg^{-1} of VK_1, 2500 mg Kg^{-1} for MSB and 2000 mg Kg^{-1} for MNB have been reported in different fish species (reviewed in Krossoy *et al*. 2011). Thus, the VK source and the amounts present in fish diets seems to depend on fish species and developmental stages.

The most common VK deficiency signs in fish are mortality, blood coagulation time, reduced growth, anemia, hemorrhages, loss of fin tissue and abnormal skeletogenesis or bone homeostasis. Based on those parameters, different studies suggested different optimal dietary VK content for a diverse set of fish species (Table 1). Although quite sensitive biomarkers for nutritional VK status in humans are commonly used at clinical level, such as the rate of circulating uncarboxylated BGP or the combined rate of uncarboxylated and dephosphorylated MGP in blood samples, no reliable, sensitive and easy to apply biomarker has been found for fish species. In this sense, GGCX activity has been previously proposed as a sensitive marker for evaluating VK status and intake (Krossoy *et al*. 2010). Atlantic salmon (*Salmo salar*) juveniles fed with increasing levels of MNB (from 0 to 50 mg Kg^{-1}) did not show differences in specific growth rate, condition factor, whole body proximate analysis, blood coagulation time, vertebra morphology or mechanical properties of vertebrae; although a positive dose-response relationship between dietary MNB and the level of MK-4 analysed in liver samples was reported (Krossoy *et al*.

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2009). However, when the enzyme activity and gene expression of GGCX was analyzed in the same samples, the authors reported a decreasing GGCX activity with the increase of VK dietary content and no differences on its gene expression (Krossoy *et al*. 2010). Taking into account those results, GGCX activity seems not to be an accurate VK biomarker since it showed differences in animals with the same biological performance. This could be due to the quantification of γ-glutamyl carboxylase by assaying the incorporation of $H^{14}CO3^-$ into synthetic peptides and subsequent quantification using liquid scintillation counting (Emson and Sutie, 1976). More sensitive and accurate quantification methods have been developed such as the described by Kaesler *et al*. (2012). Nevertheless, considering more recent results from *in vivo* and *in vitro* studies, the other two major players of the VK recycling, VKORs and PXR, have been proposed as more suitable biomarkers. Senegalese sole (*Solea senegalensis*) fed VK supplemented diets showed a better skeletal development and a lower expression of *pxr* and *vkorc1* genes (Richard *et al*., 2014). Conversely, in zebrafish larvae and osteoprogenitor cells under a VK-induced deficiency through the exposure to increased levels of warfarin (an inhibitor of VK recycling), *pxr* and *vkorc1* genes were up-regulated (Fernandez *et al*. 2014, 2015). These results, although in phylogenetically distant fish species, strengthen the value of these markers as a robust indicator of the VK status in fishes.

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Table 1. Suggested optimal dietary levels of VK in fish feeds

Fish species	Developmental stage	Criteria	Vitamer K	Total VK content*	Reference
Lake trout	Juvenile	Haematology, coagulation time	-	**0.5-1**	Poston, 1976
Salmonids	Juvenile	Growth	VK1	**0.45**	Woodward, 1994
Atlantic cod	Juvenile	Mortality, haematology, coagulation time	VK3	**0.2**	Grahl-Madsen and Lie, 1997
Salmonids	Juvenile	Growth, mortality	VK3	**1.5**	Kaushik *et al.* 1998
European seabass	Juvenile	Growth, mortality	VK3	**1.5**	Kaushik *et al.* 1998
Salmonids	Juvenile	Growth	-	**10**	Halver, 2002
Haddock	Juvenile	Growth, bone health	VK3	**20**	Roy and Lall, 2007
Atlantic salmon	Juvenile	Growth, coagulation time, bone health	VK1	**0.1**	Krossøy *et al.* 2009
Senegalese sole	Larvae	Bone health	VK1	**4.5**	Richard *et al.* 2014.

* in mg kg^{-1}

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Insights on new roles and future research needs

The requirement of VK for the blood coagulation control has been largely known. More recent works also demonstrated its requirement in fish for the prevention of soft tissues calcification (Fernandez *et al*. 2014) and for a proper skeletogenesis (Richard *et al*. 2014) and bone homeostasis (Fernandez *et al*. 2014). Further, Richard *et al*. (2014) presented evidences that VK might also be critical for a broader set of biological functions such as muscular contraction, resistance to osmotic stress, intracellular Ca^{2+} homeostasis or energetic metabolism, by applying proteomic analysis. Undoubtedly, new approaches and technologies with a high throughput like Next-Generation Sequencing (NGS) will help us to fully unveil the biological processes where VK has a key role in fish species, and confirm those already suggested in mammalian systems. In this regard, VK have an important role in the synthesis of sphingolipids that are crucial for the nervous system, and some correlation with cognition has been found (Ferland 2012). The undercarboxylated form of BGP in which Glu 13 is not carboxylated was shown to be active on β-cells and Leydig cells. In this way it is proposed that undercarboxylated BGP acts as a bone-derived hormone stimulating insulin secretion and β-cell proliferation in the pancreas, energy spending by muscles, insulin sensitivity in adipose tissues, muscles and liver, as well as by stimulating testosterone synthesis in the testis, promoting male fertility (Karsenty and Ferron 2012). Furthermore, Gray and Squires (2012), found that the transactivation of PXR in Leydig cells increased the expression of several genes involved in steroidogenesis, including *cytochrome B5A* and *cytochrome B5 reductase 1*, as well as *hydroxysteroid* (17-beta) *dehydrogenase 4* and *retinol dehydrogenase 12*. Treatment with rifampicin, an agonist of mammalian PXR but not of fish PXR (Ekins *et al*. 2008), resulted in significantly decreased sex steroid production and significantly increased production of androstene steroids. It is well known that androstene steroids are androgens controlling the development and maintenance of male characteristics in vertebrates by binding to androgen receptors, but also functioning as paracrine hormones required by the Sertoli cells to support sperm production. Further, the expression of genes involved in the biosynthesis of cholesterol and steroid hormones was found to be decreased in rats fed with VK deficient

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diets; being the mRNA levels of *cyp11a* – a rate-limiting enzyme in testosterone synthesis – positively correlated with the MK-4 concentration in testis (Shirakawa *et al*. 2006). The same authors compared testosterone concentrations among rats fed control, VK supplemented and VK deficient diets, demonstrating decreased concentrations of testosterone in plasma and testis from rats fed VK deficient diet. Similar results regarding the role of MK-4 in testis and testosterone were also reported by Ito *et al*. (2011). Thus, since VK and warfarin are able to regulate gene expression of *pxr* in fish (Richard *et al*., 2014; Fernández *et al*., 2014), and expression of PXR downstream target genes is also activated under warfarin exposure (which induces a VK-like deficiency), the VK nutritional status might affect fish gametogenesis. In this regard, although a previous report stated that VK deficiency in the parental fish did not affect the hatching rate of the eggs or the mortality of the larvae (Udagawa and Hirose 1998), little is known regarding the potential roles of VK in fish gametogenesis.

The last, but not the least, interaction of VK with other molecules should be revealed in order to find optimal nutritional levels in aquafeeds. In this sense, VK bound to its nuclear receptor can interact at the nuclear level with other fat soluble vitamins. Not only VK can activate PXR (Tabb *et al*. 2003), all forms of vitamin E are also able to activate gene expression via PXR (Landes *et al*. 2003). Since PXR form heterotetramers with retinoid X receptors (RXR; Wallace *et al*. 2013), it also might interact signaling pathways regulated by its ligands, the retinoids (vitamin A). Interestingly, vitamins A and D influence expression and synthesis of VKDPs (Darias *et al*. 2010; Fernandez *et al*. 2011), while VK is responsible for the posttranslational modification and activation of those VKDPs (Oldenburg *et al*. 2008). Another potential crosstalk between fat soluble vitamins which remains to be revealed might be the intestinal absorption mechanisms, since all fat soluble vitamins share some intestinal absorption proteins (Gonçalves and Reboul, 2011; Blomhoff and Blomhoff, 2006).

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Conclusions

Studies from the 1990's and 2000's have revealed the basal requirements of VK in blood coagulation and evidenced its role in bone development and homeostasis (reviewed by Krossoy *et al*. 2011). More recently, research efforts on determination of VK dietary requirements on early developmental stages have also suggested new biological processes where VK might has an important role such as soft tissue pathological calcification, skeletogenesis, muscular contraction and energy metabolism among others. Certainly, new functions will be identified. Nevertheless, a reliable and accurate marker of VK nutritional condition in fish species is still lacking, hampering the determination of species and developmental stage specific VK requirements. Importantly, comparative studies on the suitability of the different K vitamers for aquafeed diets is an imperative for feed producers, which should be based on thermal and chemical stability, metabolic bioavailability and on toxicological parameters, for which the identification and characterization of enzymes and transport proteins are essential.

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References

Azuma, K., Casey, S.C., Ito, M., Urano, T., Horie, K., Ouchi, Y., Kirchner, S., Blumberg, B., Inoue, S. (2010) Pregnane X receptor knockout mice display osteopenia with reduced bone formation and enhanced bone resorption. *Journal of Endocrinology* **207**, 257-263.

Blomhoff, R., and Blomhoff, H.K. (2006) Overview of retinoid metabolism and function. *Journal of Neurobiology* **66**, 606-630.

Booth, S.L. & Suttie, J.W. (1998) Dietary intake and adequacy of vitamin K. *Journal of Nutrition* **128**, 785-788.

Brenner, B., Kuperman,. A.A., Watzka,. M., Oldenburg, J. (2009) Vitamin K-dependent coagulation factors deficiency. *Seminars in Thrombosis and Hemostasis* **35**, 439-446.

Cancela, M.L., Conceição, N., Laizé, V. (2012) Gla-rich protein, a new player in tissue calcification? *Advances in Nutrition* **3**, 174-181.

Chen, Y., Tang,Y., Guo, C., Wang, J., Boral, D., Nie, D. (2012) Nuclear receptors in the multidrug resistance through the regulation of drug-metabolizing enzymes and drug transporters. *Biochemical Pharmacology* **83**, 1112-1126.

Dam H. (1935) The antihaemorrhagic vitamin of the chick. Biochemistry Journal **29**, 1273-1285.

Darias, M.J., Mazurais, D., Koumoundouros, G. Cahu, C.L., Zambonino-Infante, J.L. (2010) Dietary vitamin D3 affects digestive system ontogenesis and ossification in European sea bass (*Dicentrachus labrax*, Linnaeus, 1758). *Aquaculture* **298**, 300-307.

Ekins, S., Reschly, E., Hagey, L.R., Krasowski, M.D. (2008) Evolution of pharmacologic specificity in the pregnane X receptor. *BMC Evolutionary Biology* **8**:103.

Emson, C.T., Suttie, J.W. (1976) Vitamin K dependent carboxylase. *The Journal of Biological Chemistry* **251**, 6238-6243.

Ferland, G. (2012) Vitamin K and the nervous system: an overview of its actions. *Advances in Nutrition* **3**, 204-212.

Fernández, I., Darias, M., Andree, K.B., Mazurais, D., Zambonino-Infante, J.L., Gisbert, E., 2011. Coordinated gene expression during gilthead sea bream skeletogenesis and its disruption by nutritional hypervitaminosis A. *BMC Developmental Biology* **11**, 7.

Fernández, I., Santos, A., Cancela, M.L., Laizé, V., Gavaia, P.J. (2014) Warfarin, a potential pollutant in aquatic environment acting through Pxr signaling pathway and γ-glutamyl carboxylation of vitamin K-dependent proteins. *Environmental Pollution* **194**, 86-95.

Fernández, I., Vijayakumar, P., Marques, C., Cancela, M.L., Gavaia, P.J., Laizé, V. (2015) Zebrafish vitamin K epoxide reductases: expression *in vivo*, along extracellular matrix mineralization and under phylloquinone and warfarin *in vitro* exposure. *Fish Physiology and Biochemistry* **41**, 745-759.

Fernández, I. 2015. Vitamin K, Much More Than Blood. An Update on its Roles, Metabolism and Requirements in Fish Species. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 361-376.

Fodor, D., Albu, A., Poantã , L., Porojan, M. (2010) Vitamin K and vascular calcifications. *Acta Physiologica Hungarica* **97**, 256-266.

Goncalves, A., Reboul, E. (2011) Identification of membrane proteins involved in Vitamin K intestinal transport. *Annals of Nutrition and Metabolism* **58**, 80-80.

Graff, I.E., Krossoy, C., Gjerdevik, K. & Julshamn, K. (2010) Influence of dietary menadione nicotinamide bisulphite (vitamin K3) and phylloquinone (vitamin K1) on Atlantic salmon (*Salmo salar* L.) tissue levels, determined by high-performance liquid chromatography with fluorescence detection. *Aquaculture Nutrition* **16**, 637-647.

Grahl-Madsen, E., Lie, O. (1997) Effects of different levels of vitamin K in diets for cod (*Gadus morhua*). *Aquaculture* **151**, 269-274.

Gray, M.A., Squires, E.J. (2012) Effects of nuclear receptor transactivation on steroid hormone synthesis and gene expression in porcine Leydig cells. *Journal of Steroid Biochemistry and Molecular Biology* **133**, 93-100.

Grisdale-Helland, B., Helland, S. & Åsgård, T. (1991) Problems associated with the present use of menadione sodium bisulfite and vitamin A in diets for Atlantic salmon. *Aquaculture* **92**, 351-358.

Halver, J. (2002) The vitamins. In: Fish Nutrition, 3rd edn (Halver, J. & Hardy, R. eds.), pp. 62–142. Academic press, New York, USA.

Hammed, A., Matagrin, B., Spohn, G., Prouillac, C., Benoit, E., Lattard, V. (2013) VKORC1L1, an enzyme rescuing the vitamin K 2,3-epoxide reductase activity in some extrahepatic tissues during anticoagulation therapy. *Journal of Biological Chemistry* **288**, 28733-28742.

Harshman, S.G., Saltzman, E., Booth, S.L. (2014) Vitamin K: dietary intake and requirements in different clinical conditions. *Current Opinion in Clinical Nutrition and Metabolism Care* 17, 531-538.

Ito, A., Shirakawa, H., Takumi, N., Minegishi, Y., Ohashi, A., Howlader, Z.H., Ohsaki, Y., Sato, T., Goto, T., Komai, M. (2011) Menaquinone-4 enhances testosterone production in rats and testis-derived tumor cells. *Lipids in Health and Disease* **10**:158.

Kaesler, N., Schettgen, T., Mutucumarana, V.P., Brandenburg, V., Jahnen-Dechent, W., Schurgers, L.J., Krüger, T. (2012) A fluorescent method to determine vitamin K-dependent gamma-glutamyl carboxylase activity. *Analytical Biochemistry* **421**, 411-416.

Karsenty, G., Ferron, M., 2012. The contribution of bone to whole-organism physiology. *Nature* **481**, 314-320.

Kaushik, S.J., Gouillou-Coustans, M.F., Cho, C.Y. (1998) Application of the recommendations on vitamin requirements of finfish by NRC (1993) to salmonids and sea bass using practical and purified diets. *Aquaculture* **161**, 463-474.

Krossoy, C., Waagbo, R., Fjelldal, P.G., Wargelius, A., Lock, E.-J., Graff, I.E., Ørnsrund, R. (2009) Dietary menadione nicotinamide bisulphite (vitamin K3) does not affect growth or bone health in first feeding fry of Atlantic salmon (*Salmo salar* L). *Aquaculture Nutrition* **15**, 638-649.

Fernández, I. 2015. Vitamin K, Much More Than Blood. An Update on its Roles, Metabolism and Requirements in Fish Species. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 361-376.

Krossøy, C., Lock, E.-J., Ørnsrud, R. (2010) Vitamin K-dependent γ-glutamylcarboxylase in Atlantic salmon (*Salmo salar* L.). *Fish Physiology and Biochemistry* **36**, 627-635.

Krossøy, C., Waagbø, R., Ørnsrund, R. (2011) Vitamin K in fish nutrition. *Aquaculture Nutrition* **17**, 585-594.

Landes, N., Pfluger, P., Kluth, D., Birringer, M., Rühl, R., Böl, G.F., Glatt, H., Brigelius-Flohé, R. (2003) Vitamin E activates gene expression via the pregnane X receptor. *Biochemistry Pharmacology* **65**, 269-273.

Li, T., Chang, C.Y., Jin, D.Y., Lin, P.J., Khvorova, A., Stafford, D.W. (2004) Identification of the gene for vitamin K epoxide reductase. *Nature* **427**, 541-544.

Luo, G.B., Ducy, P., McKee, M.D., Pinero, G.J., Loyer, E., Behringer, R.R., Karsenty, G. (1997) Spontaneous calcification of arteries and cartilage in mice lacking matrix GLA protein. *Nature* **386**, 78-81.

Magnusson, S., Sottrup-Jensen, L., Petersen, T.E., Morris, H.R., Dell, A. (1974) Primary structure of the vitamin K-dependent part of prothrombin. *FEBS Letters* **44**, 189-193.

Makishima, M. (2005) Nuclear receptors as targets for drug development: regulation of cholesterol and bile acid metabolism by nuclear receptors. *Journal of Pharmacological Sciences* **97**, 177-183.

Manfioletti, G., Brancolini, C., Avanzi, G., Schneider, C. (1993) The protein encoded by a growth arrest-specific gene (Gas6) is a new member of the vitamin K-dependent proteins related to protein S, a negative coregulator in the blood coagulation cascade. *Molecular and Cellular Biology* **13,** 4976-4985.

Marchetti, M., Tossani, N., Marchetti, S., Bauce, G. (1999) Stability of crystalline and coated vitamins during manufacture and storage of fish feeds. *Aquaculture Nutrition* **5**, 115-120.

Maree, A.O., Jneid, H., Palacios, I.F., Rosenfield, K., MacRae, C.A., Fitzgerald, D.J. (2007) Growth arrest specific gene (GAS) 6 modulates platelet thrombus formation and vascular wall homeostasis and represents an attractive drug target. *Current Pharmaceutical Design* **13**, 2656-2661.

Mathers, J.C., Fernandez, F., Hill, M.J., McCarthy, P.T., Shearer, M.J., Oxley, A. (1990) Dietary modification of potential vitamin K supply from enteric bacterial menaquinones in rats. *British Journal of Nutrition* **63**, 639-652.

Nelsestuen G.L., Zytkovic, T.H. Howard, J.B. (1974) Mode of action of vitamin-k - identification of gamma-carboxyglutamic acid as a component of prothrombin. *Journal of Biological Chemistry* **249**, 6347–6350.

Oldenburg, J., Marivona, M., Müller-Reible, C., Waltzka, M. (2008) The vitamin K cycle. *Vitamins and Hormones* **78**, 35-62.

Oldenburg, J., Watzka, M., Bevans, C.G. (2015) VKORC1 and VKORC1L1: Why do Vertebrates Have Two Vitamin K 2,3-Epoxide Reductases? *Nutrients* **7**, 6250-6280.

Fernández, I. 2015. Vitamin K, Much More Than Blood. An Update on its Roles, Metabolism and Requirements in Fish Species. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 361-376.

Poston, H.A. (1976) Relative effect of two water-soluble analogues of menaquinone on coagulation and packed cell volume of blood of lake trout (*Salvelinus namaycush*). *Journal of the Fisheries Research Board of Canada* **33**, 1791-1793.

Price, P.A., Poser, J.W., Raman, N. (1976) Primary structure of gamma-carboxyglutamic acid-containing protein from bovine bone. *Proceedings of the National Academy of Sciences of the United States of America* **73**, 3374-3375.

Price, P. A., Urist, M. R., Otawara, Y. (1983). Matrix Gla-protein, a new gamma-carboxyglutamic acid-containing protein which is associated with the organic matrix of bone. *Biochemical and Biophysical Research Communications* **117**, 765-771.

Richard, N., Fernández, I., Wulff, T., Hamre, K., Cancela, M.L., Conceição, L.E.C., Gavaia, P.J. (2014) Dietary supplementation with vitamin K affects transcriptome and proteome of Senegalese sole, improving larval performance and quality. *Marine Biotechnology* **16**, 522-537.

Rost, S., Fregin, A., Ivaskevicius, V., Conzelmann, E., Hortnagel, K., Pelz, H. J., Lappegard, K., Seifried, E., Scharrer, I., Tuddenham, E. G., Muller, C. R., Strom, T. M., Oldenburg, J. (2004). Mutations in VKORC1 cause warfarin resistance and multiple coagulation factor deficiency type 2. *Nature* **427**, 537-541.

Roy, P., Lall, S. (2007) Vitamin K deficiency inhibits mineralization and enhances deformity in vertebrae of haddock (*Melanogrammus aeglefinus* L.). *Comparative Biochemistry and Physiology* **148**, 174-183.

Shearer, M.J., Newman, P. (2014) Recent trends in the metabolism and cell biology of vitamin K with special reference to vitamin K cycling and MK-4 biosynthesis. *Journal of Lipid Research* **55**, 345-362.

Shirakawa, H., Ohsaki, Y., Minegishi, Y., Takumi, N., Ohinata, K., Furukawa, Y., Mizutani, T., Komai, M., 2006. Vitamin K deficiency reduces testosterone production in the testis through down-regulation of the Cyp11a a cholesterol side chain cleavage enzyme in rats. *Biochimica et Biophysica Acta* **1760**, 1482-1488.

Stenflo, J., Fernlund, P., Egan, W., Roepstorff, P. (1974) Vitamin K dependent modifications of glutamic acid residues in prothrombin. *Proceedings of the National Academy of Sciences of the United States of America* **71**, 2730-1733.

Suttie, J.W. (1995) The importance of menaquinones in human nutrition. *Annual Review of Nutrition* **15**, 399-417.

Tabb, M.M., Sun, A., Zhou, C., Grün, F., Errandi, J., Romero, K., Pham, H., Inoue, S., Mallick, S., Lin, M., Forman, B.M., Blumberg, B. (2003) Vitamin K2 regulation of bone homeostasis is mediated by the steroid and xenobiotic receptor SXR. *Journal of Biological Chemistry* **278**, 43919-43927.

Tan, B., Mai, K. (2001) Effects of dietary vitamin K on survival, growth, and tissue concentrations of phylloquinone (PK) and menaquinone-4 (MK-4) for juvenile abalone, *haliotis discus hannai* Ino. *Journal of Experimental Marine Biology and Ecology* **256**, 229-239.

Fernández, I. 2015. Vitamin K, Much More Than Blood. An Update on its Roles, Metabolism and Requirements in Fish Species. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 361-376.

Udagawa, M., Hirose, K. (1998) Physiological abnormality and the tissue vitamin K level of mummichog *Fundulus heteroclitus* fed with a vitamin K free diet. *Fisheries Science* **64**, 612-616.

Viegas, C.S.C., Simes, D.C., Laize, V., Price, P.A., Cancela, M.L. (2008) Gla-rich protein (GRP), a new vitamin K-dependent protein identified from sturgeon cartilage and highly conserved in vertebrates. *Journal of Biological Chemistry* **283**, 36655-36664.

Wallace, B.D., Betts, L., Talmage, G., Pollet, R.M., Holman, N.S., Redinbo, M.R. (2013) Structural and Functional Analysis of the Human Nuclear Xenobiotic Receptor PXR in Complex with RXRa. *Journal of Molecular Biology* **425**, 2561-2577.

Westhofen, P., Watzka, M., Marinova, M. (2011) Human vitamin K 2,3-epoxide reductase complex subunit 1-like 1 (VKORC1L1) mediates vitamin K-dependent intracellular antioxidant function. *Journal of Biological Chemistry* **286**, 15085-15094.

Woodward, B. (1994) Dietary vitamin requirement of cultured young fish, with emphasis on quantitative estimates for salmonids. *Aquaculture* **124**, 133-168.

Wu, S.M., Cheung, W.F., Frazier, D., Stafford, D.W. (1991) Cloning and expression of the cDNA for human gamma-glutamyl carboxylase. *Science* **254**, 1634-1636.

Fernández, I. 2015. Vitamin K, Much More Than Blood. An Update on its Roles, Metabolism and Requirements in Fish Species. En: Cruz-Suárez, L.E., Ricque-Marie, D., Tapia-Salazar, M., Nieto-López, M.G., Villarreal-Cavazos, D. A., Gamboa-Delgado, J., Rivas Vega, M. y Miranda Baeza, A. (Eds), Nutrición Acuícola: Investigación y Desarrollo, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Nuevo León, México, ISBN 978-607-27-0593-7, pp. 361-376.