Norwegian University
of Life Sciences

Master's Thesis 2024 30 ECTS
Faculty of Biosciences

Influence of vitamin K_3 on Atlantic salmon (*Salmo salar* L.)

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Aquaculture

Content

Abstract

The aim of this thesis was to find the optimal level of vitamin K_3 based on general health and bone health in Atlantic salmon (*Salmo salar* L.) in early sea phase. This was done by testing six diets with increasing levels of menadione nicotinamide bisulphite (MNB) (0, 5, 10, 15, 20 and 25 mg/kg) on Atlantic salmon in early sea phase, the experiment lasted for 105 days, and the salmon were fed their respective diets for 102 days. There were two samplings, one after feeding for 74 days and the other was at the end of the study.

Growth as well as other biometric traits of the salmon were monitored. The salmon were also analysed for blood parameters, mechanical properties of vertebra, deformities, content of minerals (calcium and phosphorous) of vertebral column and gene expression of *bone Gla protein* (*BGLAP*), *gla rich protein* (*GRP*), *periostin*, *protein S* and *growth arrest specific 6* (*GAS6*).

The analyses of vitamin K_3 content in the feed revealed that diet with 0 mg/kg MNB had 0.1 mg/kg vitamin K_3.

There were conducted preliminary testing of analysing mechanical properties of vertebrae to train on the analysis and to find the proper setting to conduct this analysis on the vertebrae from the study.

There were no significant ($P>0.05$) differences between the dietary groups on growth, biometric traits, blood parameters, mechanical properties of vertebra, content of minerals (phosphorous and calcium) in vertebral column and gene expression.

The linear regression between the dietary groups on incidents of deformities, growth, mechanical properties of vertebrae and gene expression were not significant ($P>0.05$), however, linear regression of calcium content in the vertebral column was significant ($P=0.025$).

Based on the results from the present study, the optimal level of MNB for Atlantic salmon in early sea phase is 0.1 mg/kg MNB, based on the analyses conducted in the present study.

This study has contributed to more knowledge of effect of vitamin K_3 on general health and bone health, MNB do not seem to influence on general health and bone health. However, the

calcium content seemed to be influenced by MNB and it remains to further investigate the effect of more calcium in the vertebral column.

Acknowledgements

First and foremost, my gratitude goes to my supervisors, Turid Mørkøre as my internal supervisor at Norwegian University of Life Sciences and my external supervisors Ramon Fontanillas and Vibeke Vikeså at Skretting. Turid Mørkøre has suggested and helped me with analyses amongst others. I want to thank Ramon Fontanillas for all the time he has spent helping me with literature, analysation as well as providing constructive feedback. Vibeke Vikeså has provided me with a guest office as well as made sure that I felt at home at Skretting. I additionally want to thank Skretting for letting me participate in their research project and allowing it to be my master thesis.

I also want to thank Diana Urrea and the employees at the Feed Trial Plant for their technical assistance and letting me perform analysation of mechanical vertebrae there. In addition, I would like to thank Matthew Owen for his help with the deformity scoring. I also want to thank Piotr Lisik, Stefanie Lackner and Nicolay Neverdahl for their work on the gene expression analysis and their help related to the analysis and results. I also want to thank the rest of the employees at Skretting in Sjøhagen 3, Sjøhagen 6, Sjøhagen 11 and Lerang research station, for their hospitality.

In addition, I want to thank my friends and family for their support. Especially my partner has shown an enormous patience in this stressful time.

1 Introduction

Aquaculture is the world's fastest growing food production industry (Asche & Roll, 2013). The production has grown drastically from 2.6 million metric tons produced in 1970 to 87.5 million metric tons produced in 2020 (Afewerki et al., 2023). The incline of farmed salmon is higher than other species. Norway is the main producer of farmed salmon and Atlantic salmon (*Salmo salar* L.) makes up of most farmed salmon (Asche & Roll, 2013). In 2014, there was set an ambitious goal by the Norwegian government, to increase the production of farmed salmon from 1 million tons to 5 million tons by 2050 (Bailey & Eggereide, 2020).

While having such ambitions for economic growth, the matter of sustainability is of great importance (Yarkina & Logunova, 2022). Fish welfare is an important concept for the sustainable production of Atlantic salmon. Welfare is complex as it encompasses both the physical and emotional condition of the individual (Webster, 2016). Feed can have a positive effect on the physical state of the individual by preserving a normal health, maintaining a healthy animal performance in addition to prevent signs of deficiency (Oliva-Teles, 2012, p. 83).

The ingredients in salmon fish feed have shifted from marine dominating to plant dominating. The marine oils and proteins sources have been reduced steadily from 1990 to 2020 (Aas et al., 2022). This shift is explained by a shortage of fish meal an fish oil and a simultaneous growth of farmed salmon (Ytrestøyl et al., 2015). The change of ingredients has altered the nutrient composition of the diet. It has therefore changed the level of micronutrient as well as their chemical form. Additionally, the change in the diet has resulted in the presence of compounds that interact with micronutrient uptake and metabolism (Vera et al., 2020). Micronutrients are important for metabolism and maintenance of tissue function (Shenkin, 2006, p. 11). Vitamins can have a positive effect on health (Oliva-Teles, 2012, p. 92). It has additionally been seen positive effects of micronutrients above what has been recommended by National Research Council (2011) for Atlantic salmon, in diets with low levels of marine ingredients (Vera et al., 2019).

There has been a big focus on the effect of plant-derived feed ingredients on growth, feed quality, feed utilization and health. However, there are less studies examining the effect on bone health in salmon fed diets low in marine ingredients. Studying bone health is relevant as there are challenges related skeletal malformation occurring in the industry (Veterinærinstituttet,

2023, pp. 69, 262-268; Ytteborg et al., 2012, p. 329). Skeletal malformation can impair growth, have a negative effect on fillet quality and there is additionally a higher metabolic cost in fish with bone deformities (Baeverfjord et al., 2019). The skeletal malformation also has a negative consequence for welfare and health of the fish (Drábiková, 2023, p. 16). Bone health in farmed salmon is therefore of importance for monetary, environmental and ethical reasons (Baeverfjord et al., 2019).

Micronutrients including phosphorous, calcium, vitamin A and vitamin K are especially important in development of the skeleton, as it also been documented that deficiency or toxicity can lead to pathogenesis of skeletal deformities (Vera et al., 2019). Vitamin K has not received much attention, and there is limited knowledge about the mechanism of vitamin K in Atlantic salmon (Krossøy et al., 2011; Tsugawa & Shiraki, 2020, p. 1). Studies have revealed that vitamin K effect growth, level of blood cells and calcium accumulation in muscle tissue and vertebrae (Wei et al., 2023). Vitamin K effect growth, survival, bone health in Gilthead seabream (*Sparus aurata*) (Dominguez et al., 2022; Sivagurunathan et al., 2023)

Vitamin K is involved in bone metabolism (Lall & Lewis-McCrea, 2007), as the vitamin is essential for vitamin K-dependent proteins (VKDP). The VKDPs osteocalcin, Matrix Gla proteins, Gla rich proteins, Growth arrest 6, periostin and protein S have been stated to influence bone formation (Krossøy et al., 2011; Maillard et al., 1992; Stock & Schett, 2021; Tsugawa & Shiraki, 2020, p. 1; Xiao et al., 2021). In this master thesis, the aim was to determine the optimal level of vitamin K in feed for Atlantic salmon in the sea phase based on: 1) bone health and 2) general health. It was hypothesized that vertebrae from salmon fed 10 mg/kg vitamin K have a significantly stronger vertebrae than salmon fed 0 mg/kg vitamin K.

2 Theory

2.1 Vitamin K

Vitamin K appears in three forms, K_1, K_2 and K_3. Vitamin K_1 and K_2 are fat soluble, while vitamin K_3 is water soluble (Hardy & Kaushik, 2022, p. 144). Vitamin K_3 is synthetic and is the form that is mostly used as feed supplement today (Krossøy et al., 2011). The vitamins consist of 2-methyl-1, 4-napththoquionene ring, and vitamin K_1 and K_2 has a side chain at the 3-position (Isler & Wiss, 1959, pp. 54-55; Vermeer et al., 2017). It is believed that vitamin K_3 is water soluble because it has one hydrocarbon chain less than the others (Stock & Schett, 2021). However, it is also claimed that vitamin K_3 is fat soluble (Dubbs & Gupta, 1998). The required level of vitamin K varies depending on vitamin K source used (Krossøy et al., 2011). Additionally, the life stage, external factors, age and health status will impact their requirement (Krossøy et al., 2011). Vitamin K requirement is of importance to detect as vitamin K deficiency can reduce growth and prolong clotting time among others, in Amago salmon (*Onchohyncus rhodurus*) (Taveekijakarn et al., 1996).

The recommended level of vitamin K for salmon is 10 mg/kg (Graff et al., 2010). Marine proteins and oils are sources of natural occurring vitamin K (Graff et al., 2010; Krossøy et al., 2009a). In addition, vegetable oil also consist of vitamin K, however there is limited knowledge about the bioavailability (Graff et al., 2010).

It has been suggested to add a higher supplementation of vitamin K_3 above 12 mg/kg in diets for Gilthead seabream, in diets of dominating plant sourced ingredients (Dominguez et al., 2022). Contrary to this, 0.1 mg/kg vitamin K has been proven to be adequate for the minimal requirements for a normal health, growth and bone strength of juvenile salmon (Krossøy et al., 2011). While an optimal level can be health promoting by increasing growth and reduce deformities on seabream larvae, a too high level of vitamin K_3 can cause higher incidence of the deformity kyphosis, reduced mineralization and unbalances in expression of genes that are vitamin K dependent and influence bone health in seabream larvae (Sivagurunathan et al., 2023). A healthy level of vitamins are necessary for the body to function, however excessive intake of vitamins can be harmful (Shenkin, 2006, p. 11). This makes it important to determine the optimal level of Vitamin K for Atlantic salmon in sea phase as well.

2.1.1 Vitamin K and coagulation

Atlantic salmon require vitamin K for a healthy haemostasis (Salte & Norberg, 1991, p. 39). This has been known for a relative long time as it was discovered in 1936 that Vitamin K is a major constituent of blood clotting (Halder et al., 2019). Vitamin K is important for activation of both coagulation and anticoagulation proteins (Krossøy et al., 2011). This is because vitamin K carboxylate several coagulation factors that participate in the processes (Górska, 2019, p. 112).

Under a vascular injury, a haemorrhage is prevented by creating a clot. A coagulation cascade gets activated to stop the bleeding. The cascade leads to production of fibrin which seals the wound. The process of clotting must be active for a required time, so the fibrin can seal the wound (Norris, 2003, pp. 369-374). Additionally, it is necessary with a haemostatic system that quickly recognize wounds and produce a plug to end the blood loss (Nelsestuen et al., 2000, p. 359).

The coagulation cascade pathways consist of an intrinsic and an extrinsic pathway. The intrinsic pathway is induced by factors XII and XI. The extrinsic pathway consist of activated tissue factor creating a complex with factor VII (Nelsestuen et al., 2000, p. 360). When tissue factor binds to factor VII (VIIa), the coagulation process starts (Figure 1). Factor VIIa has a greater catalytic activity than factor VII. The tissue factor and VIIa further activates IX to IXa and factor X to Xa (Green, 2006). VIIIa, tissue factor and Va are cofactors, promoting the activation of zymogen. Factors V and VIII are turned into their active forms by thrombin (Nelsestuen et al., 2000, p. 360).

Factor Xa further activates factor V. Thrombin activates platelets, factor V, factor XI. Factor XI activates factor IX. Factor VIIIa, IXa and calcium ions are formed on the platelets surface which results in a high production of factor Xa. Factor Xa with factor Va and calcium ions creates a complex which produces thrombin, and this is used for the conversion of fibrinogen to fibrin. Thrombin additionally activates platelets, factor V, VIII and XI, meaning it catalyses its own formation. Thrombin additionally activates factor XIII, which promotes clot stabilization and the thrombin-activatable fibrinolysis inhibitor, regulating fibrinolysis (Green, 2006).

The factors VII, IX, X, prothrombin, protein C, protein S and protein Z are vitamin K dependent (VKD). Gla residues are additionally important for calcium binding ability to these proteins and the proteins will not function without the Gla (Nelsestuen et al., 2000, p. 356).

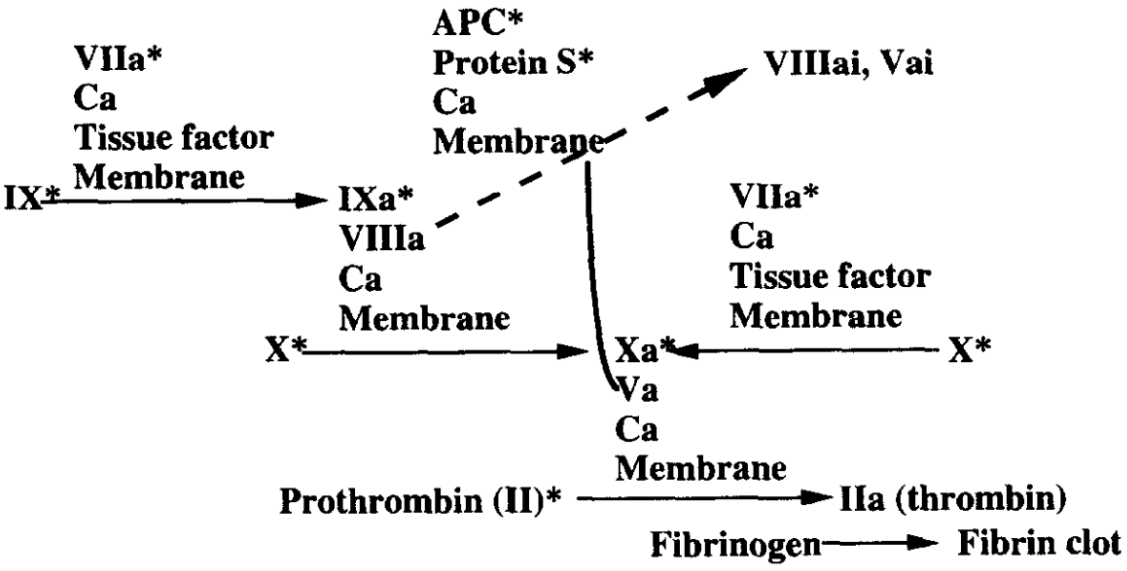

Figure 1: Schematic overview of the workflow of the factors activated in the blood clotting cascade, with emphasis on the vitamin K dependent proteins. They are marked with an asterisk (*). APC= Activated protein C. Procoagulation reactions in solid lines and inhibition reactions are presented in dashed lines. APC, Protein S, Ca membrane inactivates Va. Resulting in a fibrin clot to stop the bleeding. From: (Nelsestuen et al., 2000, p. 359).

Haematological tests are used when investigating metabolic disturbances and diseases (Sandnes et al., 1988, p. 129). Low haemoglobin (Hb) and haematocrit (HTC) values can indicate anaemia (Vangen & Hemre, 2003). A study by Rozas-Serri et al. (2022) made reference intervals for blood parameters including Hb and HTC of healthy Atlantic salmon. The reference interval of Hb value ranged from 154.32-636.29 g/L for Atlantic salmon in the smolt phase and 326.56-780.92 g/L for Atlantic salmon in the post smolt phase. A reference interval of 23.97 – 56.60 % HTC was determined for smolt and 32.62 – 60.94 % for Atlantic salmon in the post smolt phase (Rozas-Serri et al., 2022).

Lack of vitamin K can increase coagulation time and reduce HTC in Brook trout (Poston, 1964, p. 62). According to Litvinov and Weisel (2017, p. 1), red blood cells can influence blood clotting, as Hb is proteins in red blood cells and HTC is percentage of red blood cells. It could therefore be beneficial to measure the Hb and HTC in addition to clotting time.

2.1.2 Vitamin K cycle

Vitamin K plays an important role in reaction of glutamate (Glu) residues into γ-carboxyglutamate (Gla) residues by the reaction γ-carboxylation (De Vilder et al., 2017). The reaction is believed to be required for activation of Gla-proteins (Benzakour, 2008). The enzyme, named gamma glutamyl carboxylase (GGCX) together with a reduced form of vitamin K (KH_2), carbon dioxide and oxygen working as cofactors, participate in the carboxylation (Tie & Stafford, 2016) (Figure 2). GGCX serve as a catalysator to the reaction (De Vilder et al., 2017).

KH_2 is oxidized into vitamin K 2,3-epoxide (KO) simultaneously with the carboxylation of Glu to Gla. KO is transferred to KH_2 after the reaction. This occurs by a two-step reaction. The enzyme vitamin K epoxide reductase (VKOR) participates in the reaction from KO to vitamin K. The enzyme vitamin K reductase (VKR) participate in the reaction from vitamin K to KH_2 (Tie & Stafford, 2016). This makes vitamin K able to carboxylate another Glu residue (Górska, 2019, p. 113).

GGCX is an enzyme with two functions and several substrates. Carboxylation requires energy, the GGCX oxidizes KH_2 to KO to get the energy that is required. The functional side for GGCX is a propeptide binding site, glutamate binding site, vitamin K binding site, carboxylation active site, epoxidation active site and a carbon dioxide binding site. It recognizes proteins by forming a close binding to the propeptide of vitamin-K dependent proteins (Tie & Stafford, 2016). GGCX play an important role in bone formation as it influence osteocalcin (De Vilder et al., 2017). Vitamin K is not consumed in the process of γ-carboxylation, it is recycled. There is therefore a relatively low requirement for this vitamin (Stock & Schett, 2021).

There has been measured GGCX in bones in Atlantic salmon, which indicate that vitamin K can influence bone metabolism (Krossøy et al., 2010). There is limited knowledge on the bioavailability of MNB. Liver is believed to be the biggest storage organ of vitamin K (Krossøy et al., 2009a). According to Graff et al. (2010) some of the MNB is converted to menaquinone-4 (MK-4) in Atlantic salmon. MK-4 is a type of vitamin K_2. It is believed that because MNB is converted to MK-4 it is less bioavailable than vitamin K_1 and Vitamin K_2 (Graff et al., 2010; Krossøy et al., 2010). In the study by Graff et al. (2010) 1000 mg/kg MNB was added to the diets, 46.5 mg/kg MNB was measured of the analysed diet, additionally, only about 0.1 mg/kg MK-4 was measured in the liver of Atlantic salmon fed 46.5 mg/kg MNB.

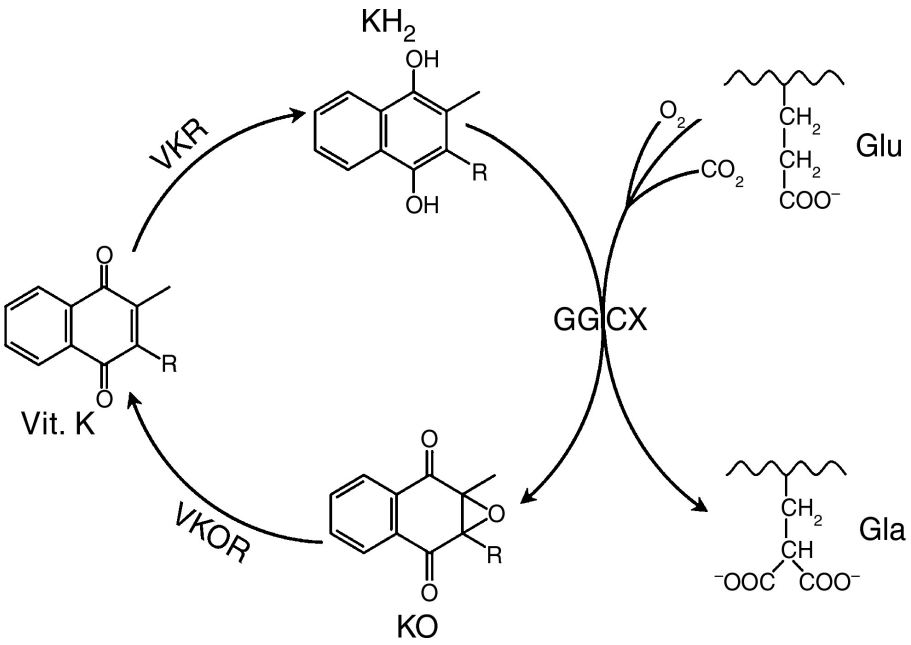

Figure 2: Vitamin K cycle. Vitamin K is reduced to KH_2 when reacting with the enzyme VKR. KH_2 is then utilized together with GGCX, oxygen and carbon dioxide to transfer Glu residues to Gla residues in vitamin K-dependent proteins. KH_2 is oxidized into KO and reacts with the enzyme VKOR into regular form of vitamin K and then reacts with a new VKR. This reaction is called γ-carboxylation. From (Tie & Stafford, 2016).

Up until the 1970s it was believed that the main function of vitamin K was as a coenzyme in the synthesis of plasma coagulation proteins (Graff et al., 2002). However it has been discovered that vitamin K additionally influence the bone metabolism (Krossøy et al., 2010).

2.2 Skeleton

The skeleton adds stiffness and toughness to the body (Currey, 2014, p. 4; Drábiková, 2023, p. 32). Stiffness refers to resilience against deformations under mechanical load and toughness refers to ability to avoid fracture when exposed to mechanical loading (Drábiková, 2023, p. 32). The bones additionally function as a site for muscle attachment and protection for vital organs and cells (Baeverfjord et al., 2019).

Important processes of the skeleton include bone matrix synthesis, matrix mineralization, resorption and remodelling. The processes are important for growth of the skeleton, biomechanics, mineral homeostasis, and bone repair (Drábiková, 2023, pp. 31 - 32). Bone consists of organic compounds made up of collagenous, non-collagenous proteins and a mineral phase. The minerals are made up of calcium and phosphate (Jähn & Bonewald, 2012, p. 1). Skeletal resorption is necessary for bone growth and remodelling in teleost species (Drábiková, 2023, p. 32). Adult teleost experience changes to the skeleton which includes mechanical

adaptions, repair, mineral homeostasis; being associated amongst other with sexual maturation and aging (Witten & Hall, 2015, p. 727).

The collagenous matrix increase the toughness while the mineralized part improves the stiffness of the bone, whereas bone matrix contributes to the structure of the bone (Drábiková, 2023, p. 32). Bone extra cellular matrix (ECM) constitutes of a mineral part consisting of hydroxyapatite and of an organic part which is mostly made up of collagen in addition to non-collagenous proteins. The non-collagenous proteins consist mainly of osteocalcin (Carvalho et al., 2021, pp. 1-5).

There is limited knowledge about the effect of non-collagenous proteins in osteogenic differentiation. It is hypothesized that non-collagenous proteins have an important role in cell attachment, differentiation, and regulation of hydroxyapatite mineral deposition. However, the mechanisms for non-collagenous proteins is still not fully understood (Carvalho et al., 2021, p. 6).

2.3 Bone development

Bone is made up of 3 main cell types: osteoblast, osteoclast and osteocytes. Osteoblasts and osteoclasts attach to ECM (Jähn & Bonewald, 2012, p. 1). Osteoclasts breakdown bone. This occurs by osteoclasts moving to the surface of the bone and form into a multinucleate cell with other osteoclasts and breakdown bone by digesting the ECM. Osteoblasts create bone, by excreting type I collagen and non-collagenous proteins into the ECM, which then mineralizes. Osteoblasts develop into osteocyte. Osteocytes communicate with other cells on the surface of the bone. It is believed that osteocytes have as function to sense distribution and amount of mechanical strain the bone is exposed to (Jähn & Bonewald, 2012, pp. 1-5).

According to Mackie et al. (2008), cartilage is of importance for creating a stable starting point for bone formation, in addition to being a source for longitudinal bone growth. Further, endochondral ossification is an important process for bone development. The process is made up of several phases. Initially, proliferation, followed by hypertrophy and death of chondrocyte which is then replaced by bone. The cartilage ECM gets infiltrated by blood vessels, osteoclasts, bone marrow and osteoblasts (Mackie et al., 2008, pp. 47-48).

According to Mackie et al. (2008), chondrocytes, in the growth cartilage, are placed in zones, reflecting their functional state. Zone furthest away from the ossification front is the zone of resting chondrocytes. Adjacent to this is the zone of proliferation. The site next to that is the transition stage known as pre-hypertrophic chondrocytes. These cells undergo hypertrophy, which results in a higher volume while simultaneously secreting ECM which subsequently mineralize. Chondrocyte proliferation and matrix secretion results in elongation of the bone. Hypertrophic chondrocytes die and the cartilage matrix surrounding them are broken down (Mackie et al., 2008, p. 47).

There is limited knowledge on the molecular mechanisms that regulate the change of distal into proliferating chondrocytes. It has been suggested that Gla rich protein (GRP) can control the differentiation process in endochondral ossification (Tagariello et al., 2008).

2.4 Vertebral column

According to Nordvik et al. (2005), the vertebral column is made up of 4 main layers. Chordacentrum, autocentrum, cartilages of the neural and haemal arches and the arcocentrum (Figure 3 A-D). Chordacentrum is the first structure to develop in the vertebral body, and they appear in the notochordal sheath. Chordacentrum is the foundation for the development of autocentrum. Autocentrum consists of sclerotomal osteoblasts, created through ossification. The arcocentrum develops from direct ossification on the surface of the cartilage of neural and haemal arches. The outer part of the vertebral column is made up of cancellous bone, attributable to a woven collagen matrix and high level of osteocyte content (Nordvik et al., 2005).

Chordocytes are notochord cells and contribute together with chordoblasts to the notochord sheath (Drábiková, 2023, p. 34). Notochord is present at early life span of salmon. It attribute with structural support to the salmon before vertebral column is developed (Wang et al., 2014). During development of the vertebral column, the notochord is present in three forms, the intervertebral ligaments, the chordacentra, the internal components of the intervertebral capsule (Kryvi et al., 2017).

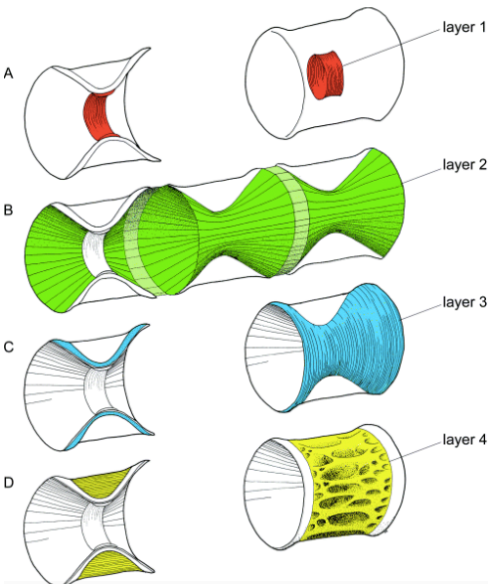

Figure 3: 4 Layers in the vertebrae in Atlantic salmon. A: Layer 1 is the chordacentrum formed during the mineralization of the notochordal sheath, B: Layer 2 is a thin layer covering the notochord, C: Layer 3 consist of the laminar bone in the amphicoel, D: Layer 4 is made up of cancellous bone. From (Nordvik et al., 2005).

Vertebral bodies are made up of vertebral centra, heamal and neural arches, haemal and neural spines and ribs (Drábiková, 2023, p. 35). According to Stiassny (2000), the teleost vertebral column is built up of vertebrae, formed as blocks. The vertebrae are connected by intervertebral joints. Teleostean vertebrae are often amphicoelous, meaning they have concave form at the ends. The intervertebral joints consist of tissue that fill the intervertebral space. The joint has two main functions, to attach to the centra of the vertebrae next to the joint and to absorb mechanical shock. Because of the functions, the structure allows for lateral flexibility and resisting compression at the same time (Stiassny, 2000, p. 114).

The vertebral column stores the spinal cord in the neural canal, according to Stiassny (2000). The canal is placed dorsal to the centra by the neural arch of each vertebra. The neural and haemal arches for most adult teleost species, become fully fused to the vertebral centra, which results in a rigid framework for muscle attachment. The structure of the column varies across the length to meet the required functions at the different sites. Posteriorly, the caudal vertebrae are developed to facilitate basal support and framework for caudal fin. The dorsal and ventral column support and is a site for muscle attachment for median fins and body musculature. The viscera is protected in the abdominal region (Stiassny, 2000, p. 114).

The spine also transports the blood vessels beneath the centra within a protective haemal canal in the caudal parts of the fish, according to Stiassny (2000). There are two main types of

vertebrae, precaudal and caudal vertebrae. Precaudal vertebrae contribute to the framework for the abdominal cavity as well as hold the parapophyses which the pleural ribs are attached to. They lack closed haemal arches and spines. The caudal vertebra lacks parapophyses and pleural ribs, and the caudal artery is in a closed haemal canal. Caudal vertebrae are often attached to well-developed haemal and neural spines. This results in an increased surface area for the attachment of the body musculature in the caudal region (Stiassny, 2000, pp. 114-115).

The fish vertebrae consist mainly of calcium, phosphate, and carbonate. Additionally, it consists of low levels of magnesium, sodium, strontium, lead, citrate, fluoride, hydroxide and sulphate. The vertebrae matrix consist a high level of non-collagenous proteins such as glycoproteins and osteocalcin (Lall & Lewis-McCrea, 2007).

Trabecular and cortical bone contribute to the mechanical functions of the bone (Burr, 2019, p. 3). Trabecular bone is a porous form of bone tissue. The lamella inside the trabeculae is arranged in single trabeculae and are not vascularized. The space between trabeculae is filled with bone marrow (McNamara, 2017, p. 211). Even though trabeculae supports to mechanical loading, cortical bone play a more important role to this function (Burr, 2019, p. 3). Cortical bone is dense and have low porosity. It forms the shell of vertebrae. Their function is additionally to facilitate movement (McNamara, 2017, p. 210).

The vertebral column consists of vertebral body and vertebral arches (Figure 4). The centrum enclose the notochord that contributes with mechanical strength to the body, while the arches have as function to enclose and protect the spinal cord and axial blood vessels (Fleming et al., 2015, p. 1733)

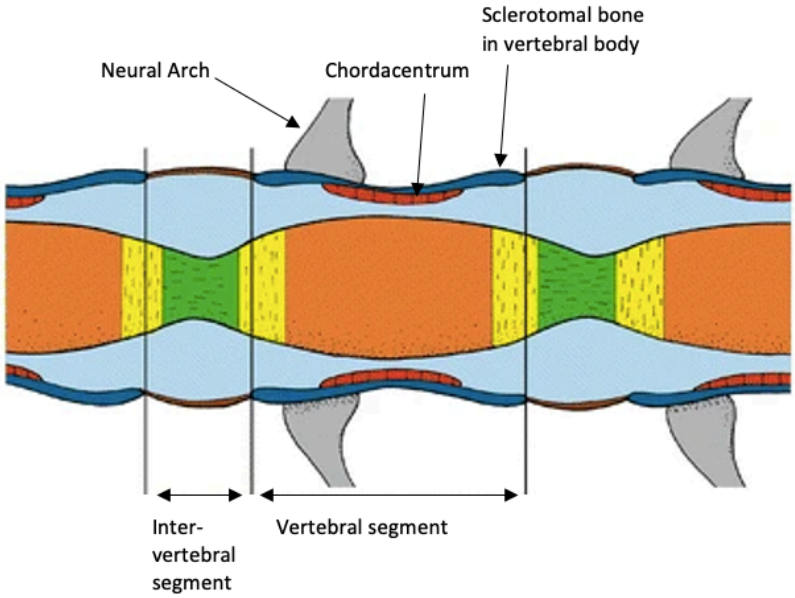

Figure 4: Vertebral column of Atlantic salmon (*Salmo salar* L.). Notochordal sheath in light blue colour, chordoblast in green colour and segmental zones within the chordoblast in yellow and orange colour. Adjusted from (Grotmol et al., 2003).

2.5 VKDPs related to bone

VDKPs consist of a wide range of different structures and functions (Shearer & Newman, 2014). In addition to the VKDPs that participate in coagulation processes that already has been described, factor VII, IX, X, prothrombin, protein C and protein Z (Nelsestuen et al., 2000, p. 356).

There has additionally been detected six VKDPs that are related to bones. This include Osteocalcin, matrix Gla protein (MGP), Gla-rich protein (GRP), periostin, protein S and growth arrest specific 6 (GAS6) (Stock & Schett, 2021). Gla residues have a high affinity to create bonds with calcium (Lall & Lewis-McCrea, 2007). There has been discovered some vitamin K dependent protein that influence growth (Krossøy et al., 2010).

Osteocalcin, also called bone GLA protein (BGLAP) is the dominating non-collagenous protein in bone. MGP and GRP are dominating in cartilage (Stock & Schett, 2021). MGP is also present in bone Hale et al. (1988, p. 5822). Periostin, protein S and GAS6 are present in skeletal tissues. The functions of these cover a wide range, including manage calcification and bone and cartilage turnover (Stock & Schett, 2021).

Vitamin K deficiency signs consist of increased blood coagulation time, reduced growth, anaemia, haemorrhages, loss of fin tissue, weak bones and occurrence of spinal curvature, short tails and even mortality in fish (Krossøy et al., 2011).

According to Krossøy et al. (2010) RT-PCR results of GGCX from vertebrae revealed low expression indicating that there is a relatively low levels. This can mean that there is a requirement for vitamin K in vertebrae, altough it is relatively low.

The functions of vitamin K and vitamin K dependent proteins (VKDPs) have been complicated to determine. The physiological consequence of non-carboxylated and undercarboxylated proteins are unknown (Krossøy et al., 2010).

2.5.1 BGLAP

BGLAP is a marker for bone formation and is particularly expressed in osteoblasts (Komori, 2020, p. 2).Gene expression of *BGLAP* has been found in vertebrae of Atlantic salmon with the osteoblast lining cell as an exception (Krossøy et al., 2009b). It has been hypothesized by Lall and Lewis-McCrea (2007) that post-translational modification of the γ-carboxylated Gla residues in the osteocalcin form a weak bond to calcium, that further allows the protein to bind to mineralized bone matrix. The binding stabilizes the secondary structure of the protein (Lall & Lewis-McCrea, 2007). Carboxylated BGLAP consists of 3 Gla residues. Carboxylated osteocalcin creates strong bonds to calcium ions, while uncarboxylated osteocalcin do not (Komori, 2020, pp. 2-3). BGLAP is the most abundant non-collagenous proteins in bones of vertebrates including bony fish (Lall & Lewis-McCrea, 2007).

The exact mechanism of the protein is still unknown, but results indicate that BGLAP have ability to activate osteoclasts and osteoblasts in early bone formation (Carvalho et al., 2021, p. 21). BGLAP is according to some considered a negative regulator of bone formation (Ducy et al., 1996; Krossøy et al., 2009a; Stock & Schett, 2021). Contradictory, other studies suggests that BGLAP increase bone formation and bone healing (Carvalho et al., 2021, pp. 21-22; Neve et al., 2013, p. 1150). According to Neve et al. (2013, p. 1151) higher intake of vitamin K have been detected to result in a higher level of carboxylated BGLAP simultaneously reduced uncarboxylated BGLAP. There has also been conducted studies indicating that BGLAP do not influence mineralization and bone quantity regulation but is of importance for bone strength (Komori, 2020, p. 10; Krossøy et al., 2009a).

2.5.2 MGP

MGP is 84 residues long and consists of 5 Gla residues. MGP and BGLAP is thought to come from a common ancestor based on the gene similarities (Price, 1988). MGP is expressed over a wider range of cells than BGLAP, chondrocytes additional to vascular smooth muscle cells and epithelial cells (Stock & Schett, 2021). MGP is an inhibitor of tissue calcification (Stock & Schett, 2021).

The mechanisms of how MGP effect bone metabolism is still unknown. It is believed that MGP prevent mineralization of cartilage. However there has also been conducted a study where the results indicated that MGP promote osteoblast proliferation, differentiation and mineralization (Carvalho et al., 2021, p. 22). Osteoblasts and odontoblasts produce MGP and it is hypothesized that it is supporting normal growth and development (Krossøy et al., 2009a).

MGP is mainly expressed in cartilage, however, it is also present in fibroblast and vascular smooth muscle (Stock & Schett, 2021). The protein has a high attraction to calcium ions and hydroxyapatite. MGP therefore work against vascular calcification. Mice with MGP deficient gene, has been documented to die due to vascular calcifications and then resulting in vessel rupture and massive bleedings. MGP additionally prevents osteoarthritis, as low serum levels of vitamin K are associated with the condition (Stock & Schett, 2021). MGP has ability to bind to calcium ions in salmon as well (U.S. National Library of Medicine, 2023).

It is required with two post-translational reactions to achieve its full calcification inhibitory ability, both glutamate carboxylation and serine phosphorylation, meaning that it depends on activated vitamin K. The inactive state of MGP is called desphospho-uncarboxylated MGP. Theoretically MGP can be seen in four different states, unmodified and inactive, only phosphorylated, only carboxylated and fully altered and activated MGP (Epstein, 2016).

2.5.3 GRP

GRP is expressed in cartilage, juvenile and resting zone chondrocytes (Stock & Schett, 2021). It is a protein with high density of γ-carboxylated protein (Cancela et al., 2012, p. 174). The chondrocytes secrete GRP to ECM. GRP has a protective function for cartilage when exposed to stressful conditions for the cartilage in adult mice (Stock & Schett, 2021). GRP contribute to cartilage protection, plausible by binding to collagen type II, IX and XI. This might attribute by interconnection of the cartilage matrix and promote its mechanical rigidity (Stock & Schett,

2021). It has been suggested that GRP play an important role in early phase of chondrocyte differentiation (Tagariello et al., 2008).

GRP gene has been identified in cartilaginous tissues and it has been suggested to play a role in chondrogenesis. However, *GRP* expression has also been observed in bone cells, arguing for having a more versatile role for the protein throughout skeletal formation (Cancela et al., 2012, p. 174).

GRP accumulates in bone, cartilage and ectopic calcification. It has been documented in zebrafish that knockout of this gene results in reduced growth, and changes in skeletal development. Chondrocyte death is increased in GRP-deficient mice, indicating that GRP protects articular cartilage by reducing chondrocyte apoptosis. GRP inhibits osteogenic differentiation, promotes skeletal homeostasis and prevents vascular calcification (Xiao et al., 2021).

2.5.4 Periostin

Periostin is thought to work on bone remodelling when exposed to mechanical loading (Bonnet et al., 2016; Kudo, 2011). It is highly expressed in connective tissue that is exposed to mechanical stress (Xiao et al., 2021).

Periostin works as a structural molecule of the bone matrix as well as a signalling molecule stimulating osteoblast functions, formation of bone and fracture healing (Bonnet et al., 2016; Stock & Schett, 2021). It participates in bone processing fibrillogenesis and cell migration. *Periostin* is expressed in osteoblasts (Stock & Schett, 2021).

According to Kudo (2011), studies have demonstrated that periostin influence regeneration of bone. There has been documented that mouse with deficient periostin have a cortical bone in femur of lower area and thickness than for mice with regular periostin. The effect of periostin in collagen cross-linking in fibrillogenesis, has been examined. Periostin has a promoting effect on the proteolytic activation of lysyl oxidase. Lysyl oxidase is the enzyme behind cross-link formation (Kudo, 2011). In a study by Zhu et al. (2009) there was found that overexpression of *periostin* increased the speed of bone formation in rats.

2.5.5 Protein S

Protein S is produced by hepatocytes, megakaryocytes and endothelial cells. It plays a crucial role in haemostasis. Two cases of protein S deficiency have been documented to be linked with

osteopenia (Maillard et al., 1992). In a study by Maillard et al. (1992), it was hypothesized that the deficiency cause osteopenia. The study found results indicating that such deficiency could be related to deficiency of protein-S secretion by osteoblasts (Maillard et al., 1992). Protein S seem to function as a cofactor to protein C (Walker, 1980, p. 5521). *Protein S* is expressed by osteoblasts (Stock & Schett, 2021).

2.5.6 GAS6

GAS6 has been documented to increase osteoclast activity, therefore increase bone resorption (Stock & Schett, 2021). It contribute to inhibiting mineral deposition by signalling to tyrosine kinases (Fernández-Fernández et al., 2008, pp. 604-607). GAS6 has a positive influence on survival and migration of vascular smooth muscle cells and vascular remodelling, strength of fibrous cap (Laurance et al., 2012).

2.6 Challenges with accuracy of vitamin K in feed

Vitamin K can be influenced by moisture, trace metals and temperature. It should therefore be avoided to mix vitamin K with minerals and the vitamin is recommended to be stored under dry conditions as well as fed as soon as doable after manufacture (Hardy & Kaushik, 2022, p. 148).

The commercial form "Menadione nicotinamide bisulphite" (MNB) is a form of vitamin K_3. This is the type used in the present thesis. This type is relatively unstable during storing and bioavailability compared with vitamin K_1 and K_2. It can be difficult to control levels of vitamins in the feed. This is because there is expected to be occur losses both during production and during storage. Method to overcome the problem with the losses is to add more vitamins than what is needed in the feed, in this way the losses is compensated (Krossøy et al., 2009a). Vitamin K is sensitive to processing conditions and can be reduced by nearly 90% within 1 year of storage (Graff et al., 2010). This makes it challenging to get the accurate level of wanted level of vitamin K in the feed.

In the study by Krossøy et al. (2009a), different levels of MNB were evaluated for Atlantic salmon at parr phase. The salmon were fed for 28 weeks, but there were not detected significant differences in specific growth rate (SGR), condition factor (CF), whole proximate composition, blood coagulation time, vertebrae morphology or mechanical properties of vertebrae, although there were detected a dose-dependent response between whole body vitamin K and dietary MNB supplementation level.

According to Krossøy et al. (2010) GGCX has been suggested as marker for vitamin K status. In the study by Krossøy et al. (2010) the effect of MNB and Phylloquinone on GGCX were compared. Phylloquinone is a type of vitamin K_1. Elevated level of Phylloquinone resulted in elevated level of GGCX resulting in a higher level of KH_2, however this was not detected in MNB. The results indicated that MNB do not act as a cofactor for GGCX in the liver of Atlantic salmon (Krossøy et al., 2010). In addition, there has also been detected a negative correlation of GGCX activity and MNB in diets, however the gene expression remained similar across the different diets (Krossøy et al., 2009a). Overall indicating that the bioavailibility of MNB might be relatively low.

2.7 Methods to assess vitamin K influence on Atlantic salmon

2.7.1 Growth

Improved fish welfare can be detected as improved growth (Sissener et al., 2021), it is therefore beneficial to monitor growth.

2.7.2 Biometric analysis

As liver is believed to be the biggest storage organ of vitamin K (Krossøy et al., 2009a). It is sensible to examine the possible toxicological effect. This can be done by analysing the liver by visual evaluation of the colour as well as weighing the liver. The liver play an important role in metabolic homeostatic functions. Pale livers can be a sign of nutritional disorder (Mørkøre et al., 2020). Healthier livers have a darker red colour (Kousoulaki et al., 2020).

2.7.3 Blood

As vitamin K is believed to reduce blood clotting time (Hardy & Kaushik, 2022, p. 145). It can be sensible to measure this. The blood clotting time is the time for fibrin clot to appear (Roshal & Reyes Gil, 2019, p. 773).

2.7.4 Vitamin K in feed

In addition to measure vitamin K_3 content in the feed, it is sensible to measure vitamin K in the liver. As it is believed that MNB must be converted to MK-4 which is a type of vitamin K_2 to become bioavailable (Krossøy et al., 2010). It is sensible to measure MK-4 in the liver.

2.7.5 Content of minerals in vertebral column

As Gla residues have a high bond affinity for bonding with calcium, it is sensible to measure the concentration of calcium in the vertebral column (Proudfoot & Shanahan, 2006). As it is known that phosphorous is also an important mineral in vertebral column (Drábiková, 2023, p. 188), it is sensible to measure this in order to control that a possible change detected in calcium level is occurring without a change in phosphorous although it is not expected that vitamin K will influence phosphorous level perse.

2.7.6 Deformity scoring

Vertebral deformation is a challenge in Atlantic salmon and other teleost species. Fusions of vertebrae is often seen. Less severe include few vertebrae and vertebrae fusion without external signs. Severe cases include vertebrae with several affected vertebrae and visible external malformation, for example shortening of the tail (Witten et al., 2009).

Electromagnetic radiation is used in x-ray. The radiation passes through the body. It is used to get an image of the structure inside the body, that is achieved as the radiation do not pass through the skeleton and tissue. The bone and tissues absorb x-ray in a varying degree, hence they appear on the x-ray with a varying shades of grey. Radiological density depends on both atomic number and density. The bone contains calcium which has a higher atomic number than most other tissues. Consequently, the bone appears whiter than other soft tissues (National Institute of Biomedical Imaging and Bioengineering, 2022). Radiography is a relatively cheap diagnostic tool with ability to differentiate between different body tissues based on X-ray photon attenuation (Paramitha et al., 2017, p. 28). X-ray is therefore a simple non-invasive method to gain insight in the vertebral column.

The method allows to see the structure of the vertebrae of fish like Atlantic salmon. As vitamin K effects some bone proteins, it is of interest to investigate the vertebral column for deformations. However, deformations of the vertebral column is not specific (Witten et al., 2009). It is therefore required to investigate for specific types of deformations. Witten et al. (2009) performed a study to find the appropriate number of categories when investigating deformation of vertebral column of Atlantic salmon. The study used 5000 samples of Atlantic salmon for over 10 years as a basis, and it was suggested that malformations could be categorized into 20 types (Figure 5, Figure 6 and Figure 7).

Nine (type 1-9) of them were linked to fusions and compression, four (type 10 – 13) of them were linked to irregular radiotranslucent or radiopaque vertebral bodies, three (type 14-16) types of bends on the spinal curve, three (type 17-19) of them were linked to symmetry abnormalities and dislocation of vertebral bodies and one (type 20) of them were linked to severe multiple malformations (Witten et al., 2009).

Placement is important when it comes to accuracy and precision of the X-ray scan (Albano et al., 2021). Radiographs of healthy vertebrae appears as a x in a squared box. Compression and fusion do not necessarily develop into curved spines. It has been suggested that these types of deformity are developmental (Perrott et al., 2018, p. 2).

If two vertebrae are fused together, Atlantic have ability to completely fuse them together so they eventually appear as one by reshaping and remodelling. In these cases, it will only be visible based on the several neural and haemal arches. The first step to all types of shortening of vertebrae starts with cartilaginous tissue in the intervertebral space replacing intervertebral notochord tissue and osteogenic tissue of the vertebral endplate growth zone. Compression of vertebrae seen in tetrapod's has been associated to notochord cells failing to maintain vertebral development, and it has been discovered that salmon notochord control vertebrae formation in early ontogeny phase. It has further been believed that failure of notochord in salmon can therefore lead to vertebrae compression (Witten et al., 2009).

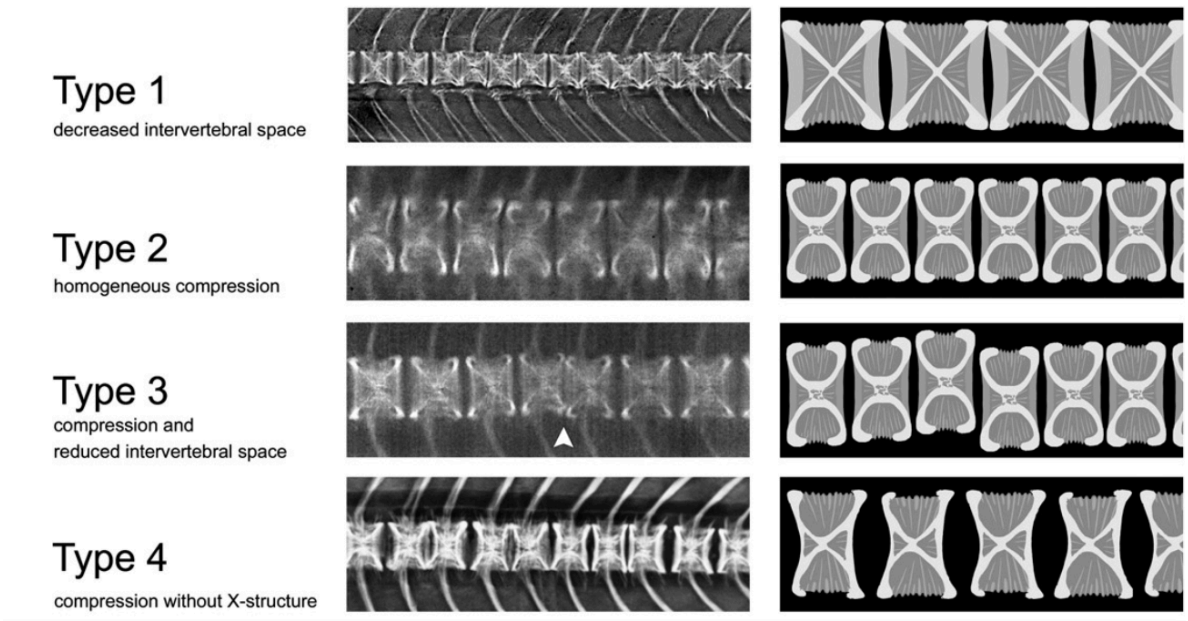

Figure 5: 4 types of deformities related to fusion and/or compression that have been detected in Atlantic salmon (*Salmo salar* L.). From (Witten et al., 2009).

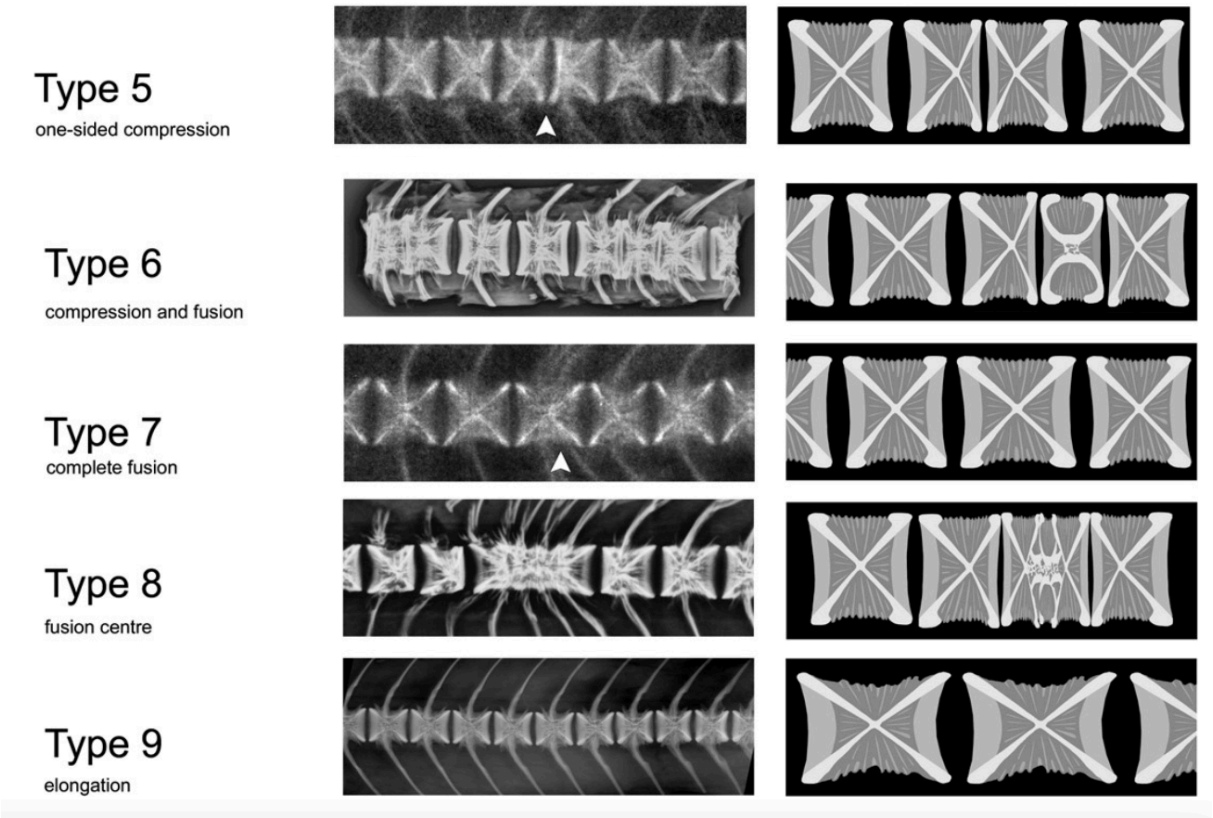

Figure 6: 5 types of deformities related to fusion and/or compression that have been detected in Atlantic salmon (*Salmo salar* L.). From (Witten et al., 2009).

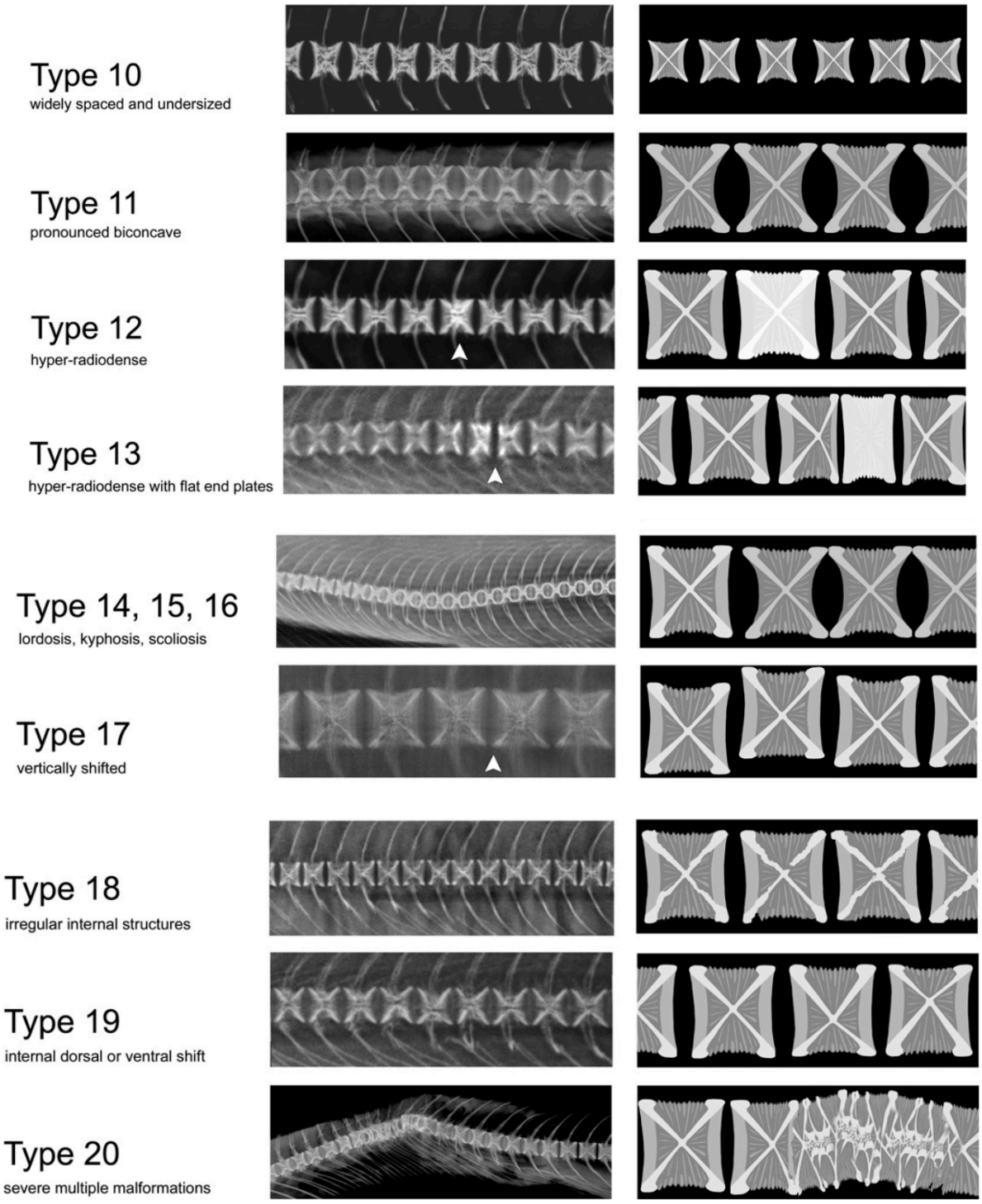

Figure 7: 10 types of deformities that have been detected in Atlantic salmon (*Salmo salar* L.) Type 10 – 13 are related to undermineralization or hyper minderlization. Type 14-16 cause abnormal shape of the vertebral column. Type17 – 19 are related to the internal structure of the vertebrae. Type 20 is severe deformity consisting of several deformities. From (Witten et al., 2009).

2.7.7 Gene expression

As vitamin K is believed to effect BGLAP, MGP, GRP, periostin, protein S and GAS6. Performing gene expression analyses are sensible to measure the proteins. One method is to measure the relative gene expression (GE) of the genes. This can be done by performing qPCR, which amplify the genes to a level that is detectable to measure. This level is called Cycles of threshold (Ct). Ct is a relative number of cycles required of DNA to be detectable (Goni et al., 2009, p. 2).

2.7.8 Mechanical properties of vertebrae

Bone quality is a complex as it consists of interdependent factors including bone strength. There exist different methods for measuring bone quality (Chappard et al., 2011). Performing a mechanical analysis of vertebrae is sensible to investigate if vitamin K effects the hardness of the bone. This can be done by measuring the force required to break a percentage of the vertebrae as has been done in master theses by Yao (2017, pp. 20-21) and Agyeman (2019, pp. 12-13) and PhD theses by Jiménez-Guerrero (2023, pp. 35-36) and Drábiková (2023, pp. 98-99).

In the master thesis by Agyeman (2019, pp. 10-21), there was detected that Atlantic salmon fed diets with vitamin K had highest compression force, area and thickness. Although the Atlantic salmon used in the experiment had a starting weight of 20 gram and weight of about 70 grams at the end, it could be expected to see a higher compression with higher vitamin K level in this thesis.

3 Material and methods

3.1 Experimental setup

The experiment was performed at Lerang Research Station (Skretting AI, Forsand, Norway). There was first an acclimation period prior to start of the experiment. On 05.12.2023, 540 of the salmon with an average weight of 265 gram were anesthetized (MS-222, Sigma-Aldrich) and evenly distributed (n=30) into eighteen 1 m diameter ($0.6m^3$) tanks supplied with a constant flow of clean seawater (salinity=33.9± 0.2 ppt, temperature=11.9±0.2°C, oxygen=103±1.6%). On 06.12.2023, eighteen Atlantic salmon (*Salmo salar* L.) were terminated by overdose (MS-222, Sigma Aldrich). The fish were starved for 2 days before they were fed their respective diets on 07.12.2023. The fish were fed to apparent satiation twice a day; feed intake was monitored throughout the experiment, and waste feed was collected. They were starved the before and on the same day as sampling (05.12.2023-06.12.2023, 19.02.2024-20.02.2024 and 20.03.2024-21.03.2024). For the duration of the experiment, the fish were kept in constant artificial light (photoperiod=24 h:0 h light:dark). Triplicate tanks were fed one of six diets with increasing level of vitamin K_3 (Diet A = 0, Diet B = 5, Diet C = 10, Diet D = 15, Diet E = 20 and Diet F = 25mg/kg), where diet A was the control diet. Vitamin K_3 was added in the form of Menadione nicotinamide bisulphite (Microvit K3 Promix MNB 96%, Adisseo, Antony Parc II, France) (MNB). All diets were extruded via a Wenger twin screw extruder at the Feed Trial Plant (Skretting AI, Stavanger, Norway). The experiment was conducted according to Norwegian legislation and approved by the Norwegian Animal Research Authority (NARA/FDU). The length of the experiment and time of samplings is illustrated in Figure 8.

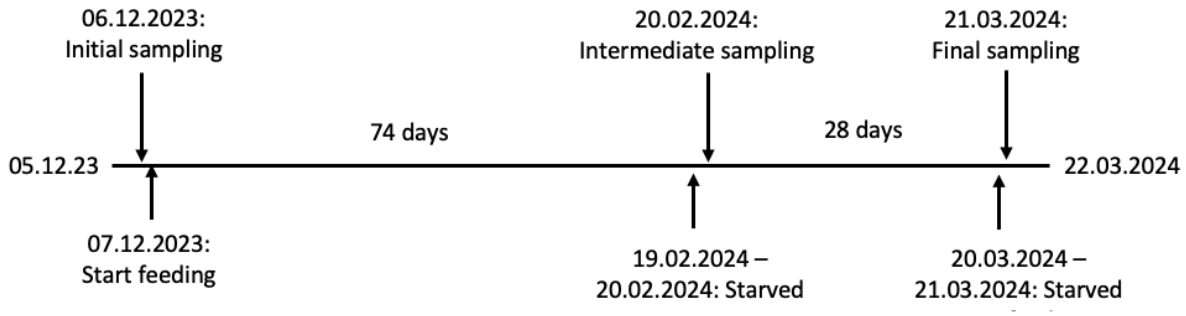

Figure 8: Atlantic salmon (*Salmo salar* L.) were fed different diets of vitamin K_3 (0, 5, 10, 15, 20 and 25 mg/kg) for 74 days before intermediate sampling, the remaining salmon were then fed for 28 more days before final sampling. A total of 90 salmon were analysed on intermediate and final sampling, 5 fish from each tank, 15 fish from each diet. All fish were weighed and measured length of at start (06.12.23) and end (21.03.24-22.03.24) of the experiment.

The master thesis included results of the following analyses: weight of whole body, and livers, body length, liver colour evaluation, blood sampling, vitamin K content of feed, mineral content of vertebral column, deformity scoring, gene expression of vertebrae and mechanical properties of vertebrae (Figure 9 and Figure 10).

The results of weight, length of whole body and mechanical properties of vertebrae were included from initial, intermediate and final samplings. The results of liver colour scoring, liver weight, deformity scoring and blood sampling were included from intermediate and final sampling. The results of mineral content and gene expression were included from intermediate sampling.

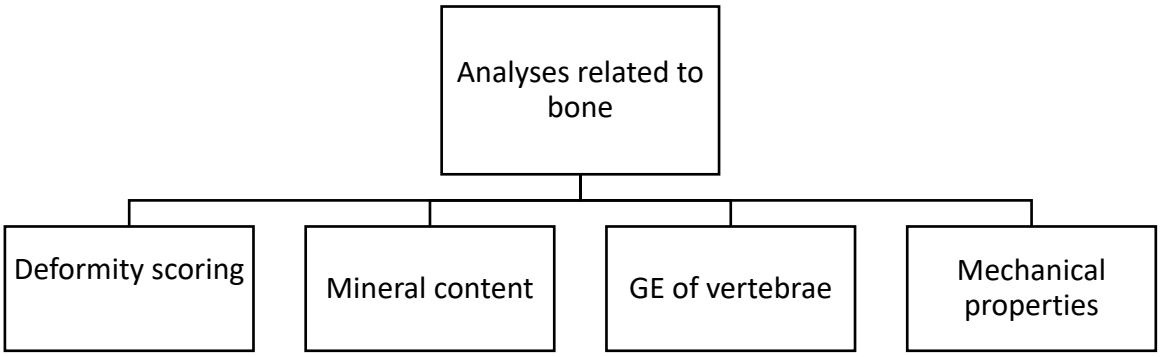

Figure 9: Overview of analyses related to bone conducted on Atlantic salmon (*Salmo salar* L.) fed different levels of vitamin K (0, 5, 10, 15, 20 and 25 mg/kg) for 74 and 102 days. GE = gene expression.

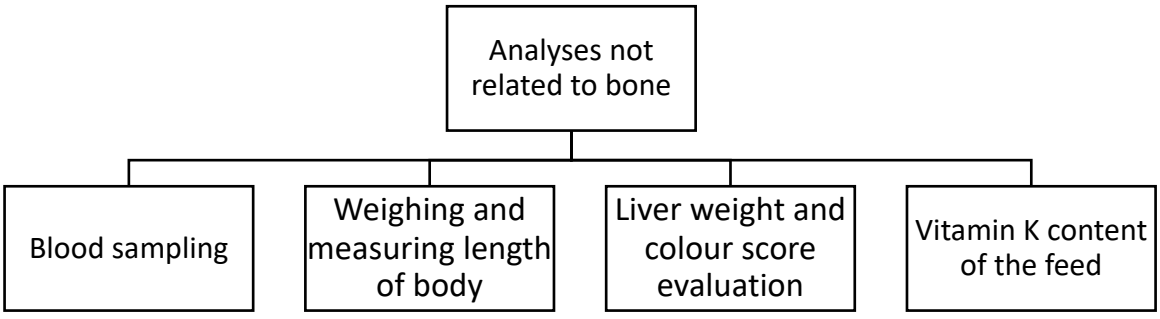

Figure 10: Overview of analyses conducted on Atlantic salmon (*Salmo salar* L.) fed different levels of vitamin K (0, 5, 10, 15, 20 and 25 mg/kg) for 74 and 102 days, and analyses of feed.

3.2 Preliminary test of mechanical properties of vertebrae

Instrument (TA-XTplus TEXTURE ANALYSER, Stable micro systems, Surrey, UK) with blade set HDP/WBR was used to measure the depth and required force to break the cortical bone (50 kg load cell, test speed 2 mm/s).

Vertebral columns of Atlantic salmon with a body weight of about 100 (n=3), 300 (n=2) and 1400 (n=2) gram were used in this preliminary test. Freshly filleted fish were used, and the vertebral columns were dissected. The traveling speed of the blade set was 2 mm/sec. The target mode was set to 5 mm distance. It became apparent that this was not an optimal instrumental setting program when looking at the results from the preliminary testing.

Based on the preliminary test, it was decided to use the following setting: target mote 70% strain, and analyses of the force time graphs. This setting was used to achieve the peak that indicated that force to break cortical bone was achieved, this was detected as the first peak (Figure 11).

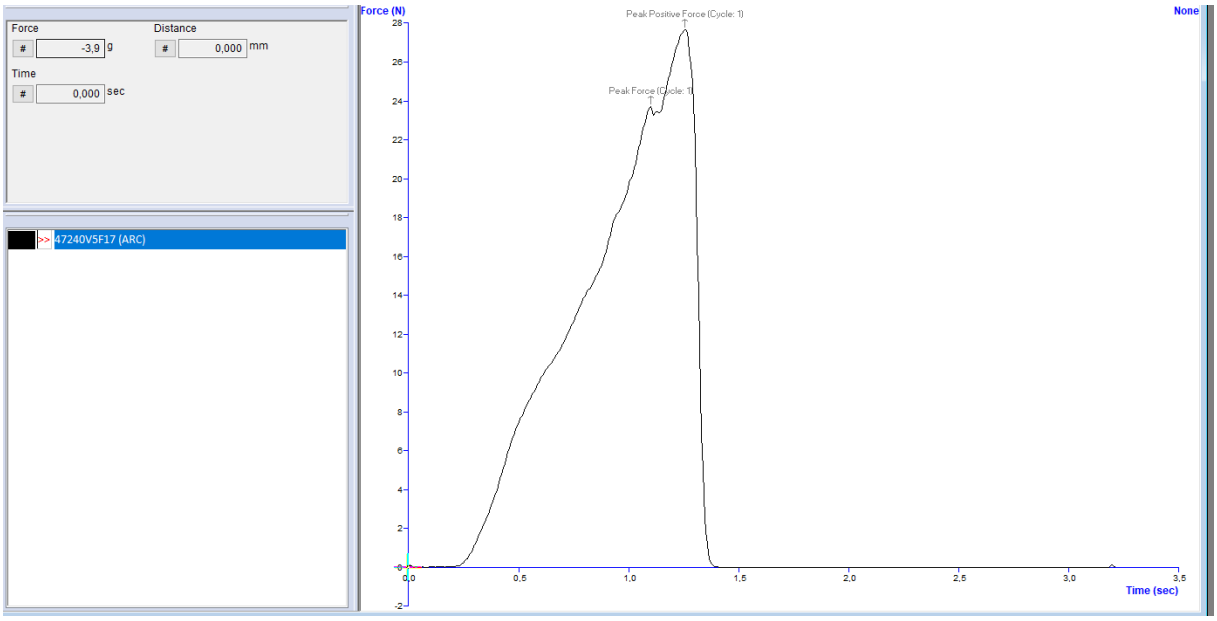

Figure 11: Force – time graphs from mechanical analysis of initial sampling, force (Newton) required to strain the vertebral column by 70% set to vertebrae 12 counted from the head of Atlantic salmon from the start of the experiment.

3.2.1 Mechanical properties of vertebrae

The samples were stored at – 20 ℃ and thawed for 16-17 hours at 3 - 4 ℃ before performing the analysis. Samples were kept in a polystyrene box with ice to keep the samples cool during sampling. The texture analyser (TA-XTplus TEXTURE ANALYSER, Stable micro systems,

Surrey, UK) with blade set, HDP/WBR was used (Figure 12 A). It was calibrated for both force and height before sampling. The target mode was set to 70% strain similar to Yao (2017).

Vertebra nr 12 counted from first vertebrae posterior to the head, was used in the analysis. The vertebral column was placed under the blade. The cranial-caudal axis was placed flat on the board (Figure 12 B).

The vertebral column was dissected using a knife and fingers. The neural and haemal arch were removed by using a scissor. Remains of flesh were removed by using the dull side of a knife. Target mode was set to 70% strain to overcome the force required to break the cortical bone in the centrum of the vertebrae. This breaking point was seen as the first peak in the curve. The aim was to measure the force to break the cortical bone and the total force required to cut 70% into the bone. The force was measured in Newton. Calculations were done using the Texture analyser software.

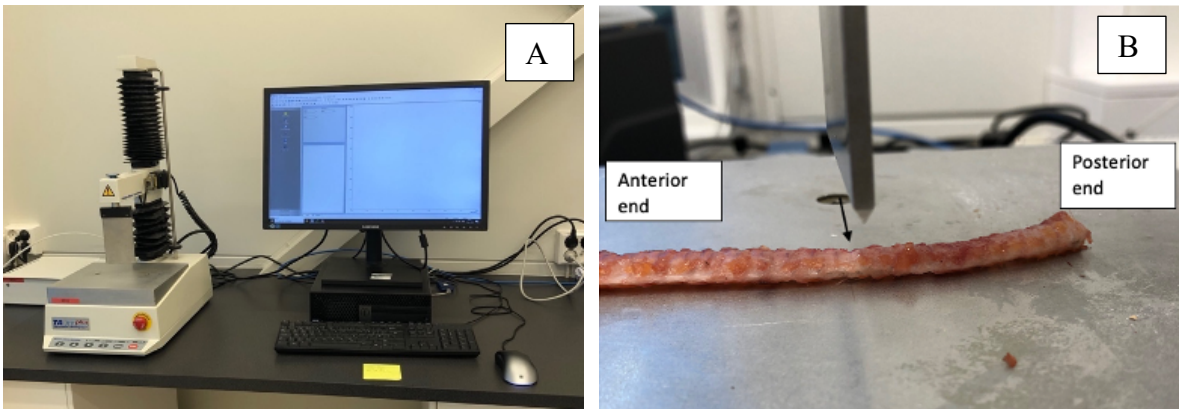

Figure 12: A: Texture analyser and computer used in this experiment to measure bone strength. B: Vertebrae 12 counted from the head was subjected to vertebrae strength measurements of. Black arrow points to vertebrae 12.

3.3 Initial sampling

18 Atlantic salmon were terminated at the start of the experiment to determine the initial status of the fish. They were weighed, measured length of, blood sampled (Hb and HTC), mechanical properties of vertebrae were analysed, and the livers were sent to MasterLab, Nutreco (Boxemeer, The Netherlands) to measure vitamin K content of the liver. The liver samples were unfortunately destroyed during transportation, and the results could not be included in the present study.

3.4 Intermediate sampling and final sampling

Five fish per tank were randomly picked out and terminated by overdose of MSS-222, before they were weighed, and measured length of, Figure 13 below illustrates the workflow after termination of the fish.

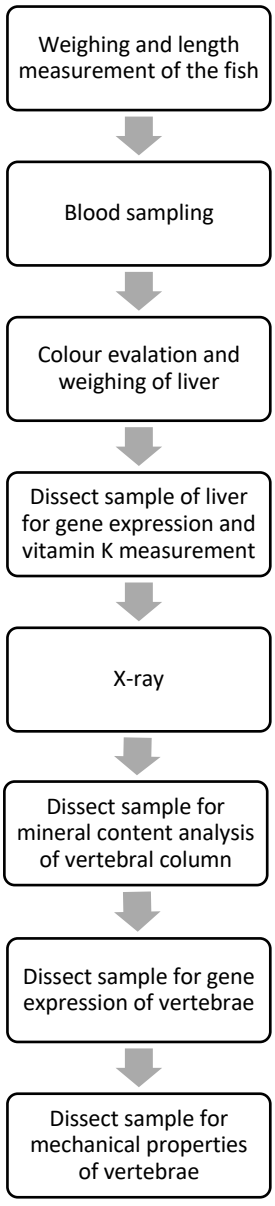

Figure 13: Schematic overview of the workflow on sampling days.

After recording the body weight and fish length, blood was collected for Hb (Hemocue Hb 201+ Analyzer, HEMOCUE, USA) and HTC determinations using Hirschmann disposable

capillary tubes into an Eppendorf tube, and time for blood clotting was measured (Coagucheck Pro II, Roche, Switzerland). A scalpel and scissor were used to dissect the fish. The scissor was inserted from the last gills in posterior direction in ventral side followed by posterior direction to the anus. The liver was then dissected for visual scoring of the liver colour, weighing and sampling of tissue for gene expression (by using the same area of livers for all samplings). The remaining part of the liver was put in a plastic bag for analyses of vitamin K content. Gene expression of the liver and vitamin K content of liver were not included in my master thesis. The fish were then prepared for x-ray examination. The gut content and the fillet on the lateral side was removed. The fish was placed in a flat position for a digital x-ray imaging (GIERTH TR90/30 peak emitter, Oehm und Rehbein Technology, Germany). The vertebral column posterior to the dorsal fin were cut into samples for mineral content and gene expression (Figure 14). This part was prepared for mineral content analysis by dissecting the vertebral column and put in a labelled plastic bag.1-2 vertebrae from the remaining posterior part was cut out for gene expression samples. The remaining part was put in a labelled bag for mechanical properties of vertebrae. All samples of the fish were put in labelled plastic bag. The bags were stored at -20 ℃ degrees before transportation. The samples for mechanical properties of vertebrae were placed flat avoid bending of the samples.

The samples were transported in polystyrene box to avoid thawing to Skretting (Norway, Sjøhagen 3) for further analyses and transportation. The following analyses were performed on Skretting, analysis of mechanical properties of vertebrae, deformity scoring and gene expressions. The remaining samples were transported to MasterLab for further analysis.

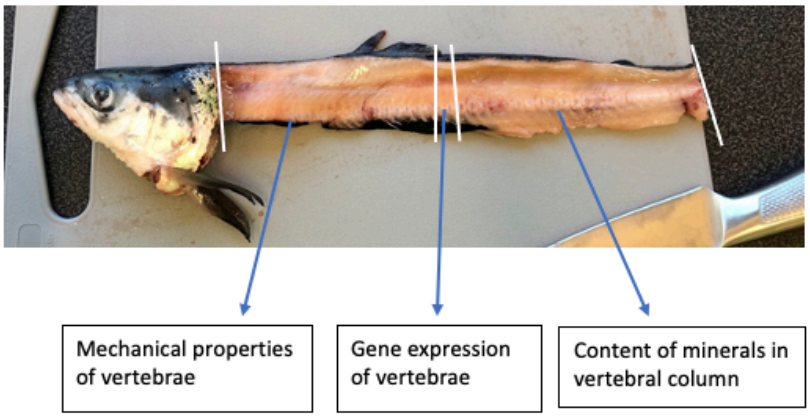

Figure 14: Vertebral columns of Atlantic salmon (*Salmo salar* L.) were used in three different analyses after feeding the salmon with diets of varying level of vitamin K (0-25 mg/kg) for 74 days. The salmon were cut according to the white lines. Analysis of mechanical properties of vertebrae were performed after the fish had been feeding their respective diet for 102 days, in addition. Head and tail were removed. Salmon from the initial sampling.

The procedure was repeated in the final sampling, with further dissection of the vertebral column for mechanical properties of vertebrae as an exception. It had not been done for the previous samplings, this was done to make the mechanical properties analysis less time consuming.

The day after the final sampling (22.03.2024), the remaining fish in the tanks were terminated followed by weighing and measuring length of.

3.5 Analyses

3.5.1 Growth

The specific growth rate (SGR) was calculated by using Equation 1, below, weight at end and start is the average value from its respective diet, t = time (days).

CF was calculated using Equation 2. The average value of length and weight per diets is calculated by using the average value per tanks.

Equation 1 $$\mathbf{SGR}\left(\frac{\%}{\mathbf{day}}\right) = (\mathbf{ln(weight\ at\ end)} - \mathbf{ln(weight\ as\ start)}) * \frac{\mathbf{100}}{\mathbf{t}}$$

Equation 2 $$\mathbf{CF} = \mathbf{100} * \left(\frac{\mathbf{weight}}{(\mathbf{length})^3}\right)$$

3.5.2 Liver analyses

The colour of the livers was evaluated according to a scale ranging from 1 to 5 (Figure 15). As it is a qualitative analysis one person did the analysis.

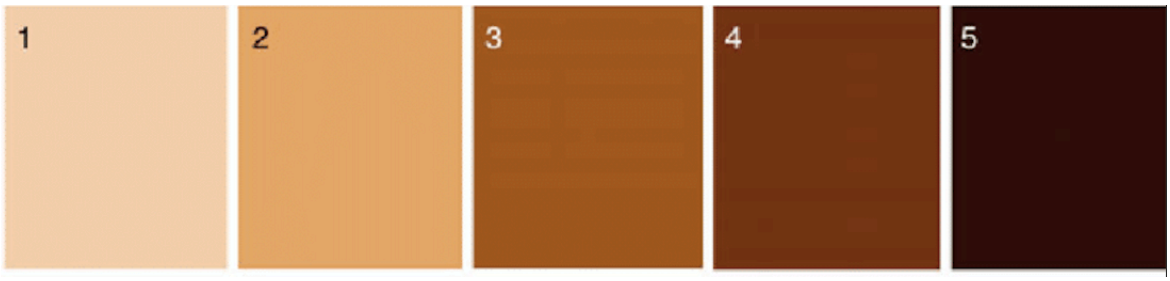

Figure 15: The colour palette used to score the liver of Atlantic salmon (*Salmo salar* L.) fed different levels of vitamin K (0, 5, 10, 15, 20 and 25 mg/kg). From (Mørkøre et al., 2020).

The hepatosomiatic index (HSI) was calculated according to Equation 3.

Equation 3

$$\textbf{HSI}\ (\%) = \frac{\textbf{liver weight}}{\textbf{body weight}} * \textbf{100}$$

3.5.3 Vitamin K content of liver

Samples of liver were sent to MasterLab, Nutreco (Boxemeer, The Netherlands). to analyze the vitamin K content. For the initial sampling the samples were thawed during transport and therefore ruined. The results from intermediate and final samples were not included in the master thesis due to not being ready in time.

3.5.4 Blood sampling

Blood sampling was performed by technician at Skretting. The blood was drawn from the lateral line, between the dorsal fin and the tail. The needle was inserted into the fish until bone was reached. The vein was located using the vertebrae as a guide. Hemocue Hb 201 (HemoCue, Sweden, Ängelhom) was used to measure Hb. Eurotrol HemoTrol (Triolab, Norway, Kjeller) was used for standardization at start of the sampling and in the end of sampling day.

Disposable capillary glass tubes (Hirschmann, Germany) were used to measure HTC. The glass tubes with the ring marked end were placed away from the blood sample and led carefully into the blood in the vacucontainer (Eppendorf tube), and the vacucontainer was carefully tilted until the container was filled with blood.

Coagulation time was measured using CoaugCheck Pro II (Roche Diagnostics, Switzerland, Rotkreuz).

3.5.5 Vitamin K content in feed

Vitamin K content in the feed were analysed by MasterLab, Nutreco (Boxemeer, The Netherlands), by using HPLC UV. This was done twice. First time was 30 days before the experiment start up and the last was two days before final sampling.

Diet A consisted of 0.1 mg/kg vitamin K on both sampling days (Table **1**). Diet B consisted of 7.2 mg/kg on first sampling day and 5.6 mg/kg on second sampling day. Diet C consisted of 15.0 mg/kg on first sampling day and 14.2 mg/kg on second sampling day. Diet D consisted of 27.0 mg/kg on first sampling day and 25.4 mg/kg on second sampling day. Diet E consisted of 35.0 mg/kg on first sampling day and 33.2 mg/kg on second sampling day. Diet F consisted of 44.0 mg/kg on first sampling day and 39.8 mg/kg on second sampling day. Diet B-F had a reduction of 5 – 22% from first sampling to second sampling.

Table 1: Formulated and analysed vitamin K level (mg/kg) in diets A-F used to feed Atlantic salmon (*Salmo salar* L.) for 102 days.

	Diet A (mg/kg)	**Diet B (mg/kg)**	**Diet C (mg/kg)**	**Diet D (mg/kg)**	**Diet E (mg/kg)**	**Diet F (mg/kg)**
Formulated	0.0	5.0	10.0	15.0	20.0	25.0
First sampling	0.1	7.2	15.0	27.0	35.0	44.0
Second sampling	0.1	5.6	14.2	25.4	33.2	39.8
Difference (mg/kg)	0.0	- 1.6	- 0.8	-1.6	- 1.8	- 4.2
Difference (%)	0.0	22.2	5.3	5.9	5.1	9.5

3.5.6 Content of minerals in vertebral column

The posterior part of the vertebral column (Figure 14) was analysed for phosphorous and calcium content by MasterLab, Nutreco (Boxemeer, The Netherlands). In accordance with NEN-EN 15510(EN);2017

3.5.7 Deformity scoring

The vertebral columns were investigated for the 20 different deformations given by Witten et al. (2009) (Figure 5, Figure 6 and Figure 7). Vertebrae in the occipital region and vertebrae in the urostyle were considered as non-pathological and therefore not considered in the deformity count (Drábiková, 2023, p. 60). As this is a qualitative analysis, one person got trained to perform this analysis. Fusion in caudal region was overlooked as it can normal (Witten et al., 2009).

3.5.8 Gene expression of vertebrae

Gene expressions were performed by lab technician at the bubble Skretting (Stavanger, Norway). Vertebrae were stored at – 80 ℃ until analysis. Gene expression analysis of vertebrae were performed in following steps: Cell lysis, homogenization, RNA isolation, concentration measurements, cDNA synthesis and qPCR analysis (Figure 16).

For RNA isolation, the QIAcube connect HT (QIAGEN, Netherlands) was used with the RNAEASY 96 kit (QIAGEN, Netherlands). The Tissuelyser II (QIAGEN, Netherlands) was used to lyse the samples in preparation for total RNA extraction. The cell lysis, homogenization and RNA isolation was performed according to Rneasy 96 kit for QIAcube HT (Qiagen, Netherlands), according to their protocol (QIAGEN Netherlands, 2023) (Appendix A).

However, the protocol deviated some from the standard reagents used with QIAcube HT. Normally, the RNA lysis and extraction reagent Qiazol together with chloroform is used for RNA purification. To avoid using these hazardous chemicals, an alternative method employing the tissue lysis reagent RLT-buffer and DTT was used for lysate preparation. In addition, protein digestion in the lysates was performed by incubation with proteinase K and vacuum pump was used. Otherwise, the instructions in the RNAeasy 96 kit handbook were followed.

Buffer from RNeasy Kit Part 1 tissue, equipment from Qiagen cube.

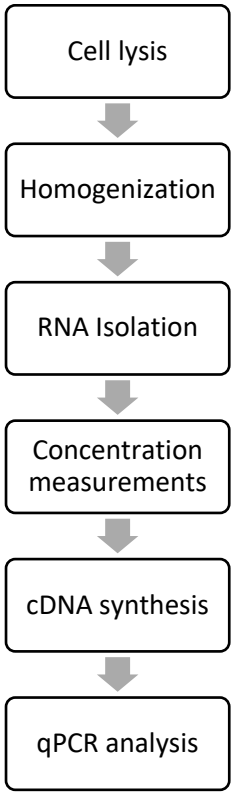

Figure 16: Workflow of gene expression of vertebrae.

The samples were stored at -80 ℃ overnight, before further steps. RNA concentration was checked by adding 1 µL sample to Nanodrop (Thermo Fischer Scientific, USA) to control quantity and quality.

cDNA synthesis was performed according to protocol QuantiTect Reverse Transcription Kit (QIAGEN, 2016) (

Appendix B). Wipeout buffer was used to remove genomic DNA (gDNA). Machine PCR machine (T100 Thermocycler, BIO-RAD, USA) was used to synthesize cDNA. The plates for qPCR analyses were then prepared using Qiagility (Qiagen, Netherlands) followed by qPCR (Quantstudio 5 PCR system) with master mix (PowerUp SYBR Green Master Mix). The qPCR measured the mean value of the Ct of each gene. The qPCR was run in duplicates. All the primers used in this study were validated by controlling their amplification and melt curve. Requirement to primer efficiency was between 90-110% and was obtained by performing a standard curve analysis with series dilution of cDNA sample.

Two housekeeping genes were used as a reference: Actin and EF1α . Expression of five genes of interest related to vitamin K metabolism were studied (*BGLAP*, *GRP*, *periostin*, *protein S*

and *GAS6)* (primer sequences in Appendix C). Primer used for *MGP* did not meet the efficiency requirement for primer and was therefore excluded from the master thesis.

The results were then converted to Excel where Equation 4 and Equation 5 were solved. Ct = mean values of the respective genes.

Equation 4 $$\Delta Ct = Ct\ (gene\ of\ interest) - Ct\ (housekeeping\ genes)$$

Equation 5 $$Relative\ gene\ expression = 2^{-\Delta Ct}$$

3.5.9 Statistical analyses

The results from gene expression were tested using D'Agostino Normality Test for normality to determine if a one-way ANOVA or Kruskal Wallis should be used. The distribution was considered normal if measure of skewness was around 0 and not significant (P>0.05) and measure of kurtosis was around 3 and not significant (P>0.05). For the other analyses a normal distribution was assumed, and one-way ANOVA was used. Statistical significance was set to 0.05. When ANOVA and Kruskal Wallis were of significance (P<0.05), further statical analysis were conducted to examine for the significant grouping between diets. Tukey test was used to get the significant grouping between the diets when ANOVA analysis was used. Multiple comparisons were used to examine for the significant grouping between the diets when Kruskal Wallis was used.

Linear regression line was used on SGR, liver scoring, deformity scoring, mechanical properties of vertebrae, gene expression and calcium content of vertebral column. It was performed to estimate the correlation between vitamin K and the other parameters. R^2 was calculated to examine how much of the variance in the parameters analysed where explained by dietary vitamin K inclusion. Between 0.00-1.00, where R^2=1.00 linear regression describes 100% of the results, and R^2=0.00 linear regression describes 0% of the results.

Statskingdom.com was used to perform the Kruskal Wallis analysis with no correction and Mann Whitney U as multiple comparison method. R studio was used for ANOVA, Tukey test and linear regression analyses.

4 Results

A total of three fish from three different tanks died during the experiment. One was given diet A (0 mg/kg vitamin K) and two were given diet B (5 mg/kg vitamin K). The results of one fish from diet B was excluded from this thesis, as it was a looser fish with weight of 262 g after 102 days of feeding. It was assumed that the incidents of looser fish of mortalities were not correlated to the vitamin K level as there was relatively few incidents.

4.1 Growth

The initial length averaged 28 cm. The final mean length was between 40.8 (diet C) and 41.2 (diet A and D) (Table **2**), there were no significant differences of mean length between the dietary groups (P=0.95) at the end of the present study.

The initial averaged CF was 1.16, whereas the final mean CF was between 1.28 (diet E) and 1.32 (the remaining diets). There were no significant differences between the dietary groups (P=0.52).

The initial weight averaged 265 g. The final mean weight was between 899 and 934 g. There were no significant dietary differences (P=0.09) of body weight at the end of the study. The weight increase corresponded with an SGR of 1.19, with no significant differences between the dietary group (P=0.94). The SGR was calculated to be 1.18 at the lowest (diet C) and 1.21 at the highest (1.21). The correlation between vitamin K and SGR was not significant (P=0.88). The SGR was estimated to increase with 0.00013 per increase of 1 mg/kg vitamin K (R^2=0.001) (Table **3**).

Table 2: Weight, Body length, CF and SGR (%/day) of Atlantic salmon (*Salmo salar* L.) before and after feeding diets with varying vitamin K content. Results are shown as mean (SD), (start; n=540, end; n=75 fish, except for in diet A n=74 fish and diet B n =72 fish).

Diet	**Growth parameters**						
	Weight		Length		CF		SGR (%/day)
	Days of feeding						
	0 days	102 days	0 days	102 days	0 days	102 days	102 days
A: 0 mg/kg	265 (4)	934 (21)	28.3 (1.3)	41.2 (0.2)	1.16 (0.07)	1.32 (0.10)	1.19 (0.02)
B: 5 mg/kg		927 (58)		41.1 (1.0)		1.32 (0.10)	1.20 (0.06)
C: 10 mg/kg		899 (35)		40.8 (0.8)		1.32 (0.10)	1.18 (0.05)
D: 15 mg/kg		924 (15)		41.2 (0.2)		1.32 (0.09)	1.21 (0.02)
E: 20 mg/kg		927 (9)		41.1 (0.4)		1.28 (0.14)	1.20 (0.02)
F: 25 mg/kg		923 (52)		41.1 (0.6)		1.32 (0.08)	1.19 (0.04)
Mean		918 (16)		41.0 (0.2)		1.310.10)	1.19 (0.03)

Table 3: Estimated regression line of SGR (%/day) with p-value and R^2 of Atlantic salmon (*Salmo salar* L.) fed different levels vitamin K (0-25 mg/kg) for 102 days.

	Y	**P-value**	**R^2**
SGR (%/day)	1.2 + 0.00013*vitamin K (mg/kg)	0.88	0.0014

4.2 Biometric traits of fish sampled for analyses

The body weight after 74 days of feeding (range 685 - 727 g) were similar between the dietary groups (P=0.24) and after 102 days of feeding (range 893- 955 g) also similar between the dietary groups (P=0.73) (Table 4).

The length after 74 days of feeding (range 37.8 - 38.8 cm) were similar between the dietary groups (P=0.26). The length after 102 days of feeding (range 37.8 cm - 38.8 cm) were similar between the dietary groups(P=0.63).

The CF after 74 days of feeding (range 1.23 - 1.29) were similar between the dietary groups (P=0.45). The CF after 102 days of feeding (range 1.23 - 1.29) were similar between the dietary groups (P=0.38).

The liver score after 74 days of feeding (range 3.1 - 3.4) were similar as the correlation for liver scoring was not significant (P=1.00). Liver weight after 74 days of feeding (range 8.15 - 9.02 g) were similar between the dietary groups (P=0.86).

The liver score after 102 days of feeding (range 3.1 - 3.3) were similar as the correlation was not significant (P=0.95). The liver weight after 102 days (range 5.98 - 6.86) were similar between the dietary groups (P=0.68).

The HSI (%) after 74 days of feeding (range 0.88 % - 0.94 %) were similar between the dietary groups (P=0.87). The HSI after 102 days of feeding (range 0.89 - 0.95) were similar between the dietary groups (P=0.59).

Table 4: Body weight, body length, CF, liver weight, HSI (%), and liver colour score from Atlantic salmon (*Salmo salar* L.) fed diets with varying level of vitamin K for 74 and 102 days. Results are shown as mean (SD) (n=3; five individuals per tank, except one looser fish was excluded from diet B after 102 days of feeding).

Diet	**Biometric traits**											
	Weight (g)		Length (cm)		CF		Liver colour score		Liver weight (g)		HSI (%/body weight)	
	Days of feeding											
	74 days	102 days	74 days	102 days	74 days	102 days	74 days	102 days	74 days	102 days	74 days	102 days
A: 0 mg/kg	727 (31)	953 (69)	38.8 (0.8)	41.2 (0.8)	1.23 (0.03)	1.35 (0.03)	3.3 (0.04)	3.4 (0.6)	6.6 (0.6)	9.02 (0.80)	0.90 (0.04)	0.95 (0.01)
B: 5 mg /kg	707 (23)	932 (9)	38.1 (0.6)	41.2 (0.4)	1.28 (0.04)	1.33 (0.02)	3.1 (0.1)	3.2 (0.2)	6.7 (0.4)	8.84 (0.56)	0.94 (0.06)	0.96 (0.17)
C:10 mg/kg	685 (19)	924 (41)	37.8 (0.3)	40.5 (0.8)	1.27 (0.03)	1.39 (0.03)	3.0 (0.2)	3.1 (0.1)	6.0 (0.2)	8.49 (0.27)	0.88 (0.05)	0.92 (0.03)
D:15 mg/kg	753 (33)	893 (60)	38.7 (0.8)	40.5 (0.8)	1.29 (0.05)	1.34 (0.03)	3.3 (0.1)	3.2 (0.0)	6.9 (0.6)	8.15 (0.67)	0.90 (0.07)	0.91 (0.04)
E: 20 mg/kg	724 (17)	955 (25)	38.5 (0.2)	41.3 (0.5)	1.26 (0.01)	1.36 (0.03)	3.1 (0.2)	3.2 (0.2)	6.5 (0.5)	8.66 (0.34)	0.91 (0.07)	0.91 (0.05)
F: 25 mg/kg	711 (51)	936 (75)	38.6 (0.5)	40.9 (0.9)	1.23 (0.05)	1.36 (0.05)	3.3 (0.1)	3.1 (0.1)	6.4 (0.5)	8.47 (1.04)	0.90 (0.04)	0.90 (0.03)

4.3 Blood parameters

The measured Hb after 74 days of feeding varied from 7.0 g/dL (diet A) to 7.4 g/dL (diet E) (Table **5**). There were no significant differences (P=0.75) between the dietary groups. After 102 days of feeding the Hb varied from 7.7 g/dL (diet F) to 7.9 g/dL (diet D). There were no significant differences (P=0.81) between the dietary groups.

The measured HTC after 74 days of feeding varied from 38.1 % (diet D) to 40.5 % (diet E). There were no significant differences (P=0.61) between the dietary groups. The measured HTC after 102 days of feeding varied from 40.2 % (diet C) to 41.0 % (diet E), there were no significant differences (P=0.99) between the dietary groups.

The clotting machine was meant for humans, it had a time limit of 96 seconds. Several individuals that had a longer clotting time than this after feeding for 74 (Figure **17** A) and 102 days (Figure **17** B). This resulted in results with low accuracy as it is unknown what their real clotting time is. It was further not possible to conduct statistical analysis on the results.

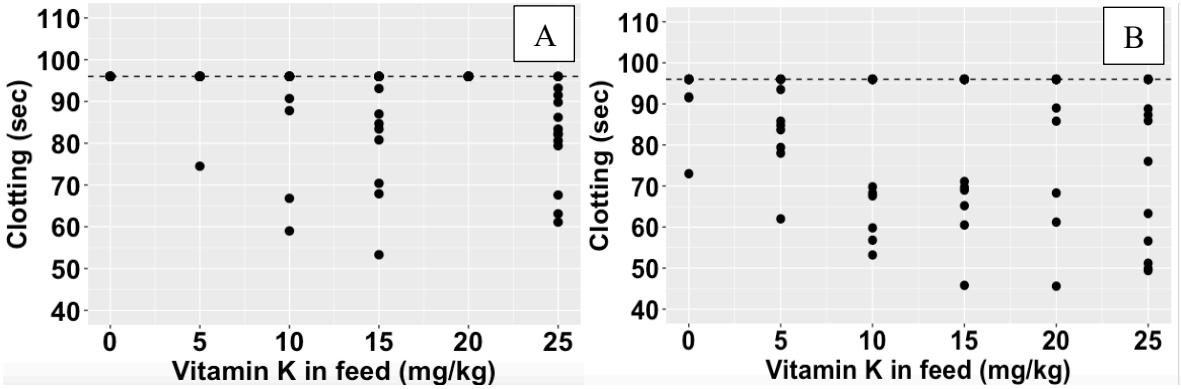

Figure 17: Clotting times for Atlantic salmon (*Salmo salar* L.) for 74 days (A) and 102 days (B), black dots are the individual value, n=15 fish per diet, except for diet 5 mg/kg fed for 102 days, where n =14. The dashed line is the maximum detection time (96 seconds), the dots at this value are 96 or more.

Table 5: Haemoglobin, haematocrit and clotting in seconds for Atlantic salmon (*Salmo salar* L.) fed diets with varying level of vitamin K for 74 and 102 days. Results are shown as mean (SD) (n=3; five individuals per tank, one looser fish was excluded from diet B after feeding for 102 days).

Diet	**Blood parameters**			
	Haemoglobin (g/dL)		Haematocrit (%)	
	Days of feeding			
	74 days	102 days	74 days	102 days
A: 0 mg/kg	7.0 (0.3)	7.7 (0.3)	38.2 (1.0)	40.3 (1.2)
B: 5 mg/kg	7.1 (0.3)	7.7 (0.3)	40.0 (1.9)	40.3 (1.5)
C:10mg/kg	7.4 (0.8)	7.8 (0.3)	40.3 (3.5)	40.2 (1.9)
D 15 mg/kg	7.2 (0.4)	7.9 (0.1)	38.1 (1.4)	40.6 (0.5)
E 20 mg/kg	7.4 (0.4)	7.8 (0.3)	40.5 (3.4)	41.0 (2.4)
F 25 mg/kg	7.1 (0.1)	7.7 (0.3)	38.3(1.4)	40.3 (1.6)

4.4 Content of minerals in the vertebral column

The calcium content varied from 31.8 g/kg (diet A) to 38.9 g/kg (diet D) (Figure **18**). There were no significant differences (P=0.37) between the dietary groups. The estimated correlation was significant (P=0.025); (R^2=0.28).

The phosphorous content ranged from 16.7 g/kg (diet A) to 18.5 (diet D), there was no significance difference between the dietary groups (P=0.347) (Table 6).

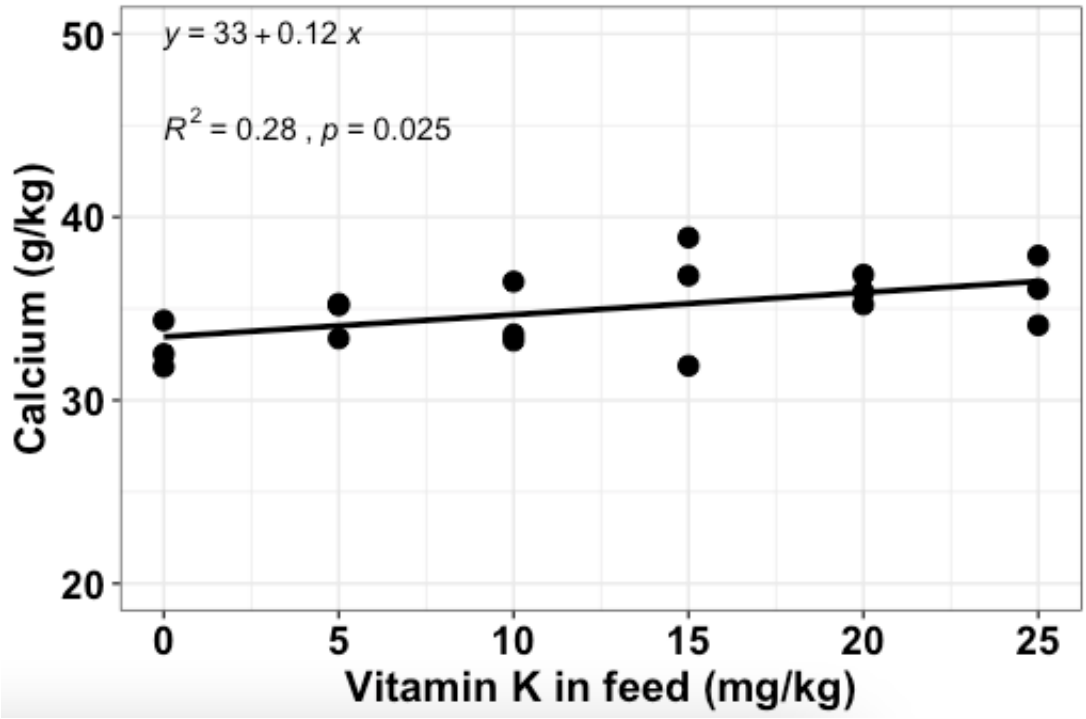

Figure 18: Calcium (g/kg) in vertebral column from Atlantic salmon (*Salmo salar* L.) fed different levels of vitamin K for 74 days. The black dots indicate the mean of tank with the respective diet (n=5 fish per tank, one looser fish was excluded from diet B), y=linear regression, x = vitamin K (mg/kg).

Table 6: Content of phosphorous in the vertebral column of Atlantic salmon (*Salmo salar* L.) fed diets with different level of vitamin K for 74 days. Results shown as mean (SD), (n=3; five fish per tank).

Minerals	**Diet A 0 mg/kg**	**Diet B 5 mg/kg**	**Diet C 10 mg/kg**	**Diet D 15 mg/kg**	**Diet E 20 mg/kg**	**Diet F 25 mg/kg**
Phosphorous (g/kg)	16.72 (0.83)	17.76 (0.54)	17.75 (0.90)	18.49 (1.83)	18.30 (0.51)	17.97 (0.37)

4.5 Deformity scoring

The fish were qualitatively assessed for deformities. There were not detected any severe cases of fish which is recognized by an abnormal body shape, either by curves of shorter or more compressed than normal salmon (Gjerde et al., 2005, p. 78).

From the intermediate and final samplings, the type 11, pronounced biconcave and type 18, irregular internal structure were prevalent in almost all fish, but they were not considered severe and therefore excluded from the results. Type 11 is a common artefacts in the tail region, hence it can be caused by increased deviation of the X-ray beam to the ends of the X-ray plate, amplificant of this deviation by bending the tail downward and less soft tissue in the region (Witten et al., 2009). It is possible that this artefact occurs in other places of the fish if it is not placed in flat position, which was assumed as this deformity type was detected in so many fish. Figure 19 A-E, below shows examples of all deformity types that were detected.

Deformities included were type 1: decreased intervertebral space, type 2: homogeneous compression, type 3: compression and reduced intervertebral space, type 4: compression without X-structure, type 5: one-sided compression, type 6: compression and fusion, type 7: complete fusion, type 8: fusion centre, type 9: elongation, type 10: widely spread and undersized, type 12 hyper-radiodense: type 13: hyper-radiodense with flat end plates, type 14 lordosis: type 15: kyphosis, type 16 scoliosis: type 17 vertically shifted: type 19: internal dorsal or ventral shift and type 20: severe multiple malformation (Figure 5, Figure 6 and Figure 7).

Result of deformity types detected after 74 days feeding and 102 days feeding are shown for the different diets in Table 7.

In Atlantic salmon fed diet A (0 mg/kg vitamin K) for 74 days, 13.33 % of the individuals had at least one deformity. 13.33 % of the individuals fed diet A for 102 days had at least one deformity.

In Atlantic salmon fed diet B for 74 days, 26.67 % individuals showed sign of at least one deformity. In salmon fed diet B for 102 days, 13.33 % of the individuals showed sign of at least one deformity.

In Atlantic salmon fed diet C (10 mg/kg) for 74 days, 13.33 % of the individuals showed signs of at least one deformity. In salmon fed diet C for 102 days, 13.33 % of the individuals showed sign of at least one deformity.

In Atlantic salmon fed diet D (15 mg/kg) for 74 days, 6.67 % of the individuals showed sign of at least one deformity. In salmon fed diet D for 102 days, 33.33 % of the individuals showed sign of at least one deformity.

In Atlantic salmon fed diet E (20 mg/kg) for 74 days, 13.33 % of the individuals showed sign of at least one deformity. In salmon fed diet E for 102 days, 6.67 % of the individuals showed sign of at least one deformity.

In Atlantic salmon fed diet F (25 mg/kg) for 74 days, 26.67 % of the individuals showed sign of at least one deformity. In salmon fed diet F for 102 days, 6.67 % of the individuals showed sign of at least one deformity.

Table 7: Number of fish with at least one deformity in Atlantic salmon (*Salmo salar* L.) fed with diets of varying level of vitamin K, (n=15 fish; per diet each sampling time, one looser fish was excluded from diet B after feeding for 102 days). Deformity rate = percentage of the 15 fish from the sampling time with minimum one deformity.

Diet	**Deformity type**												**Deformity rate (%)**	
	1: Decreased intervertebral space		2: Homogenous compression		5: One-sided compression		6: Compression and fusion		12: Hyper-radiodense		19: Internal dorsal or ventral shift			
	Days of feeding													
	74 days	102 days	74 days	102 days	74 days	102 days	74 days	102 days	74 days	102 days	74 days	102 days	74 days	102 days
A: 0 mg/kg					1		2	1		1			13.33	13.33
B: 5 mg/kg					1	1			2	1	1		26.67	13.33
C: 10 mg/kg				1	2	1		1					13.33	13.33
D: 15 mg/kg				1		2			1	3			6.67	33.33
E: 20 mg/kg	1				1	1							13.33	6.67
F: 25 mg/kg					3				1	1			26.67	6.67

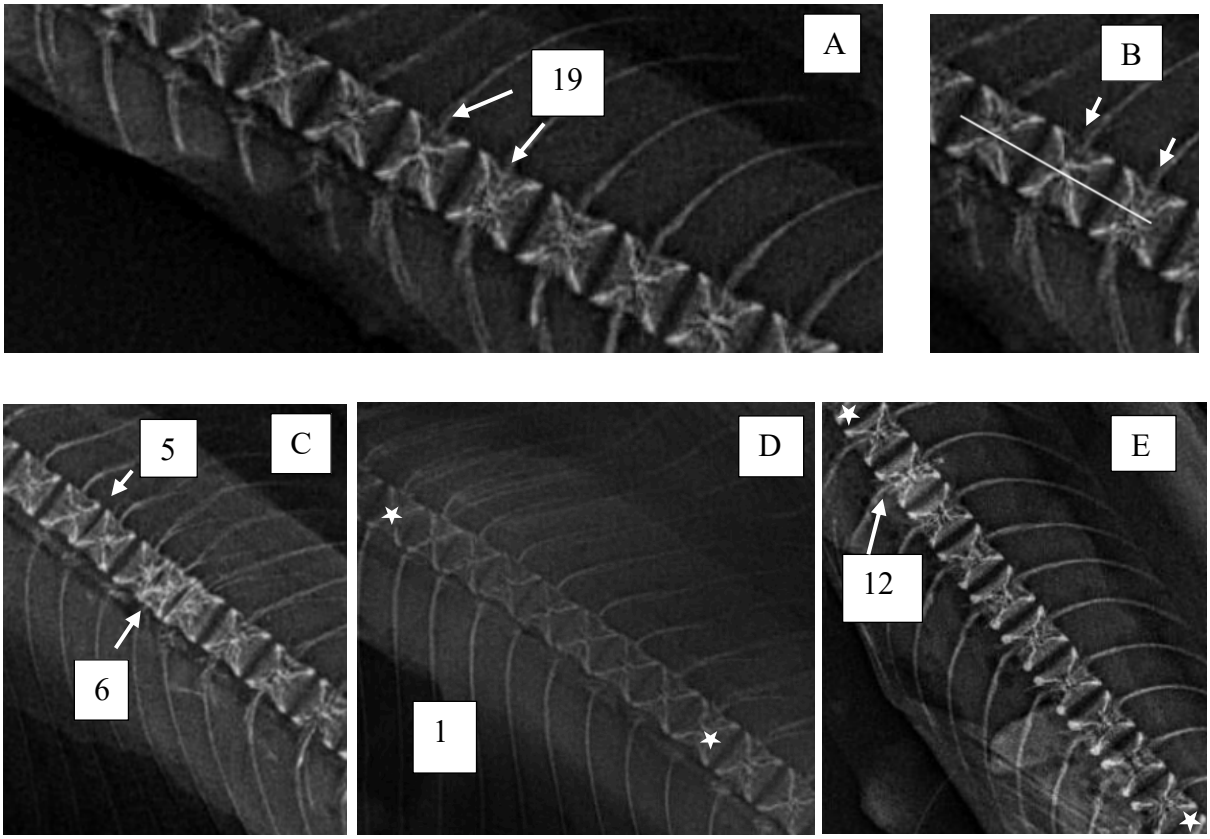

Figure 19: X-ray of the vertebral column from eight Atlantic salmon fed different levels of vitamin K (0 - 25 mg/kg vitamin K). A = Arrows pointing to deformity type 19, internal dorsal or ventral shift. B = Vertebrae of the same individual as in figure 19 A with deformity type 19. White line to illustrate the centre of the vertebral bodies if they had been symmetrical. The centre of the vertebral bodies the arrows points to, one is shifted dorsally, and therefore above the line while the other is ventrally and therefore under the line. C = Arrows pointing to deformity type 5, one-sided compression and type 6, compression and fusion. D = deformity type 1, decreased intervertebral space in vertebrae between the stars. E = Arrow pointing to deformity type 12, hyper-radiodense and type 2, homogeneous compression between stars.

The correlation between deformity and vitamin K was not of significance (P=0.86); (R^2=0.0021) after feeding for 74 days (Table 8). The correlation between deformity was not significant (P=0.44); (R^2 = 0.037) after feeding for 102 days.

Table 8: Linear regression of mean value of Atlantic salmon (*Salmo salar* L.) with minimum 1 deformity (0-1) fed diet with different levels of vitamin K for 74 and 102 days.

	Linear regression	**P-value**	**R^2**
After 74 days of feeding	Y = 0.15+0.0011*vitamin K (mg/kg)	P=0.86	R^2 =0.0021

After 102 days of feeding	Y = 0.16 – 0.0027 *vitamin K (mg/kg)	P=0.44	R^2 = 0.037

4.6 Gene expression of vertebrae

The *BGLAP* ranged from 0.003 to 3.23 (Figure **20** A). The results of *BGLAP* expression were not normally distributed but had a significant skewness value (P<0.01) and significant kurtosis (P=0.01). There were no overall significant differences between the dietary groups (P=0.27). The calculated correlation was not significant (P=0.37).

The expression of *GRP* ranged from 0.04 to 0.17 (Figure **20** B). The expression of *GRP* were not normally distributed, but had a significant skewness (P<0.01) and kurtosis (P=0.01). There were no overall significant differences between the dietary groups (P=0.92). The calculated correlation was not significant (P=0.38).

The expression of *periostin* ranged from 0.04 to 0.13 (Figure **20** C). *Periostin* were not normally distributed but had a significant skewness (P<0.01). There were no overall significant differences between the dietary groups (P=0.14). The calculated correlation was not significant (P=0.36).

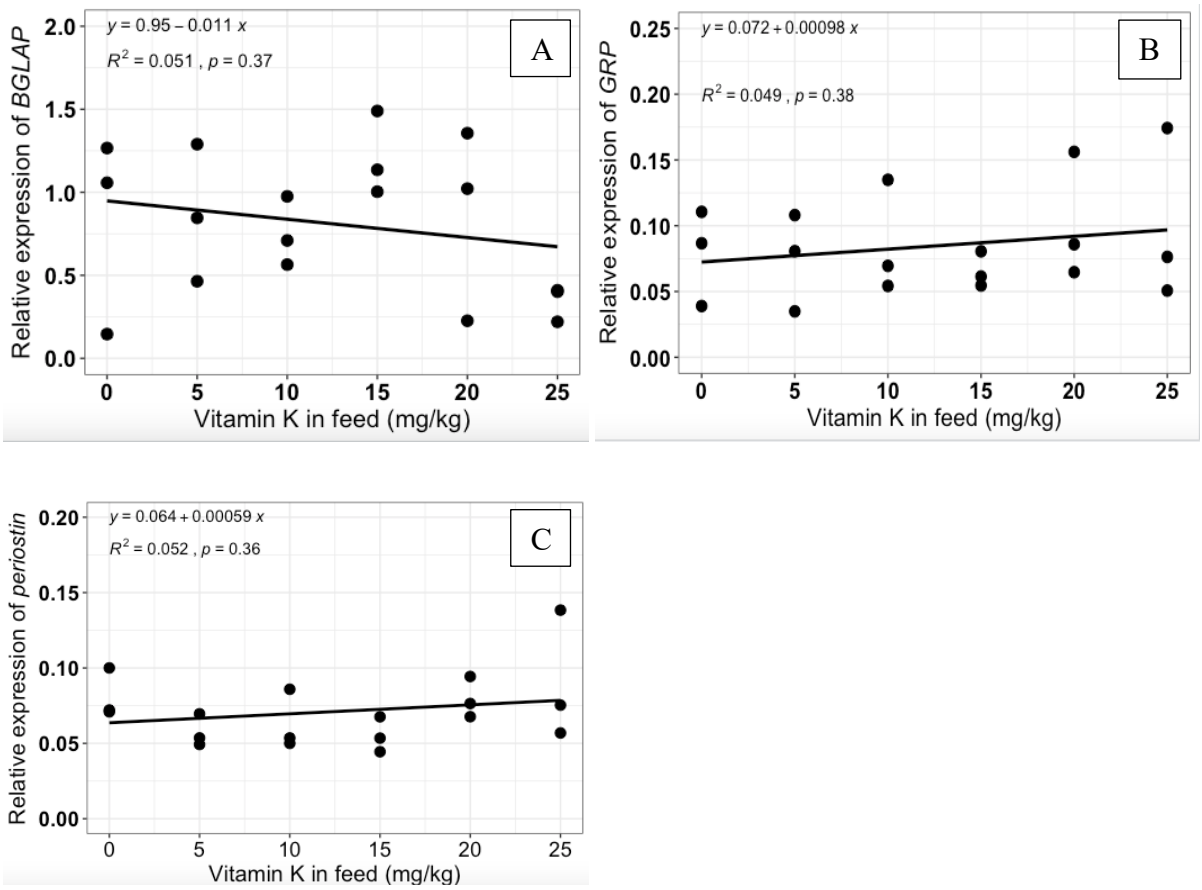

Figure 20: Gene expression of *BGLAP* (A), *GRP* (B) and *periostin* (C) of vertebrae of Atlantic salmon (*Salmo salar* L) fed different levels of vitamin K for 74 days. The black dots represent mean of tank with the respective diet (n=5 fish per tank, one looser fish was excluded from diet B). y=linear regression, x=vitamin K (mg/kg).

The expression of *protein S* ranged from 0.003 to 0.005 (Figure 21 A). The expression of *protein S* were not normally distributed but were significantly skewed (P=0.02). There was no significance difference between the dietary groups (P=0.66). The correlation was not significant (P=0.94).

The expression of *GAS6* ranged from 0.0002 to 0.0003 (Figure 21 B). The expression of *GAS6* were normally distributed as there were no statistical significance for skewness (P=0.8336) or kurtosis (P=0.7913). There were no significance differences (P=0.268) between the dietary groups. The regression line was not significant (P=0.99).

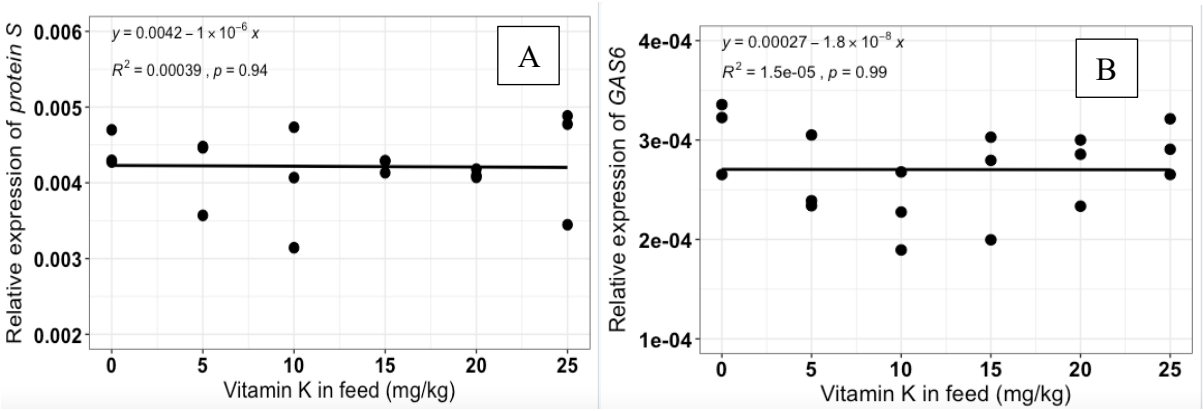

Figure 21: Gene expression of *Protein S* (A) *GAS6* (B) of vertebrae of Atlantic salmon (*Salmo* salar L.) fed different levels of vitamin K for 74 days. The black dots indicate the mean of tank with respective diet (n=5 fish per tank), y =linear regression, x = vitamin K (mg/kg).

4.7 Mechanical properties of vertebrae

4.7.1 Mechanical properties of vertebrae from initial sampling

The breaking force (N) from the initial sampling varied from 4.0 (fish nr 10) to 29.5 N (fish nr 3) (Figure 22 A), the mean was calculated to be 18.5 N. The total force (N) varied between 21.8 N (fish nr 14) and 40.2 N (fish nr 11) (Figure 22 B), the mean was calculated to be 28.8 N.

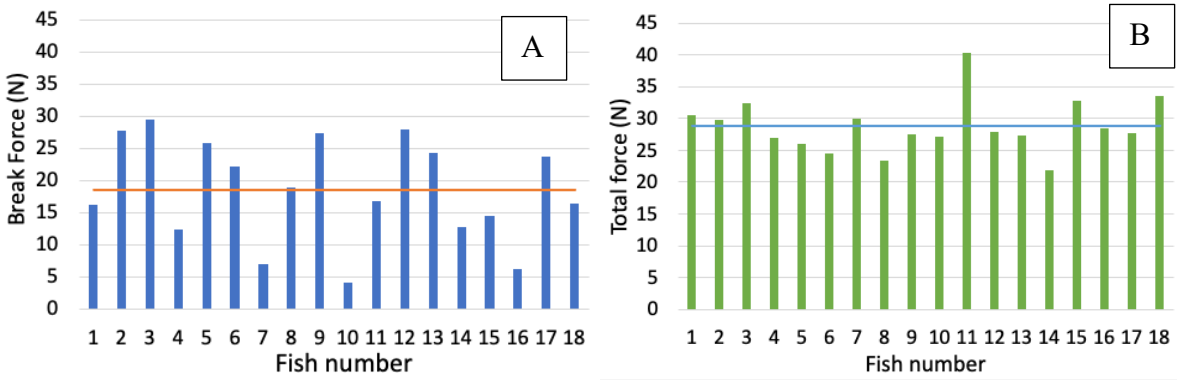

Figure 22: A: Break force of vertebrae number 12 counted from the head in Atlantic salmon (*Salmo salar* L.) before the fish were exposed to diets with different levels of vitamin K. The orange line is the mean value of the 18 individuals. B: Total force of vertebrae number 12 counted from the head in Atlantic salmon (*Salmo salar* L.) before the fish were exposed to diets with different levels of vitamin K. The blue line is the average of the total peak force between the individuals.

4.7.2 Mechanical properties of vertebrae after 74 and 102 days of feeding

The results of two individuals fed diet A were excluded due to technical errors with the texture analyser machine, resulting in unacceptable low accuracy.

After 74 days of feeding, the breaking force (ranged from 40 – 54 N) with no significant differences between the dietary groups (P=0.30). The correlation was not significant (P=0.97); ($R^2 = 7.4 * 10^{-5}$) (Figure 23 A). The maximum force (ranged from 40 – 59 N) with no significant differences between the dietary groups (P=0.751). The correlation was not significant (P=0.91); (R^2=0.00078) (Figure 23 B).

After 102 days of feeding, the breaking force (ranged from 49 - 66 N) with no significant differences between the dietary groups (P=0.43). The correlation was not significant (P=0.22); (R^2=0.094) (Figure 23 C). The maximum force (ranged from 52 - 71 N) with no significant differences between the dietary groups (P=0.54). The correlation was not significant (P=0.14); (R^2=0.13) (Figure 23 D).

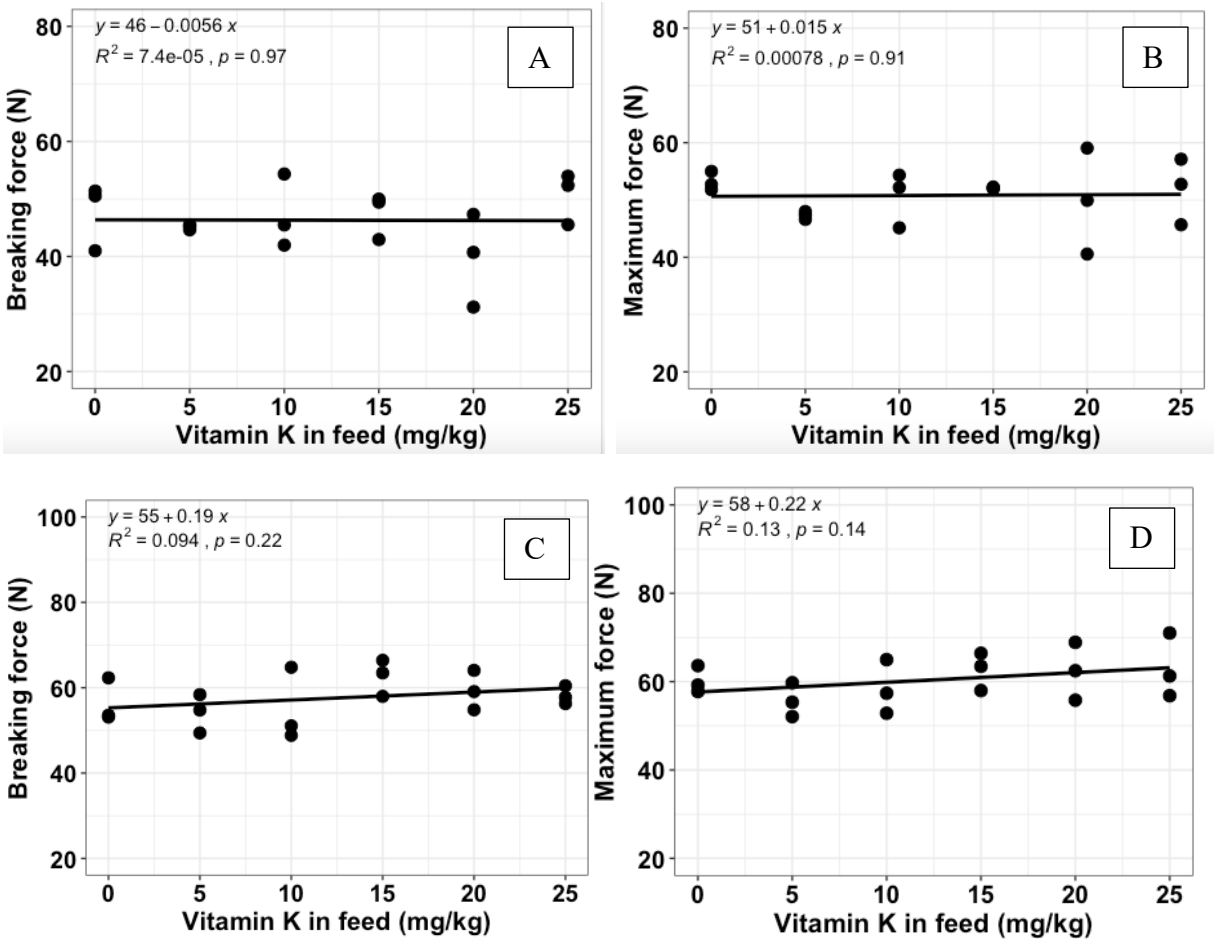

Figure 23: Breaking force (N) of vertebrae of Atlantic salmon (*Salmo salar* L.) fed different levels of vitamin K for 74 days (A) and 102 days (C). Maximum force (N) of vertebrae of Atlantic salmon (*Salmo salar* L.) fed different levels of vitamin K for 74 days (B) and 102 days (D). The black dots represent mean for each respective tank (n=5 fish per tank, one looser fish was excluded from diet B after feeding for 102 days). y=linear regression, x=vitamin K (mg/kg).

5 Discussion

Vitamin K is a cofactor in the vitamin K cycle (De Vilder et al., 2017; Tie & Stafford, 2016). It is known that it influences the vitamin K dependent proteins that participate in blood clotting (Halder et al., 2019). In addition, there has also been discovered some vitamin K dependent proteins that influence the bone such as BGLAP, GRP, periostin, protein S and GAS6 (Krossøy et al., 2011; Maillard et al., 1992; Stock & Schett, 2021; Tsugawa & Shiraki, 2020, p. 1; Xiao et al., 2021). There was therefore a need to investigate the effect of vitamin K on overall and bone health on Atlantic salmon. The aims were to investigate the influence of vitamin K on: 1) growth, 2) biometric traits, 3) blood parameters, 4) gene expressions of the mentioned genes, 5) mechanical properties of vertebrae, 6) incidents of deformities and 7) content of minerals in vertebral column, to gain knowledge on how vitamin K_3 influence bone.

5.1 Vitamin K in feed

There was analysed a higher amount of vitamin K in diet B-F than what was added. However, as expected the vitamin K level in most diets decreased from first sampling to second sampling (reduction by 5 – 22 %), with diet A as an exception. There were detected relative high reductions in both diet B and F, while there were reductions of around 5% in diet C, D and E.

5.2 Growth

Based on the estimated correlation of the SGR and MNB in the diet, there is no indication that MNB in the diets (≥ 0.1 mg/kg vitamin K_3) affects growth of Atlantic salmon in early sea phase. This is in accordance with similarly studies on Atlantic salmon by Graff et al. (2002) and Krossøy et al. (2009a), where menadione sodium bisulphite (MSB) (a type of vitamin K_3) was used in the study by Graff et al. (2002) and MNB was used in the study by Krossøy et al. (2009a). However, this is not in accordance with the results from the study by Taveekijakarn et al. (1996) using menadione (type of vitamin K_3) on young Amago salmon, where it was detected a positive effect on growth and studies on Gilthead seabream by Sivagurunathan et al. (2023) using menadione and (Dominguez et al., 2022) using MSB where there were detected a positive effect on growth up to a level of 4.98 mg/kg vitamin K_3, while levels above that had a negative effect on growth.

It is possible there would have been detected a significant influence on growth if vitamin K_1 had been used or if a higher level of vitamin K_3 had been used, as was seen in a study by

Grisdale-Helland et al. (1991) on Atlantic salmon and Richard et al. (2014) on Sengalese sole larvae. According to the results from studies by Graff et al. (2010) and Krossøy et al. (2010) vitamin K_1 has been proven to have a higher bioavailability than vitamin K_3.

5.3 Biometric traits

There was no indication that MNB levels in the diets (≥ 0.1 mg/kg vitamin K_3) influence the biometric traits. It was expected to not detect any significant difference of the status of the liver between the dietary groups, as there has been conducted a study by Graff et al. (2010), where a level of 46.5 mg/kg MNB was present in diets to Atlantic salmon and it resulted in about 0.1 mg/kg increase of MK-4 in the liver. Due to the low bioavailability of MNB seen in the study, it was expected that vitamin K used in present study, would not be toxic or lead to malnutrition. Furthermore, no correlation between liver scoring or HSI and dietary MNB inclusion was observed.

5.4 Blood parameters

The measured Hb values were above the level for smolts reported by Rozas-Serri et al. (2022), however they fit well with the intervals for post smolts. This was a bit surprising, as it was expected for these values to be withing the range for smolts. The HTC levels fit with the reference intervals for smolts reported by Rozas-Serri et al. (2022). The absence by the dietary effects was expected. The relatively consistent results of Hb and HTC and while some numerical decrease in clotting time, can indicate that vitamin K had some influence on clotting time. As the methodology used in this study was based on human clotting measurements, one cannot conclude on whether vitamin K influence clotting time. However, the results seemed to indicate that MNB supplementation reduced clotting time as expected.

As there seemed to be a numerical increase of fish with clotting time below 96 seconds. It would be beneficial to repeat the analysis of clotting time with a machine that can measure a longer clotting time for over 96 seconds.

5.5 Content of minerals

There is indication that MNB levels in the diets (≥ 0.1 mg/kg vitamin K_3) influence the calcium content in the vertebrae column as the correlation was significant, and the linear regression describes 28 % of the variance. On the other hand, there were no significant differences between

the dietary groups. Based on the correlation, it seems likely that a higher level of MNB than used in present study, can result in a significant difference of calcium content in the vertebral column between the dietary groups.

At a level of 50 and 250 mg/kg vitamin K_1, there has been seen a positive effect on incidents of deformity and calcium content on Senegalese Sole in study by Richard et al. (2014). It is possible that a higher level of MNB in the diet would have resulted in the same, but it is more likely that vitamin K_1 would lead to more similar results because of the believed higher bioavailability in that type of vitamin K (Krossøy et al., 2010).

Similar to calcium content, the content of phosphorous also increased numerically in accordance with the increase of calcium. The highest phosphorous content was measured in diet D, and like calcium content, the highest standard deviation was also detected in diet D, however there were no significant differences between the dietary groups.

5.6 Deformity scoring

From the intermediate and final samplings, the type 11, pronounced biconcave and type 18, irregular internal structure was prevalent in almost all fish, but they were excluded from the results, as they were regarded to be more likely caused by artefacts. Type 11, pronounced biconcave is a common artefacts in the tail region, hence it can be caused by increased deviation of the X-ray beam to the ends of the X-ray plate, amplificant of this deviation by bending the tail downward and less soft tissue in the region (Witten et al., 2009). It is possible that this artefact occurs in other places of the fish, if it is not placed in flat position.

Pronounced biconcave deformity can be a sign of early mineral deficiency (Luyer et al., 2015, p. 2). However, as the fish did not show any other indications of early mineral deficiency, based on the mineral content of phosphorous and calcium. It was assumed that the incidents of pronounced biconcave was caused by an artefact and it seemed likely to be due to positioning of the salmon. Type 18, irregular internal structure was also assumed to be caused by an artefact due to the high prevalence.

There were detected a somewhat consistent incidents of deformities between the dietary groups, there was most often detected 13.33 % of the individuals with at least one deformity but also 6.67 % and 26.67 % of the individuals with at least one deformity. There were relatively many type 5, one-sided compressions between the dietary groups. However, the number of incidents

of deformity 5 were relatively stable (1 to 2) of the 15 individuals per diet, with diet F as an exception where 3 incidents were detected. Due to the fluctuation in incidents between dietary groups, there do not seem to be an indication that MNB influences incidents of deformity type 5, one-sided compression.

Even though the detected deformities were relatively mild deformity types, there were detected some incidents of deformity 6, compression and fusion. This was most prevalent in diet A after 74 and 102 days of feeding, but also detected one incident in diet C. It is possible that vitamin K might have a positive effect on this deformity, however, this would need to be further investigated.

As type 1, decreased intervertebral space and type 2, homogenous compression are often considered to be related to shortening of the vertebral column and thus also shortening the body (Witten et al., 2009). One would expect to detect the deformities based on their external appearance of the fish. However, this was not detected in any of the fish, this could possibly support that the deformity was not severe. As the incidents were detected in diets with C, D and E (10, 15 and 20 mg/kg MNB), this was believed to indicate that MNB do not influence the incidents of the deformities, as a numerically increasing or decreasing trend would have been expected to detect.

There were detected several incidents of type 12, hyper-radio dense deformities. This can be due to hyper mineralization to heal from mechanical stress, but not necessarily (Witten et al., 2009). There were not detected a numerical decreasing or increasing trend, instead there were detected a relatively stable number across the dietary groups (1-2), however, diet D (15 mg/kg MNB) was an exception with 3 incidents after 102 days of feeding. However, there were still believed to not be a clear indication that MNB influenced the incidents.

There was detected one incident of type 19, internal dorsal or ventral shift, as there was only detected one incident of this, there is no indication that MNB influence the incidents of this deformity.

There was no indication that MNB levels in the diets ($\geq$ 0.1 mg/kg vitamin K_3) influence the incidents of deformities as the correlations were not significant and was estimated to be positive after 74 days of feeding and negative after 102 days of feeding.

5.7 Gene expression

As there was a relative high variation in diet A and E (0 and 20 mg/kg MNB), it was difficult to determine a trend of expression of *BGLAP*. As there were no significant differences between the dietary groups and the regression line was not significant either, vitamin K do not seem to influence expression of *BGLAP*. Because of the relative high variance, it seems likely that other factors influence the expression.

In study by Sivagurunathan et al. (2023) there was detected a numerical reduction of expression of *BGLAP* in larvae fed diets with highest vitamin K_3 (58.51 mg/kg) for 14 days. This seem to be in accordance with the results from the present study. Furthermore, in the study by Sivagurunathan et al. (2023), there was detected a high correlation between *BGLAP* and gene expression of sox9, for further studies analysis of sox9 and *BGLAP* could perhaps be something to investigate to gain better understanding of *BGLAP*. According to Sivagurunathan et al. (2023) too high intake of vitamin K_3 can reduce mineralization as it can decrease the affinity to hydroxyapatite. However, this was not detected in the present study.

In a study by Krossøy et al. (2009a) on Atlantic salmon fed diets with and without MNB, the expression of *BGLAP* differed from week 14 to 23 and 28. Fish fed diets without MNB had highest expression in week 14, whereas the diets without and diet with highest content of MNB had an relatively equal expression in week 23 and fish fed diets with highest content of MNB had highest expression in week 28. It is possible the same would have been seen if the experiment lasted for a longer time. However, as Atlantic salmon parr was used in the aforementioned study, it is also possible that Atlantic salmon is more influenced by vitamin K in a younger phase.

Vitamin K_2 is found to regulate expression of *BGLAP*, through steroid and xenobiotic receptor signalling pathway (Krossøy et al., 2009a). It is possible that there were not detected a significant trend in expressed *BGLAP* due to low bioavailability of vitamin K_3, as it is converted into MK-4 to participate in the vitamin K cycle (Krossøy et al., 2010).

GRP is believed to indicate calcification activity, and has a high ability to bind calcium (Viegas et al., 2009). It was expected with an elevated level of *GRP* from diet A to F due to the expectation of more calcium being bound. As the there were no significant differences between the dietary groups and the correlation was not significant, there is no indication that MNB used in present study influence *GRP* expression. The correlation was numerically slightly positive,

it is therefore possible that with a higher level of MNB in the diets than used in present study, could result in a significant difference between the dietary groups. However, due to the high variation, it seems unlikely.

The correlation of *periostin* and MNB was numerically slightly positive. However, as there were no statistical significance difference between the dietary groups, and regression line was not significant, there is no indication that vitamin K_3 influences *periostin* expression with MNB levels used in the present study and when salmon are exposed to the same condition they were in this present study.

As it is believed according to Bonnet et al. (2016) that *periostin* has a positive influence on bone formation, it is possible that significant differences between the dietary groups would have been detected if the salmon had been exposed to mechanically stressful situations (Bonnet et al., 2016; Kudo, 2011). Transportation and handling is stressful for salmon (Sigholt et al., 1997, p. 898). It is possible there would have been detected significant differences between the dietary groups is the salmon had experienced more handling and transportation. This is possible as the salmon might have a higher need for bone formation to compensate for the stress.

The results from gene expression of *protein S* across the different diets were not significant different from each other and as the regression line was not significant, there were no indication that MNB used in present study influence expression of *protein S*.

The results did not indicate that MNB influence the expression of *GAS6* as there were no significant differences between the dietary groups and the regression line was not significant. It was surprising that there was a slightly negative correlation between MNB and expression of *GAS6*, as it was rather expected with a positive correlation.

There was no indication that MNB levels in the diets ($\geq$ 0.1 mg/kg vitamin K_3) influence the gene expression of the genes investigated (*BGLAP*, *GRP*, *periostin*, *protein S* and *GAS6*). The detected variation between diets and within diets indicate that there were other unknown factors present in the present study had a bigger influence on the expression than the diets.

5.8 Mechanical properties of vertebrae

In attempt to measure mechanical properties of the vertebrae, there were times the vertebrae were not positioned correct. This resulted in values with low accuracy, to compensate for this,

vertebrae number 13 or 14 were used instead as there was not expected to be high variance between those vertebras as they were so close to each other.

It was surprising that there was a negative correlation between breaking force and MNB, after 74 days of feeding. There was a numerically, slight stronger correlation between the dietary groups after 102 days of feeding compared to the results after 74 days of feeding, although none of them were significant. The results of mechanical properties of vertebrae after 74 days of feeding were likely to some degree inaccurate, due to errors made during measuring, including missing the centra and measure the mechanical properties of intervertebral space instead.

The results of correlation of mechanical properties and MNB after 74 days of feeding were less significant than compared to the correlations after 102 days of feeding. This could indicate that correlation increases with time, however, as the correlation is not of significant, there was still no indication of that levels of MNB used in the present study influence the mechanical properties of the vertebrae. The results of mechanical properties in present study were not in accordance with the result in master thesis by Agyeman (2019, pp. 10-21) younger Atlantic salmon were used in the thesis. This can indicate that Atlantic salmon is more influential in a younger phase.

Additionally, according to Fjelldal et al. (2004) there was detected difference in mechanical properties of vertebrae between deformed (scoliosis and ventral-dorsal spinal curvatures) and nondeformed. It is possible that there not detected significant differences, as none of the vertebral columns were severely deformed.

Overall, there was no indication that MNB levels in the diets (≥ 0.1 mg/kg vitamin K_3) influence the breaking force of cortical bone of the vertebrae or maximum force at centrum of vertebrae, as there were no significant differences between the dietary groups, and the correlations were not significant. The results of mechanical properties seem to be in accordance with the deformity scoring and gene expressions as there were not detected any significant effect of vitamin K on those parameters.

6 Conclusion

There were no significant (P>0.05) differences between the dietary groups on specific growth rate (SGR).

There were no significant differences on the biometric traits on the analysed salmon between the dietary groups, after 74 and 102 days of feeding (P>0.05).

There were no significant differences between Hb and HTC of salmon after 74 and 102 days of feeding (P>0.05).

The linear regression of calcium content in vertebral column was significant (P=0.025) after 74 days of feeding, however there were no significant (P>0.05) differences between the dietary groups of calcium content in the vertebral column. There were no significant (P>0.05) differences between the dietary groups of phosphorous content.

The linear regression of incidents of deformities were not significant after 74 days (P>0.05) and 102 days (P>0.05) of feeding.

There were no significant (P>0.05) differences between the gene expression, and the linear regression of the measured genes (*BGLAP*, *GRP*, *periostin*, *protein S* and *GAS6*) were not significant (P>0.05) between the dietary groups.

There were no significant (P>0.05) differences between the dietary groups on mechanical properties of the vertebrae after 74 and 102 days.

The results from the mechanical properties of vertebrae, deformity scoring and gene expression, is not supporting the hypothesis as there were no significant differences between the dietary groups. The optimal level based on general health and bone health seem to be 0.1 mg/kg MNB for Atlantic salmon in early seawater phase.

7 Future work

As there was a positive correlation between calcium content and MNB in the diets, it could be interesting to further investigate the effect of more calcium content in the vertebral column.

Although level of vitamin K_3 was controlled, it could be interesting to measure level of vitamin K_1 that is naturally present in the feed as there is some from the fish oil, fish meal and vegetable oil (Graff et al., 2010). It is possible that there is enough vitamin K_1 naturally in the feed to support a normal health.

It is additionally unknown how much of the MNB was converted into MK-4 in the liver. It is expected that a relatively low concentration of MNB was converted to MK-4 as seen in the study by Graff et al. (2010). However, it still would be beneficial to conduct this analysis as well.

It could be interesting to examine the effect of MNB on Atlantic salmon experiencing stressful situations including transportation and handling, to examine if the expression of *periostin* is more affected in those situations.

Histological analysis could also be beneficial to perform to get conclusive results on deformities on MNB influence incidents of deformities, this could be done according to the protocol by Goldschlager et al. (2010). Although, based on the results from present study there is not a clear indication that one would detect significant differences between the dietary groups.

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Appendix A

Q Protocol

RNeasy® 96 QIAcube® HT Total RNA Tissue Protocol

For use with RNeasy 96 QIAcube HT Kit and QIAcube HT Plasticware

This protocol is intended for the automated purification of RNA from tissue using the QIAcube HT instrument. The procedure yields high-quality RNA that performs well in RT-PCR and other downstream reactions. The RNeasy 96 QIAcube HT Kit combines the selective binding properties of a silica-based membrane with a high-throughput 96-well format, and is designed for fully automated, simultaneous processing of 24–96 samples on the QIAcube HT instrument. This protocol also requires QIAzol® Lysis Reagent (cat. no. 79306), Collection Microtubes (cat. no. 19560), and Collection Microtube Caps (cat. no. 19566).

Q Protocol description:

- Run file: **RNeasy 96 QIAcube HT total RNA Tissue V1.qsp**
- Purification of RNA using silica technology
- Automated processing using vacuum filtration for bind, wash, and elute steps

Protocol specifications

Specification	Description
Number of samples	24–96 samples (to be processed in increments of 8)
Sample input volume	350 µl aqueous phase from up to 40 mg frozen or 20 mg stabilized tissue homogenized in 750 µl QIAzol Lysis Reagent
Elution volume	110 µl
Duration	96 samples in approximately 73 minutes 24 samples in approximately 34 minutes Each additional 8 samples extend the processing time by 5 minutes

Worktable setup

For detailed protocol and worktable setup information, see the pre-run report and the *RNeasy 96 QIAcube HT Handbook*.

Reagents

Note: Each Reagent Trough must be loaded on the worktable in a Reagent Trough Adapter.

Reagent	Plasticware	Worktable position
Wash Buffer RWT	Reagent Trough 170 ml	R1
Wash Buffer RPE	Reagent Trough 170 ml	R1
Binding reagent (70% Ethanol)	Reagent Trough 70 ml	R1
Elution reagent (RNase-free water)	Reagent Trough 70 ml	C1
TopElute Fluid	Reagent Trough 70 ml	C1

Consumables

Consumables	Worktable position
Filter-Tips, OnCor C, 200 µl	B2 and C2 (2 x tip racks)
RNeasy 96 well plate	A1 (waste (left) compartment of vacuum chamber)
Elution Microtubes RS	A1 (elution (right) compartment of vacuum chamber)

Samples

Samples	Worktable position
S-Block, 96-Well	B1

Ordering Information

Product	Contents	Cat. no.
RNeasy 96 QIAcube HT Kit (5)	For 480 preps: RNeasy plates, RNase-free water, buffers	74171
QIAcube HT Plasticware	For 480 preps: 5 S-Blocks, 5 EMTR RS, 2 x 50 Caps for EMTR, 9 x 96 Filter-Tips OnCor C, TapePad	950067
Elution Microtubes RS	24 x 96 Elution Microtubes, racks of 96; includes cap strips	120008
S-Blocks	24 x 96-well blocks with 2.2 ml wells	19585
QIAzol Lysis Reagent	200 ml QIAzol Lysis Reagent	79306
TissueLyser		
TissueLyser II	Bead mill, 100–120/220–240 V, 50/60 Hz; requires the TissueLyser Adapter Set, 2 x 24 or TissueLyser Adapter Set 2 x 96 (available separately)*	85300
TissueLyser Adapter Set 2 x 24	2 sets of adapter plates and 2 racks for use with 2 ml microcentrifuge tubes on the TissueLyser II	69982
TissueLyser Adapter Set 2 x 96	2 sets of adapter plates for use with Collection Microtubes (racked) on the TissueLyser II	69984
TissueLyser LT	Compact bead mill, 100-240 V AC, 50–60 Hz; requires the TissueLyser LT Adapter, 12-Tube (available separately)†	85600
TissueLyser LT Adapter, 12-Tube	Adapter for disruption of up to 12 samples in 2 ml microcentrifuge tubes on the TissueLyser LT	69980
Stainless Steel Beads, 5 mm (200)	200 stainless steel beads (5 mm diameter), suitable for use with TissueLyser systems	69989
Collection Microtubes (racked, 10 x 96)	Nonsterile polypropylene tubes (1.2 ml), 960 in racks of 96	19560
Collection Microtube Caps (120 x 8)	Nonsterile polypropylene caps for collection microtubes (1.2 ml) and round-well blocks, 960 in strips of 8	19566

* The TissueLyser II must be used in combination with the TissueLyser Adapter Set 2 x 24 or TissueLyser Adapter Set 2 x 96.
† The TissueLyser LT must be used in combination with the TissueLyser LT Adapter, 12-Tube.

QIAcube HT instrument		
QIAcube HT system	Robotic workstation with UV lamp, HEPA filter, laptop, QIAcube HT operating software, start-up pack, installation and training, 1-year warranty on parts and labor	9001793
Accessories Pack, QXT	Upgrade kit for QIAxtractor® instrument; adapter set to use dedicated QIAcube HT kits on the QIAxtractor Contains: Transfer Carriage (9022654), Riser Block EMTR (9022655), and Channeling Adapter (9022656)	9022649

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at www.qiagen.com or can be requested from QIAGEN Technical Services or your local distributor.

For a complete list of accessories, visit www.qiagen.com/p/QIAcubeHT.

www.qiagen.com
Australia ■ 1-800-243-800
Austria ■ 0800-281011
Belgium ■ 0800-79612
Brazil ■ 0800-557779
Canada ■ 800-572-9613
China ■ 800-988-0325
Denmark ■ 80-885945
Finland ■ 0800-914416
France ■ 01-60-920-930
Germany ■ 02103-29-12000
Hong Kong ■ 800 933 965
India ■ 1-800-102-4114
Ireland ■ 1800 555 049
Italy ■ 800-787980
Japan ■ 03-6890-7300
Korea (South) ■ 080-000-7145
Luxembourg ■ 8002 2076
Mexico ■ 01-800-7742-436
The Netherlands ■ 0800-0229592
Norway ■ 800-18859
Singapore ■ 1800-742-4368
Spain ■ 91-630-7050
Sweden ■ 020-790282
Switzerland ■ 055-254-22-11
Taiwan ■ 0080-665-1947
UK ■ 0808-2343665
USA ■ 800-426-8157

Sample & Assay Technologies

Appendix B

Quick-Start Protocol March 2016

QuantiTect® Reverse Transcription Kit

The QuantiTect Reverse Transcription Kit (cat. nos. 205310, 205311, 205313 and 205314) should be stored immediately upon receipt at –30 to –15°C in a constant-temperature freezer.

Further information

- *QuantiTect Reverse Transcription Handbook*: **www.qiagen.com/HB-0189**
- Safety Data Sheets: **www.qiagen.com/safety**
- Technical assistance: **support.qiagen.com**

Notes before starting

- Dissolve any precipitates in gDNA Wipeout Buffer by vortexing. If necessary, briefly incubate at 37°C until the precipitates dissolve.
- Set up all reactions on ice to minimize the risk of RNA degradation.
- RNase inhibitor and dNTPs are already included in the kit components. Do not add additional RNase inhibitor or dNTPs.
- RT Primer Mix (supplied) or gene-specific primers (not supplied) should be used. RT Primer Mix is optimized to provide high cDNA yields for all regions of RNA transcripts. For gene-specific primers, we recommend using a final concentration of 0.7 µM.
- Separate denaturation and annealing steps are not necessary before starting the reverse-transcription reaction.
- If using a reaction volume of 200 µl or greater for reverse transcription, make sure the reaction tube is efficiently heated (e.g., if using a heating block, carefully fill each well with a drop of water so that heat can be efficiently transferred from the block to the tube).
- After reverse transcription, the reaction must be inactivated by incubation at 95°C for 3 min.

Sample to Insight

- If working with RNA for the first time, refer to Appendix A of the *QuantiTect Reverse Transcription Handbook*.
- If you have purchased the QuantiTect Reverse Transcription Kit in order to perform additional reverse-transcription reactions with the FastLane® Cell cDNA Kit, follow the protocol in the *FastLane Cell cDNA Handbook*. Do not follow this protocol.

1. Thaw template RNA on ice. Thaw gDNA Wipeout Buffer, Quantiscript® Reverse Transcriptase, Quantiscript RT Buffer, RT Primer Mix and RNase-free water at room temperature (15–25°C). Mix each solution by flicking the tubes. Centrifuge briefly to collect residual liquid from the sides of the tubes, and then keep on ice.
2. Prepare the genomic DNA elimination reaction on ice according to Table 1. Mix and then keep on ice.

   Note: If setting up more than one reaction, prepare a master mix of gDNA Wipeout Buffer and RNase-free water with a volume 10% greater than that required for the total number of reactions to be performed. Distribute the appropriate volume of master mix into individual tubes, followed by each RNA sample.

   Note: The protocol is for use with 10 pg to 1 µg RNA. If using >1 µg RNA, scale up the reaction linearly. For example, if using 2 µg RNA, double the volumes of all reaction components for a final 28 µl reaction volume.

Table 1. Genomic DNA elimination reaction components

Component	Volume/reaction
gDNA Wipeout Buffer, 7x	2 µl
Template RNA, up to 1 µg*	Variable
RNase-free water	Variable
Total reaction volume	14 µl

* This amount corresponds to the entire amount of RNA present, including any rRNA, mRNA, viral RNA and carrier RNA present, and regardless of the primers used or cDNA analyzed.

3. Incubate for 2 min at 42°C, then place immediately on ice.

   Note: Do not incubate at 42°C for longer than 10 min.

4. Prepare the reverse-transcription master mix on ice according to Table 2. Mix and then keep on ice. The reverse-transcription master mix contains all components required for first-strand cDNA synthesis except template RNA.

   Note: If setting up more than one reaction, prepare a volume of master mix 10% greater than that required for the total number of reactions to be performed. Distribute the appropriate volume into individual tubes.

   Note: If using >1 µg RNA, scale up the reaction linearly. For example, if using 2 µg RNA, double the volumes of all reaction components for a final 40 µl reaction volume.

Table 2. Reverse-transcription reaction components

Component	Volume/reaction
Reverse-transcription master mix	
Quantiscript Reverse Transcriptase*	1 µl
Quantiscript RT Buffer, 5x†‡	4 µl
RT Primer Mix‡	1 µl
Template RNA	
Entire genomic DNA elimination reaction (step 3)	14 µl (added at step 5)
Total reaction volume	20 µl

* Also contains RNase inhibitor.

† Includes Mg^{2+} and dNTPs.

‡ For convenience, premix RT Primer Mix and 5x Quantiscript RT Buffer in a 1:4 ratio if RT Primer Mix will be used routinely for reverse transcription. This premix is stable when stored at –20°C. Use 5 µl of the premix per 20 µl reaction.

5. Add template RNA from step 3 (14 µl) to each tube containing reverse-transcription master mix. Mix and then store on ice.
6. Incubate for 15 min at 42°C.

   Note: In some rare cases (e.g., if the RT-PCR product is longer than 200 bp or if analyzing RNAs with a very high degree of secondary structure), increasing the incubation time up to 30 min may increase cDNA yields.

7. Incubate for 3 min at 95°C to inactivate Quantiscript Reverse Transcriptase.
8. Place the reverse-transcription reactions on ice and proceed directly with real-time PCR. For long-term storage, store reverse-transcription reactions at –20°C.

   Note: For details on performing real-time PCR after reverse transcription, refer to Appendix C of the *QuantiTect Reverse Transcription Handbook*. For details on appropriate controls, see Appendix D. We recommend using a Rotor-Gene® Kit, QuantiFast® Kit or QuantiTect Kit for real-time PCR.

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Appendix C

Gene name	Accession	Forward primer	Reverse primer
BGLAP	NM_001136551.2	TCAGCTGGAGAGTCTGAGGG	AGTTGGTTTGTGGAGTGGGTT
UCMA	XM_014147860.2	CTGTCCCCGATGATAAGGGC	TGCCTCTGTTCAGCGTCAAT
Matrix Gla	XM_014203686.2	GAAACAGATGGGTGGGGG	TCGGTCTCTTGGTCTGCCTA
Protein S	XM_045699644.1	GAGAACTACCCAGAGACGGAAT	CTGAGGTTTGAAGGGACGCT
Periostin	XM_045690215.1	ATTCTTTACCCCGGGGACCT	AGGGGGATTTCGGTGTAGGA
GAS6	XM_045698411.1	CCGAGGTTTGACGGCTGTAT	ACTGGGACTCTGCGTATGTG

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