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Discolouration of the dorsal white musculature of farmed Atlantic salmon (*Salmo salar* L.) in Norway, characterised by histological and transcriptional methods

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Acknowledgment

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Abstract

Focal melanisation in the white muscle of farmed Atlantic salmon (*Salmo salar* L.*)* has become a common finding in Norway. Depending on the magnitude of such changes, muscle tissue (fillets) are graded to lower quality, leading to immense economic losses. In 2010, the cost to Norwegian aquaculture of melanised changes in fish fillets was estimated to approximately NOK 500 million (Hjeltnes et al., 2016). In particular, major costs are associated with removing melanised changes in fillets.

The purpose of the present work was to investigate the nature of focal melanised changes located in the dorsal muscle of seawater-farmed Atlantic salmon, and to compare these with similar changes in the muscle of the abdominal wall. The material was sampled from two fish farms, one in Western and one in Mid Norway. All the fish investigated had been vaccinated intraperitoneally with oil-adjuvanted vaccines. Gross pathological inspection, histological and immunohistochemical methods were used to characterize the changes. In addition, infectious agents were searched for by molecular approaches.

Focal and diffuse melanised changes, which were observed in dorsal musculature, were compared with focal melanised abdominal changes. The results showed non-distinguishable pathological processes in both muscle regions. Presence of *piscine orthoreovirus* was identified in the different changes. No bacteria or fungi were detected in the investigated material.

These results suggest that melanised changes in dorsal and abdominal areas are of the same nature as concerns origin and manifestation.

Keywords: *Atlantic salmon, HSMI, melanin, piscine orthoreovirus, PRV*.

Contents

List of abbreviations

BKD – Bacterial kidney disease (due to infection with *Renibacterium salmoninarum*)

BSA – Bovine serum albumin

HE – Haematoxylin and eosin

HSMI – heart and skeletal muscle inflammation

IPNV – Infectious pancreatic necrosis virus.

ISAV – Infectious salmon anaemia virus

MSB – Martius, Scarlet and Blue

PAS – Periodic acid-Schiff

PBS – Phosphate-buffered saline

PCNA – Proliferative cell nuclear antigen

PD – Pancreas disease

PMCV – Piscine myocarditis virus

PRV – Piscine Reo-virus

SAV – Salmon alpha virus (cause of PD)

Se-E – Selenium-vitamin E

TBS – Tris-buffered saline

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1. Introduction

1.1 Challenges and priorities of aquaculture concerning the environment

Today's aquaculture industry plays an important and increasingly dominant role as a source of protein for global human consumption, given the increasing demand for fish products worldwide combined with a stable or diminishing catch from wild fisheries. Globally, the Atlantic salmon (*Salmo salar*) is one of the most intensely-farmed and highly-valued fish species. Farmed Atlantic salmon is also a biologically-efficient source of food and animal protein (Pettersen et al., 2015). The majority of the World's production takes place in Chile, the United Kingdom, Canada and Norway.

The Norwegian marine aquaculture industry is the largest in the World and has an ambition to grow even larger. It currently generates more than a million tonnes of fish per year, with an export value of 4 billion euros (OECD). The industry produces mainly Atlantic salmon and rainbow trout (*Oncorhynchus mykiss*) and has locations all along the coast, however the main areas of production extend from the west coast northwards. Despite the effectiveness of the aquaculture industry, it faces a number of challenges related to environmental sustainability. Persistent disease outbreaks, safety and quality control in aquaculture all require additional research and improvement. *"A* ***Disease outbreak*** *defined by the OIE Aquatic Animal Health Code, means clinical or non-clinical infection with one or more aetiological agents, with or without clinical signs"* (OIE, 2016). In addition, this expanding industry needs more knowledge on curbing environmental impacts (Sandersen and Kvalvik, 2014).

The global production of salmon is constantly under the threat of emerging infectious diseases. Diseases such as heart and skeletal muscle inflammation, pancreas disease, cold-water vibriosis, bacterial kidney disease, and others, can cause serious problems and reduced profitability for the industry. Therefore, one of the priorities of aquaculture in Norway internationally is the control of disease outbreaks and establishment of vaccines (Pettersen et al., 2015).

The development of effective vaccines for farmed Atlantic salmon was the main reason for the reduction of antibiotics usage in the 1990s and is today one of the most important tools to prevent outbreaks of many bacterial and viral diseases. Today, almost all farmed Atlantic salmon are routinely vaccinated against a number of bacterial and viral diseases such as furunculosis caused by *Aeromonas salmonicida ssp*. *salmonicida*, vibriosis and infection pancreas necrosis. However, the oil-adjuvanted vaccines commonly in use today may have negative side effects, including reduced appetite and growth. Furthermore, adhesions between intraperitoneal organs, and melanin deposits on internal organs and the abdominal wall may occur, leading in the most severe cases to reduced carcase quality at slaughter. These side-effects are generally considered to result from prolonged inflammation caused by persistent antigens from the vaccine. In addition, systemic autoimmune reactions to oil-adjuvant vaccines are quite a common finding. The negative effects of vaccines can depend on the time of vaccination, vaccine formulation, water temperature and fish condition (Koppang et al., 2008, Haugarvoll et al., 2010). However, the benefits of vaccines outweigh their negative effects (Timmerhaus, 2011). Bacterial and viral disease agents mutate and evolve with time, necessitating the continuous improvement of existing vaccines and development of new ones.

A number of important infectious diseases have emerged in intensive salmon aquaculture and this trend is likely to continue. Close contact with marine fauna, hydrodynamic and management links between farms, and movement of fish are the main conditions that determine the level of disease emergence. The consequences of some disease epidemics have played a significant role in the rate of development of the salmon industry. Thus, the control of disease is essential for the establishment of a sustainable industry (Pettersen et al., 2015).

Like the rest of the world, the Norwegian fish farming industry struggles with disease control. Infectious diseases caused by parasites, viruses or bacteria impair fish welfare, lead to profit losses and may have negative influence for the development of aquaculture in Norway (Stormoen, 2013). Threats from disease still exist because of a lack of knowledge of the causality and treatment of fish diseases. Research into these subjects takes a considerable time because it has to be done in methodical stages: sampling, laboratory work, analysis, etc. This thesis will

cover some of these steps and contribute to the research of one type of virus diseases that influence on the muscle structure and consequently on the fillets quality.

1.2 Quality of fish fillets as the primary task of aquaculture

Today there is a stable demand for fish products as a healthy food, and the most practical way of meeting this demand is fish farming. In Norway, the Atlantic salmon has been farmed for decades, and other species, such as the Atlantic cod (*Gadus morhua*), are emerging. After slaughter, however, the carcases of different farmed species respond differently to the different treatments and storage conditions they may be exposed to during processing. The consumer's continued acceptance of fishery products depends on perceived food quality. Important properties of fish products include safety, freshness, nutrition, flavour, texture, colour (especially of pigmented species) and appearance and the suitability of the raw material for processing and preservation. Production cycles on fish farms have a significant influence on fish quality. Many fish species exhibit changes in textural properties during storage after slaughter, long before they are spoilt as result in flesh softening and gaping. A perceived unfavourable appearance may result in downgrading of a carcase and its return to the producer, with accompanying loss of reputation and economical losses. Moreover, the fillets may fall apart in the skinning operation if they have become too soft to tolerate mechanical processing. Therefore, it is important to understand the mechanism(s) of loss of quality in order to avoid it or even use it beneficially (Hultmann, 2003).

In addition to factors within the farm production cycle, a vast number of pathogens - viral, bacterial, parasitic and fungal - may affect the quality of the fish fillet. For instance, the disease of heart- and skeletal muscle inflammation, caused by the agent *piscine orthoreovirus* (PRV), may lead to the development of melanised, focal changes in the white muscles (Bjorgen et al., 2015). Another viral disease that changes muscle structure of fish with inflammation and fibrosis is pancreas disease (Lerfall et al., 2012). The skeletal muscle disorders in Atlantic salmon caused by these two diseases impact directly on the quality of fish flesh. According to Norwegian statistical data by Nofima for 2015, losses of fish fillet due to muscle inflammation-related damage is around 20% (Mørkøre et al., 2015).

2. Diseases in relation to muscle changes

The following section contains: a definition of diseases, infections, aetiology, etc; and brief information about agents and their influence on human and animals and more detailed description of fish muscle changing diseases.

Muscle disease is any of the diseases and disorders that affect musculature. Diseases and disorders resulting from pathological changes in the muscles in question are called primary muscle diseases. Due to the fact that muscles and nerves supplying muscle operate as a functional unit, disease of one or both of these systems leads to muscular atrophy and paralysis.

"*A **disease** is a morbid entity ordinarily characterised by two or more of the following criteria: a recognised aetiological agent(s), an identifiable group of signs and symptoms, or consistent anatomic alterations*" (Dictionary, 2006).

***"Infection** is the presence of pathogenic molecules, virions, cells or organisms in host tissue or associated with host surface, initially inactive, non-replicating and latent, although later on replicating, developing or infection-producing units"* (Dictionary, 2006).

The development of disease involves the themes of aetiology, factors and agents. *"**Aetiology** is used for the cause of disease". "**Factors** are any characteristics that affect the health of a population"* (Dictionary, 2006). *"**Agent** is a factor such as a microorganism, chemical substance, or a form of radiation, the presence or absence of which (as in deficiency diseases) result in disease or in more advanced form of disease"* (Dictionary, 2006).

*"**Manifestation** is the display or disclosure of characteristics signs or symptoms of an illness"* (Dictionary, 2006).

There are two types of muscle disorders: primary and secondary. The term **primary** is applied to any disorder which can be attributed to primary morphological, biochemical or electrical changes in muscle fibres or related interstitial tissue, and in which there is no evidence that such changes are in any way secondary to changed function in the central and peripheral nervous system

(Walton and Gardner-Medwin, 1981). **Secondary** muscle disorder occurs in infectious disease, endocrine disorders, metabolic disorders, immunological disease, vascular diseases, haematological disorders, and malignancies (Finsterer et al., 2016).

The invasion and multiplication of microorganisms such as bacteria, viruses, parasites and fungi within muscle tissue can cause inflammation (Crum-Cianflone, 2008). However, muscle inflammation can also result from non-infectious sources. In total, muscle disorder/inflammation may have many different aetiologies including infection, autoimmune conditions, genetic disorders, adverse medication, electrolyte disturbance, and diseases of the endocrine system.

Inflammation of the muscles is defined as *myositis* and may result in muscle weakness due to inflammation and damage to muscle fibres. Muscular manifestations maybe classified as acute, subacute, or chronic (Finsterer et al., 2016).

The most common inflammatory myopathies (myo- Greek μυο "muscle" + pathos -pathy Greek "suffering") with distinct clinicopathologic features and clinical course include dermatomyositis, polymyositis, necrotising myositis and inclusion body myositis. Dermatomyositis presents with proximal muscle weakness accompanied or preceded by typical skin changes. Polymyositis is rare as a single entity and presents subacutely with proximal muscle weakness and creatine kinase elevation. Necrotising myopathy has a multifactorial aetiology and may have an acute or subacute onset, can be severe, also may have a seasonal variation or cancer association. Inclusion body myositis has a distinct clinical phenotype characterised by weakness and atrophy of certain distal and proximal muscles (Dalakas, 2010).

In man, a broad spectrum of infectious agents can affect muscles and cause a primary or secondary inflammatory response. Inflammatory myopathies, defined as chronic and acute inflammation in muscles, are characterised clinically by a wide variety of symptoms including muscle tenderness, weakness, swelling and pain, and include a large number of aetiologies such as infectious, toxic and endocrine (Gan and Miller, 2011).

In mammals many infectious agents are frequently the cause of an inflammatory or degenerative response of muscle tissue. Some of them give more or less the classical primary inflammatory reaction of the vascular connective tissue (myositis). Others give rise to only (often mild) degenerative reactions of muscle cells (myopathy). On the other hand, an inflammatory reaction induces secondary degenerative changes, while primary degenerative changes activate an inflammatory reaction. Therefore, especially in subacute to chronic cases, a clear distinction between the two is often not possible. In many cases the cause of a myositis is evident, in some others the aetiology is more complex or unknown (Goedegebuure, 1987).

In fish, a large range of both infectious and non-infections conditions affect skeletal muscle, leading to an often-fatal loss of muscle function. Currently, main types of causes which provoke muscle disorders have already been identified. For instance, most of diseases of bacterial nature were defined. However, many factors should be considered when determining the aetiology of diseases. For instance, environmental and dietary factors can also cause muscle disorders. Several viral infections which influence muscle structure are new for aquaculture and aetiology and manifestation of them remain unknown. At present time, these diseases are heart- and skeletal muscle inflammation, pancreas diseases and cardiomyopathy syndrome.

2.1 Viral diseases

Certain human infectious diseases can be associated with muscle disorders, with or without myositis, for example influenza, dengue, toxoplasmosis, Lyme disease (*Borrelia burgdorferi*), infective endocarditis, Rocky Mountain spotted fever (*Rickettsia rickettsii*), AIDS, HTLV-1, and other virus diseases (Crum-Cianflone, 2006). Rarely, hepatitis viral infections have been also related with a diversity of musculoskeletal syndromes, including polyarthritis (Crum-Cianflone, 2008), the clinical signs of which are most frequently related to infection with influenza or parainfluenza virus. Symptoms may present myalgia, weakness, impaired ambulation and gait disturbance (Cardin et al., 2015).

Viral diseases of domestic mammals have the same nature as diseases of humans. However, there are several viral muscle disorders that prevail in mammals. Examples include feline

infectious peritonitis in the cat, Akabane disease and three-day fever (*Ephemerovirus*) in cattle, as well as diseases caused by the *Parvoviridae* in dogs. Symptoms vary depending on the species of animal, time of infection and have a variety of muscle disorders.

The aqueous environment is ideal for the spreading of infectious diseases. Farmed fish are exposed to new and already-known viral diseases and outbreaks (Rimstad, 2011). *Piscine reovirus* (PRV), *salmon alphavirus* (SAV) and piscine myocarditis virus (PMCV) are novel viruses which cause muscle changes and/or similar pathological changes. Moreover, a number of diseases caused by viruses are not associated directly with disorders of muscle structure, but can lead to muscle disorders in chronic stages. For instance, diseases caused by the family *Rhabdoviridae,* such as infectious haematopoietic necrosis virus, viral haemorrhagic septicaemia virus, hirame rhabdovirus and others (Bruno et al., 2013). The present part of viral disease will describe viruses PRV, SAV, PMCV.

2.1.1 Piscine Reovirus

One of the most widespread diseases of farmed and wild fish in Norwegian aquaculture is heart and skeletal muscle inflammation (HSMI) (Kongtorp et al., 2004), which is caused by *piscine orthoreovirus* (PRV), a novel reovirus (Palacios et al., 2010). HSMI was observed in farmed Atlantic salmon, in Norway in 1999 (Kongtorp et al., 2004). Outbreaks have been recorded along the Norwegian coast the whole year round and is widely distributed in both farmed and wild Atlantic salmon. Although the HSMI pathological changes were well described, the pathogenesis of HSMI remains unknown.

Piscine reovirus (PRV) is part of the *Reoviridae* family. The *Reoviridae* family (collectively called reoviruses) - ***R***espiratory ***E***nteric ***O***rphan (Sabin, 1959). The *Reoviridae* family has a wide host range, including vertebrates, invertebrates, plants, and fungi (Urbano and Urbano, 1994). The family *Reoviridae* is divided into two subfamilies: *Spinareovirinae* and *Sedoreovirinae*. It contains 15 genera, 5 of which—*Orthoreovirus*, *Coltivirus*, *Rotavirus*, *Aquareovirus* and *Orbivirus* - can infect humans, animals and fish. Other genera can infect plants and insects (Attoui H et al., 2012). The most common infection in humans is *Rotavirus,* causing vomiting,

diarrhoea and fever in infants and young children. However, *Rotavirus* also infects animals, and is a pathogen of livestock (Kapikian and Shope, 1996).

The genome of PRV was identified in 2010 using bioinformatic tools after high-throughput sequestering (Palacios et al., 2010) and the virus was detected in a tissue sample by immunohistochemistry (Finstad et al., 2012). PRV belongs to the family *Reoviridae*, genus *Orthoreovirus*. However, phylogenetic analysis of this novel fish *orthoreovirus* shows that the virus was also related to *Aquareovirus* inside the *Reoviridae* family (Nibert and Duncan, 2013).

PRV virus contains 10 RNAs segments - 3 large (L), 3 medium (M), 4 small (S) - and has a double-stranded RNA structure (dsRNA), total length of 23.3kb, with a size of 80nm in diameter. The capsid has a double shell. In the shell is included σ1, μ1 and σ3 proteins. Important serotype-specific antigen protein is σ1. The antigen indication of this protein is the basis for three major serotypes of *Mammalian orthorevirus* and 5-11 serotypes of A*quareovirus* (Markussen et al., 2013).

Clinical signs of HSMI appear in the period 5-9 months after sea transfer. The signs include abnormal swimming behaviour, anorexia and mortality that can reach more than 20 % (Kongtorp et al., 2004). Lesions are predominantly present in the heart and skeletal muscle. The heart muscle and the lining of the heart become inflamed, with epi-, endo and myocardial necrosis. The heart lesions are of inflammatory character and include immune cells/leukocytes. Muscles are infiltrated with inflammatory cells, myositis and necrosis. Another type of damage of organs includes focal liver necrosis, circulatory disturbances and erythrocyte accumulation in several organs. Further, histopathological analysis of spleen and head kidney lesions showed high loads of PRV (Kongtorp et al., 2004).

Diagnosis of HSMI is similar to PD (McLoughlin and Graham, 2007) and CMS (Lovoll et al., 2010). But HSMI has another distribution of the cardiac and skeletal muscle lesions. Further analysis by viral agent helps to distinguish HSMI and define correct diagnosis (Kongtorp et al., 2004). However, heart disorders and lesions can also be related to non-infectious aetiologies

such as: a variety of food supply, low oxygen levels, stress, temperature variations and restriction of activity (Ferguson, 2006).

Prevention by vaccines against HSMI is not available at present. However, for *Mammalian orthorevirus* monoclonal antibodies directed against outer capsid protein σ1, σ3 and μ1C and core protein can neutralise the virus (Tyler et al., 1993). Therefore, homologous protein in PRV could be a candidate for a vaccine target (Finstad, 2014).

2.1.2 Salmon alphaviruses

Salmon alphavirus is the causative agent of pancreas disease (PD) and is responsible for other serious disease also in rainbow trout, *Oncorhynchus mykiss* (Walbaum) (Nelson et al., 1995). Virus was first isolated from Atlantic salmon in Scotland (Nelson et al., 1995). Subsequently, disease was discovered in Norway, Ireland, USA, Spain, and other countries (Kent and Elston, 1987, Poppe et al., 1989, Murphy et al., 1992, Boucher and Baudin Laurencin, 1994).

Salmon alphavirus was identified in 1995, and physicochemical characteristics and morphology supposedly belonged to *Togaviridae* family (Nelson et al., 1995). SAV has also been isolated from rainbow trout, *Oncorhynchus mykiss* (Walbaum), with sleeping disease in France (Castric et al., 1997). I In 1999 it was confirmed that this virus belongs to the genus *Alphavirus* within the family *Togaviridae* (Weston et al., 1999). Later in 2000 the agent causing sleeping disease in rainbow trout was identified and confirmed as an atypical alphavirus (Villoing et al., 2000).

The virus family *togaviridae* contains the subfamilies *Alphavirus* and *Rubivirus*. The *Alphaviruses* have single positive sense strand RNA of total range between 11,000-12,000 nucleotides. Subtypes SAV 1, SAV 2, and SAV 3 are the most important causative agents of PD in farmed Atlantic salmon in Europe. However, at the present time at least six SAV subtypes have been distinguished and they are divided by geographical areas. In Norway the most prevalent are SAV 3 and marine SAV 2 (Fringuelli et al., 2008). The SAV 3 has been detected only in Norway (Hodneland et al., 2005).

Alphaviruses are classified as arboviruses (arthropod-borne virus) and have a range of hosts such as humans, rodents, fish, birds, and larger mammals as well as invertebrates. Viruses are transmitted between species and individuals, mostly by mosquitoes (Strauss and Strauss, 1994). However, there is some evidence that SAV can also be transmitted without insects (Jansen et al., 2016).With SAV, morbidity usually appears during the first year at sea, during the summer and early autumn (Rodger and Mitchell, 2007).

In Norway PD has been endemic since 2003, with the highest number of outbreaks in 2007. During of 2012-2013, outbreaks were reported from 236 fish farms (Gjevre et al., 2013).

Clinical signs associated with PD are inappetence, lethargy, faecal casts, mortality, slow growth and "sleeping behaviour". Histopathological examination shows changes in the structure of pancreas, heart tissue and skeletal musculature. Changes include necrosis of the entire exocrine pancreas, degeneration with further invasion of leukocytes and degeneration and necrosis of cardiomyocytes. However, distributions of the lesions depend on the time after initial infection. The first lesions are an acute stage of pancreatic acinar cell necrosis, inflammatory response and/or fibrosis in the periacinar tissue. Simultaneously, histological investigation shows heart lesions, a severe degeneration with multifocal cardiomyocytic necrosis. In the chronic phase, inflammation and fibrosis can be observed in both red and white skeletal muscles (McLoughlin et al., 2002).

The diagnosis of PD is possible by a combination of histopathological examination and virus culture or PCR detection using samples of heart and kidney (Bruno et al., 2013).

A basic principle of prevention of disease caused by SAV is a reduction in stress in production cycles. In addition, all necessary measures to prevent horizontal transmission from infected to non-infected fish populations. Moreover, vaccines against SAV has a positive effect, decreasing the incidence of disease in populations (Bang Jensen et al., 2012)

2.1.3 Cardiomyopathy syndrome

Cardiomyopathy syndrome (CMS) was first identified as a diseases in sea-farmed Atlantic salmon in Norway in 1985 (Ferguson et al., 1990). Further, disease was diagnosed in the Faroe Islands and Scotland (Poppe and Sande, 1994, Rodger and Turnbull, 2000). The diseases was also diagnosed in wild population of Atlantic salmon in Norway (Poppe and Seierstad, 2003).

Casual virus agent for CMS likely belongs to the family *Totiviridae.* Virus was named piscine myocarditis virus (PMCV) and associated with a double-stranded RNA. Total base pars are 6,668, Virus includes three open reading frames (ORFs). ORF1 probably encodes the major capsid protein (Haugland et al., 2011).

Clinical signs usually manifest during the second year at sea and the disease develops over several months. Symptoms includes lethargy, swollen abdomen, ventral petecchiation and exophthalmia. Fish with this disease have a ventral skin scale-pocket oedema and haemorrhage. At necropsy, the typical manifestations are haemopericardium and/or blood clots in the pericardial cavity. Haemorrhage is the result of a small or larger rupture of the atrium or sinus venosus resulting from severe congestion. Furthermore, severe haemorrhage may occur in the anterior part of abdominal cavity when the sinus venous ruptures caudally to the septum transversum. The accumulation of fluid in the peritoneal cavity causes abdominal swelling-ascites. Ascites may also be observed in the peritoneal cavity. The liver is mottled, with a fibrinous capsule and general congestion. Acute death is the result (Ferguson et al., 1990, Poppe and Seierstad, 2003). Histological investigation also shows degeneration and necrosis of the inner, spongious myocardium of the ventricle and the atrium, infiltration with lymphocytes, macrophages, and proliferation of the endocardium (endo- and myocarditis) (Poppe and Seierstad, 2003, Rodger et al., 2014).

CMS is often a chronic disease, with a long-lasting, moderately elevated mortality rate, which may increase with stress (e.g. due to predators, superimposed diseases, grading, treatments and

transportation). However, sometimes there is an acute, dramatic increase in mortality (Brun et al., 2003).

In Norway this disease of fish is severe and has a significant influence on aquaculture. Due to the fact that this chronic, non-treatable disease affects the fish in the last stages of the production cycle, it leads to large economic losses (Brun et al., 2003).

Decrease of stress in production cycles could provide effective prevention. Selective breeding can improve growth and survival rates, robustness and quality of the farmed fish. In addition, good environment at production farms and balanced nutrition lead to a decrease of this disease. (Timmerhaus, 2011).

2.2 Bacterial diseases

Bacterial diseases include any type of illness caused by bacteria. Bacteria are a type of single-celled microorganism that can only be seen with a microscope. Millions of bacteria live in the soil, water and air. The majority of bacteria are not harmful, some of them are helpful and even necessary for living organisms. However, some categories of bacteria called pathogenic, meaning that they can cause infection and disease. They have an ability to reproduce themselves very quickly, give off chemical toxins and damage tissue and organs. Pathogenic bacteria can also affect the skeletal musculature. For example, Gram-positive and negative bacteria, aerobic, and anaerobic may all cause muscle inflammation (Crum-Cianflone, 2008).

In analogy to virus diseases described above, bacterial diseases also affect humans, animals and aquatic organisms. Like viruses, bacteria can cause hundreds of illnesses in the human body. Some do this immediately after they enter the body, while others can be present without doing harm. Bacterial infection may be localised and only harm particular organs, internal or external. Bacteria cause a range of musculoskeletal infectious diseases such as psoas abscess, gas gangrene, group A streptococcal necrotizing myositis and anaerobic streptococcal myonecrosis. Other bacterial diseases may also sporadically cause myalgias, myositis, myopathy, or acute rhabdomyolysis. Examples of the most widespread bacterial diseases are syphilis (*Treponema*

pallidum) (Durston and Jefferiss, 1975), Rocky Mountain spotted fever (*Rickettsia rickettsia*) (Behar and Ben-Ami, 2001) and Lime diseases (*Borrelia burgdorferi*) (Holmgren and Matteson, 2006). Localised bacterial infections and the first stages of generalised bacterial diseases can be successfully treated with antibiotics. Later stages may require surgical procedures.

Animals are also susceptible to bacterial diseases. Bacterial infections in the animal body follow a course similar to that of human infection. The most widespread bacterial diseases related to muscle changes as primary and secondary agents are acute suppurative myositis caused by *Corynebacterium pyogenes* in ruminants and swine, and *Streptococcus equi* in horses (Traub-Dargatz et al., 1994) ; clostridial myositis and myonecrosis (gas gangrene) caused by gram-positive, anaerobic, spore-forming bacilli and wooden tongue of cattle caused by *Actinobacillus lignieresi*.

Histological signs of bacterial disease in skeletal muscles include degeneration and necrosis, oedema, haemorrhages and gas formation (Zachary et al., 2011). Early treatment and antibiotic therapy may halt the course of the disease (Meeusen et al., 2007).

In marine fish culture, bacterial diseases are mainly represented by Gram-negative organisms such as *Aeromonas salmonicida*, *Listonella anguillarum* and *Yersinia ruckeri,* while Gram positive, for instance *Renibacterium salmoninarum* are fewer. The clinical signs caused by each pathogen depend on the host species and fish age. Disease outbreaks may be acute, chronic or of subclinical nature (Toranzo et al., 2005). The most common diseases which relate to muscle structure changes in salmonid are classic vibriosis (infection by *Vibrio (Listonella) anguillarum*), cold water vibriosis (infection by *Vibrio salmonicida*), winter ulcers (infection by *Moritella viscosa*) and bacterial kidney disease (infection by *Renibacterium salmoninarum*) (Bruno, 1986, Lunder, 1992, Bruno et al., 2013, Kashulin et al., 2016).

2.2.1 Vibriosis

Vibriosis is one of the most prevalent fish diseases and is caused by bacteria within the *Vibrionaceae* family. The species causing the most economically-serious diseases in aquaculture are *Listonella* (*Vibrio) anguillarum, Vibrio ardalii, Vibrio salmonicida* and *Vibrio vulnificus*

biotype 2, which all produce similar clinical and pathological manifestations (Bruno et al., 2013). The first description of this pathogen was made by Bergman from diseased eel (*Anguilla anguilla*) in Swedish coastal waters in 1909 (Egidius, 1987).

Listonella (*Vibrio) anguillarum* is the aetiological agent of classical vibriosis. Bacteria affect a wide variety of warm and cold water fish species, such as Pacific and Atlantic salmon (*Oncorhynchus spp*. and *Salmo salar*), rainbow trout (*Oncorhynchus mykiss*), turbot (*Scophthalmus maximus*), sea bass (*Dicentrarchus labrax*), sea bream (*Sparus aurata*), striped bass (*Morone saxatilis*), cod (*Gadus morhua*), Japanese and European eel (*Anguilla japonica* and *Anguilla anguilla*) and ayu (*Plecoglossus altivelis*) (Toranzo et al., 2005).

Fish with classical vibriosis show the clinical signs of generalised septicaemia, exophthalmia and corneal opacity. The disease often breaks out in the summer, when water temperature is above 10 °C. The clinical sings and pathological lesions may vary, but are similar to those in diseases caused by several other Gram negative, and are dependent of temperature of water, fish age and pathogen virulence. *Listonella anguillarum* may be present among the normal gut microflora of healthy fish, with outbreaks of disease triggered by stress-activated virulent strains present in the gastrointestinal tract. Poor water quality and rapid temperature changes can also activate infection and contact between fish seems to be an important factor for the spread of this pathogen (Bruno et al., 2013).

The external clinical signs of disease can include dark skin coloration, anorexia, pale gills with excessive mucous, periorbital oedema and haemorrhage near the base of the pectoral and pelvic fins. In most of the cases, the muscles and skin of sick fish have haemorrhage or multifocal liquefactive, muscle necrosis, especially in the subacute or chronic stages of the disease with large numbers of bacteria. Also dermal or subdermal skin lesions are frequently coupled with hyperaemia and haemorrhage, and may be linked to occasional haemorrhagic ‘boil’ lesions in the muscle. The liver is usually swollen and some fish show petechiae (Zachary and McGavin, 2012, Bruno et al., 2013)

Histologically, the anterior part of the digestive tract can show vasodilatation and extensive necrosis of the mucosa and muscularis (Bruno et al., 2013). Necrosis and oedema can also be found in haematopoietic tissue (Frans et al., 2011).

Listonella anguillarum is halophilic with bipolar staining, Gram-negative, slightly curved, flagellated motile rod. The bacterium grows well at an optimum temperature at between 25-30 °C on rich media containing 1.5-2% sodium chloride (Frans et al., 2011).

Diagnosis is based upon classical pathology and isolation of *Listonella anguillarum* on NaCl-supplemented blood agar or TSA at room temperature and verification by the enzyme-linked immunosorbent assay (ELISA). Serologically identification may be accomplished using a rapid agglutination test kit (Bruno et al., 2013). In addition, a PCR-based approach was described for the accurate detection of *Listonella anguillarum* in infected fish tissues (Toranzo et al., 2005).

Oil-adjuvant multivalent vaccines usually provide excellent protection against the disease. In Norway and other Nordic countries different polyvalent oil-based vaccines including distinct combinations of *Listonella* (*Vibrio) anguillarum* with other pathogens such as *Vibrio ordalii, Vibrio salmonicida* and *Moritella viscosa* are available (Toranzo et al., 2005).

2.2.2 Cold water vibriosis

Cold water vibriosis is a septicaemic condition of farmed salmon caused by the bacterium *Aliivibrio (Vibrio) salmonicida*. In Norway the first records of this disease date from 1977, although it existed in commercial aquaculture facilities for years before that. In 1980, due to the rapid growth in the marine fish-farming industry, the disease had become a serious threat. Outbreaks were also recorded in Scotland, Faroe Islands, Iceland and the east coast of USA and Canada (Egidius et al., 1986).

The first DNA analysis of *A. salmonicida* showed that it differed substantially from other known Vibrio species. Thus, bacteria of this type was identified as a new species of Vibrio (Wiik and Egidius, 1986).

Signs of the disease normally appear during late autumn, winter and early spring, with lethargic behaviour, appearance of dark spots, erratic swimming and inappetence. The disease has signs of anaemia and extended haemorrhages, especially in the integument surrounding the internal organs of the fish. The pathology displayed depends on the stage of disease process. External lesions include exophthalmia, swollen and haemorrhagic vent, petechial haemorrhage under the belly and at the base of the pectoral and pelvic fins. Internal lesions can reveal a yellowish liver, petechial or ecchymotic haemorrhage, ascites, splenomegaly, haemorrhagic enteritis and a generalised oedema. Histopathological lesions are characterised by large numbers of bacteria in blood vessels, followed by the heart, kidney, muscle and spleen. Histological investigation also shows congestion with arteriolar mural necrosis and thrombi, as well as kidney tubular necrosis and myolysis of skeletal muscle in the later stages (Bruno et al., 2013).

The diagnosis of cold-water vibriosis is based on gross lesions, histopathology and isolation of *A. salmonicida*. The bacterium is a psychrophilic, moderately halophilic Gram-negative curved or straight rod, and is motile with up to 9 polar sheathed flagella. The temperature has a significant impact on the growth rates of the microorganism. It can be grown at 15°C on NaCl-supplemented blood agar where the salt concentration is 1.5 % (Colquhoun and Sorum, 2002).

Vaccination has proved to have immensely favourable results. However, evaluation of the duration of protection provided by the injectable vaccines showed that their protection declines with time, but lasts longer than the protection provided by the immersion immunisation (Lillehaug, 1991). The vaccine is available in a commercial, polyvalent oil-adjuvanted form (Sommerset et al., 2005). Cold-water vibriosis may be treated with antibiotics. Oxolinic acid, florfenicol or flumequine are presently the types most frequently used to treat the disease (Hjeltnes et al., 2015).

2.2.3 Winter ulcers

Winter ulcer disease of sea-farmed Atlantic salmon, in analogy to cold water vibriosis, originates in cold temperatures, and therefore hits salmon and rainbow trout farms mainly during the coldest winter months. This disease is caused by other psychrotropic Vibrio species termed

Moritella viscosa. The illness was diagnosed in Norway in 1990 (Salte et al., 1994), Island (Benediktsdottir et al., 1998), Faroe Islands and Scotland (Bruno et al., 1998). Infection increases mortality rates and leads to lower quality meat, and results in significant economic loss due to downgrade of the fish at slaughter.

The visible sign of the disease is small raised skin lesions. The area of skin affected increases in size, and the skin gradually breaks, exposing the underlying muscle. The edges of the wounds are typically rounded or oval with a white demarcation area to normal skin, which may heal with increasing temperature, leaving scar tissue and sometimes with melanisation in the area of the lesion. Extensive petechial haemorrhage may also develop on the ventral body surface. Internal investigation shows haemorrhage in the peritoneum, adipose tissue, pyloric region and liver (Bruno et al., 2013).

Histopathologic changes depend on the length of the period of ulcer development. The early stages of disease are characterised by oedema down to the compact layer of the dermis, and some inflammatory cell invasion. With the progression of the disease, lesions can be found in the white muscle, with inflammatory infiltrates between muscle bundles, haemorrhage and thrombosis of small vessels. The chronic stages are characterised by a severe inflammation of the dermis and of the interstitial muscle tissue. Bacteria are usually detected near the edges of the lesions. In the reparative phase, granulation tissue covers the ulcers, starting from the edges, followed by gradual replacement by new epidermal and dermal layers without scales (Lunder et al., 1995, Bruno et al., 2013).

Moritella viscosa is a psychrophilic, Gram-negative, motile, flagellated curved rod (Hoffman et al., 2012). Diagnosis can be made by clinical signs and bacterial isolation with identification of the bacterium and real-time PCR bacterial detection (Grove et al., 2008).

Moritella viscosa antigens are included in most of the multivalent injection vaccines used on sea-farmed salmon, but the protection is apparently variable (Bruno et al., 2013). However, in Norway, winter ulcer is the main bacterial disease in salmonids and the disease does not seem to be fully controlled with existing vaccines (Grove et al., 2008).

2.2.4 Bacterial kidney disease

Bacterial kidney disease (BKD), caused by the Gram positive diplobacillus *Renibacterium salmoninarum*. BKD is a serious disease that usually becomes chronic in wild and farmed salmonids, causing an increase of mortality in farmed fish in fresh and sea water the world over.

The first reports of BKD as a new disease in Atlantic salmon came from the rivers Aberdeenshire Dee and Spey in Scotland (Munro et al., 1984). The disease is transmitted horizontally by co-habitation, and vertically via the eggs directly from ovarian tissue prior to ovulation (Bruno et al., 2013).

Clinical signs and external lesions are variable. Infected fish show loss of balance, exophthalmia, superficial blebs or blisters in the skin, and even the formation of cavitations in the musculature. The blebs and cavitations are reported to contain a white, yellowish or haemorrhagic fluid. Pale gills, distended abdomen, petechiae and haemorrhagic areas around the fins and the lateral line are also common external indications of BKD. Superficial blisters with vesicle formation, ulceration and abscesses may develop in the integument. At necropsy, the gills and internal organs are pale, giving the impression of anaemia. Internal lesion usually includes a swollen kidney that may show greyish-white nodular lesions. Similar nodules likewise occur in heart, liver and spleen. Petechial haemorrhage of the muscle and peritoneum are observed, along with ascites (Bruno, 1986).

Diagnosis usually occurs with the help of internal observation of organs, for instance the enlarged greyish kidney and the occurrence of white nodules in internal organs such as kidney, heart, liver and spleen. Also bacterial isolation with identification, an ELISA and a real time PCR are commonly used for diagnosis (Bruno et al., 2013).

Disease prevention can be accomplished with the commercial vaccine “Renogen”. This vaccine provides significant long-time protection of Atlantic salmon against BKD (Toranzo et al., 2005).

2.3 Parasitic diseases

A parasitic disease is a type of infectious disease caused by a parasite. Human and domestic animals have a variety of parasitic infections with different severities of impact. Some types of parasites may not cause any disease.

The main injuries by parasites in humans related to muscle structural changes result from encystment of parasites in the musculature. The most frequently reported parasitic causes of myositis are *Trichinella spp*. (trichinosis), *Taenia solium* (cysticercosis) and *Toxoplasma gondii* (toxoplasmosis). However, there is a range of other, less common parasitic infections which can also lead to myalgias or myositis, such as *Trypanosoma cruzi* (Chagas disease), *Sarcocystis spp*., *Microsporidia spp*., *Toxocara canis*, *Schistosoma spp*., *Echinococcus spp*., *Entamoeba histolytica*, *Spirometra mansonoides* (sparganosis), *Plasmodium falciparum* (malaria) and *Onchocerca volvulus* (Crum-Cianflone, 2008).

The most widespread parasite for mammals is *Trichinella spp*. The disease caused by *Trichinella spp.* parasite is Trichinosis. The disease occurs after the ingestion of undercooked muscles of domestic or wild animals containing *Trichinella spp*.-encysted larvae. After ingestion of raw or undercooked meats, the gastric enzymes digest the muscle and release the larvae. Larvae are resistant to acid and pass into the small intestine. In the small intestine they burrow underneath the epithelium and develop into adult worms, which may mate and release new-born larvae. Spreading of new-born larvae occurs via the lymphatic system and bloodstream to striated muscles, where the larvae encyst (Crum-Cianflone, 2008). Therefore, one of the obvious methods of disease prevention is proper food control.

Symptoms are haemorrhages, and periorbital oedema. Muscle invasion may cause myalgias, swelling, and weakness. Myositis initially occurs in the extraocular muscles, followed by the masseters and muscles of the diaphragm, neck, and larynx as well as the limbs; any striated muscle can be involved (Capó and Despommier, 1996, Crum-Cianflone, 2008).

Diagnosis can be established by the clinical history, presence of eosinophilia, laboratory tests leukocytosis, elevated immunoglobulin E, and serologic testing. In addition, biopsy of superficial skeletal muscle and a PCR test can also confirm presence of parasites (Capó and Despommier, 1996, Crum-Cianflone, 2008).

Treatment is the use of antihelminthic medications.(Crum-Cianflone, 2008) Meanwhile, one of the most efficient means of disease prevention is proper meat preparation. Meat should be cooked to at least 60°C for 4 or more minutes or frozen to -15°C for 20 hours.

Several protist parasites affect the muscular structure of fish and are capable of causing great damage and significant mortality in fish populations. *Ichthyophonus hoferi (Mesomycetozoea)*, *Kabatana takedai (Microsporidia), Kudoa thyrsites (Myxozoa),* and *Anisakis simplex* commonly locate in musculature (Woo, 2006).

2.3.1 Ichthyophonus hoferi

Ichthyophonus hoferi is a protozoan parasite that phylogenetically belongs to *Mesomycetozoea*. This parasite does serious damage to cultured fisheries because it causes a reduction of growth rates and death and, hence, requires large expenditure for the sanitation of fish farms. However, this parasite is not infectious to human so it is harmless in food hygiene.

In August 1991, the disease was detected in the North Sea autumn spawning stock between Denmark and Sweden and also in the Norwegian spring spawning herring feeding in the Norwegian Sea (Skagen, 2010).

Clinical signs and pathology have been reported in salmon as behavioural anomalies, lethargy and uncoordinated swimming movements. At necropsy, whitish nodules can be seen in many organs, primarily the heart, muscle, kidney, liver and spleen. Microscopically, a heavy granulomatous response is characteristic, frequently followed by large numbers of macrophages and multinucleated giant cells. Few developmental stages can be observed, but a spore or resting

stage is common in several organs. Germinating spores of *Ichthyophonus hoferi* can also be observed histologically (Bruno et al., 2013).

Infection occurs probably through the ingestion of infected material. The further spread of parasite occurs through penetrating the gastric mucosa, entering the bloodstream and spreading to several organs where secondary cysts are formed (Bruno et al., 2013).

Diagnosis is based on pathological and histological findings. Preventive measures include isolating infected fish and avoiding feeding raw fish products.

2.3.2 Kabatana takedai

Kabatana takedai (formally *Microsporidium takedi*) infects the skeletal muscle and heart of wild and cultured salmonids in Japan and Sakhalin, Russia (Lom et al., 2001). Mainly it affects such species as chum, masou, pink and sockeye salmon, rainbow and brown trout. A seasonal prevalence of *Kabatana takedai* is documented during the summer period with an overall temperature around 15° C.

Light stages of infection do not show any external clinical signs, while highly-depressed body size and enlarged heart are evident in heavy infection. Acute cases lead to high mortality and are characterised by a massive occurrence of whitish, spindle to ovoid-shaped proliferating microsporidian, which measures 2.5-4.0 μm. The same scenario is seen in histology. The early phase of infection does not show any reactions around a mass of parasite developing in the endocardium. Eventually, tissue becomes granulomatous and spores are phagocytised by macrophages, followed by degeneration of the myofibrils and proliferation of connective tissue Fibrinoid degeneration occurs in the marginal areas of foci (Dykova and Lom, 2000).

Diagnosis is based on autopsy and gross examination of infected musculature and confirmed by PCR or by microscopical examination of the cysts. Transmission routes are still unknown (Bruno et al., 2013).

2.3.3 Kudoa thyrsites

Kudoa thyrsites has been implicated as a cause of soft flesh syndrome in farmed Atlantic salmon (*Salmo salar*) and coho salmon (*Oncorhynchus kisutch)* reared in North American seawater. *Kudoa thyrsites* belongs to the *Myxozoa*, a class of microscopic parasites *Myxosporea*. The parasite has a broad host range and is distributed worldwide (Moran et al., 1999). Damage and discolouration of musculature caused by Kudoa results in significant economic losses post-harvest, as infected fish are rejected during processing.

Moribund fish are dark-coloured, but generally no clinical signs are apparent until post-mortem. An autopsy helps to define anaemia and pale liver. The intramuscular stage begins with a single parasite in the muscle sarcolemma forming nodules or pseudocysts. The dorsal musculature lesions show a multifocal, intracellular infection with associated inflammatory response in the pericardium and myocardium. A high loading of *Kudoa* within the red and white muscle leads to necrosis, fibrosis and inflammation, and then a chronic, active myositis with myolysis. In severely-affected fish, the kidneys display a swollen renal interstitium, with giant cells (Bruno et al., 2013).

Stained sections or squashed preparations of muscle tissue stained with Gram or Giemsa allows to see the spores and make a diagnosis. The Kudoa spores are stellate in shape and characterised by four valves and four polar capsules, each containing a polar filament (Bruno et al., 2013).

Prevention and/or control of Kudoa infections is challenging especially in open water. Currently medicine does not have any available treatments. One approach of prevention may be to disrupt the life cycle in some way thereby minimizing the likelihood of infection.

2.3.4 Anisakis simplex

Larval nematodes of the genus *Anisakis* belongs to *Nematoda*: *Anisakidae*. *Anisakis simplex* is a very widespread parasite and it can be found almost in all commercially exploited species in North Atlantic waters. The life cycle of *Anisakis simplex* is complex and includes few stages. It

involves marine mammals (eg cetaceans) as the definitive host, and fish, squids and planktonic crustaceans as intermediate hosts for the larval stages. *Anisakiasis* is also an important fish-borne zoonosis (Audicana and Kennedy, 2008, Mo et al., 2010, Bruno et al., 2013).

By investigation, both encapsulated and nonencapsulated larvae can be seen around the hind gut, inside the discrete space towards the skin, between this region and the urethra and between the hindgut and the genital cavity. *Anisakis* can also be identified deep within the skeletal musculature above the vent area and sometimes within the lumen of the genital cavity.

Histological analysis shows severely affected tissues of the vent show, scale loss and absence or a detached epidermal layer. Capillary dilation, blood congestion, haemorrhage and moderate to severe dermal inflammation associated with non-encapsulated migrating larvae also can observed. An inflammatory reaction dominated by eosinophilic granular cells, melano-macrophages and multinucleated giant cells have also been reported (Noguera et al., 2009, Bruno et al., 2013)

2.4 Fungi

A fungus is a living organism, a member of the eukaryotic group that includes yeasts, molds and mushrooms. These organisms belong to the kingdom Fungi. One significant parameter that differentiates fungi from plants, bacteria and some protists is that its cell walls contain chitin.

Although fungi are widely used in food and medicine, the oomycetes (fungal-like) microorganisms can cause infection. The symptoms of fungal myositis often overlap bacterial myositis. Fungal myositis may be due to *Cryptococcus neoformans, Candida spp., Coccidioides spp., Aspergillus spp., Pneumocystis jiroveci*, *Histoplasma capsulatum* and *Fusarium spp*. Biopsy with culture is usually required to confirm the diagnosis of the fungal aetiology. The diagnosis of a fungal pathogen is not initially measured and is discovered by histopathologic examination or culture of the muscle tissue. Treatment can be accomplished with surgical debridement and systemic antifungal agents (Crum-Cianflone, 2008).

The most usually-reported cause of fungal myositis is *Candida spp*. Myositis may develop in the setting of systemic candidiasis. In the 1970s was described the first case in a patient with acute lymphoblastic leukemia which involved diffuse myositis (Diggs et al., 1976).

The disease is progressive, with such symptoms as fever, rash, and muscle myositis. Most often the muscle involvement contains diffuse and multiple micro-abscesses. Muscle biopsy confirms the diagnosis by revealing budding yeasts and pseudohyphae; blood cultures may be positive, especially in the setting of systemic infection. Therapy normally involves antifungal agents such as amphotericin B, an azole, or an echinocandin. Focal infectious nodes should be surgically drained. Early diagnosis and treatment is recommended. Myopathy appears to be autoimmune in nature, rather than directly caused by the fungal pathogen (Diggs et al., 1976, Crum-Cianflone, 2008).

Some animals suffer from fungal infection caused by *Coccidioidomycosis* (Valley Fever). The disease varies from infections with several or no signs to progressive. In horses, the disease is characterised by respiratory, dermatological, musculoskeletal, neurological, and ophthalmological clinical signs. This agent can cause a disease in other domestic animals such as cattle, sheep, pigs, dogs, cats (Graupmann-Kuzma et al., 2008, Cafarchia et al., 2013).

Fish are susceptible to agents of the fungal family *Saprolegniaceae* (pathogen *Saprolegnia spp.)* which can lead to muscle disorders and influence the quality of fillets in severe stages. *Saprolegnia spp* (*saprolegniosis*) is represented by two species: *S. parasitica* and *S. diclina*. They are able to affect wild and farmed fish at all developmental stages in fresh water (Bruno et al., 2013).

Clinical signs of *saprolegniosis* in adult fish include a superficial “cotton like” growth with a white growth of mycelia on the fish skin, commonly around the head, caudal and dorsal fins, gills, and in the muscular layer and internal organs. Additional symptoms are lethargy, a loss of equilibrium and respiratory difficulties (Sandoval-Sierra et al., 2014).

Infection provokes rapid degenerative changes in the muscle that leads to diffuse oedema. Further, *Saprolegnia spp* destroys epidermis, and consequently hyphae can penetrate the basement membrane, with growth continuing into the dermis, hypodermis and musculature (Woo and Bruno, 2011).

Identification of *Saprolegnia* species usually requires the use of molecular approaches such as PCR methods (Sandoval-Sierra et al., 2014).

2.5 Nutritional diseases

In animals and humans, a wide variety of pathological muscle disorders are related to deficiency of selenium and/or vitamin E (Se-E). Selenium (Se), a metalloid mineral micronutrient, is essential for adequate and healthy life in humans, animals, archaea, and some other microorganisms (Hatfield et al., 2011). Selenium is a trace element nutrient that functions as co-factor for glutathione peroxidases and certain forms of thioredoxin reductase (Vella, 1995). Classical features of Se deficiency in mammals are liver necrosis, white muscle disease and cardiac and skeletal muscle degeneration (Hatfield et al., 2011). Vitamin E activity in food derives from a series of compounds of plant origin and has many biological functions - antioxidant, enzymatic activity regulator (Schneider, 2005) and in gene expression (Devaraj et al., 2001). It protects lipids and prevents the oxidation of polyunsaturated fatty acids. All in all, the properties of vitamin E are most important biologically (Bell, 1987, McDowell, 1989).

The aetiology of selenium-vitamin E (Se-E) deficiency disease may be complex. Both Vitamin E and selenium-containing enzymes (the glutathione peroxidase-glutathione reductase system) are required in muscle cells and many other cells as physiologic antagonists to free radicals (products of normal cell function) (Goedegebuure, 1987). Many of the syndromes comprise combined deficiency of selenium and vitamin E. Selenium gets into the animal and human food chain from soil and plants, which may contain inadequate amounts of the nutrients in a lot of areas of the world. Vitamin E may be in low concentration in many animal feeds unless supplements are added (Van Vleet and Ferrans, 1992).

Se deficiency provokes various diseases. In humans it leads to Keshan disease, an endemic cardiomyopathy in China. Animals, such as calves, lambs, pigs, turkey poults, and ducklings suffer from myocardial lesions, necrosis of skeletal muscle and necrosis of smooth muscle of the gizzard and intestine. Other Se-E deficiency lesions include hepatic necrosis, gastric ulceration, intestinal and uterine lipofuscinosis, pancreatic damage, steatitis, exudative diathesis, encephalomalacia, and testicular necrosis (Van Vleet and Ferrans, 1992).

Histological lesions in animal tissue are characterised by hypercontraction, hyaline, floccular and granular degeneration and fragmentation of muscle fibres, with or without abundant calcification and phagocytosis (Goedegebuure, 1987).

In some fish species, including Atlantic salmon, deficiency of Se and vitamin E causes muscle myopathy (Le et al., 2014). Se and vitamin E may to some extent compensate for the lack of each other to prevent muscle myopathy. Myopathy occurs in diets deficient in both Se and vitamin E, but not in single deficiency (Gatlin et al. 1986). Fish with deficiency of both vitamin E and selenium may show severe dystrophic lesions of the muscles; muscle fibre bundles may be enlarged and be invaded by giant cells. Consequently, alimentation with supplementation of both vitamin E and selenium can significantly prevent degeneration of skeletal muscles (Poston et al., 1976).

3 Muscle pathological processes

The section of muscle pathological processes includes overall understanding of muscle anatomy and pathology, as well as several pathological processes in muscle.

3.1 Muscle anatomy

The musculature consists of individual muscles which are mainly built up of muscle cells (muscle fibres, myocytes, myotubes), and its main function is to move and support the bones of the skeleton. Skeletal muscles are under the control of the central nervous system, the spinal cord

and motor nerves. In addition to voluntary motor function, which is the elementary task of the organ, muscle is also required for involuntary motor control, stabilisation of joints, and heat production. It has also immunological and endocrine functions (Jensen-Jarolim, 2013). In humans, the musculature is the largest organ, consisting of 640 single muscles. It makes up 50 % of the body weight, moves 200 bones, and has 2,200 points of attachment.

There are three types of muscles: skeletal, smooth and cardiac (Fig.3.1.1) (Jensen-Jarolim, 2013).

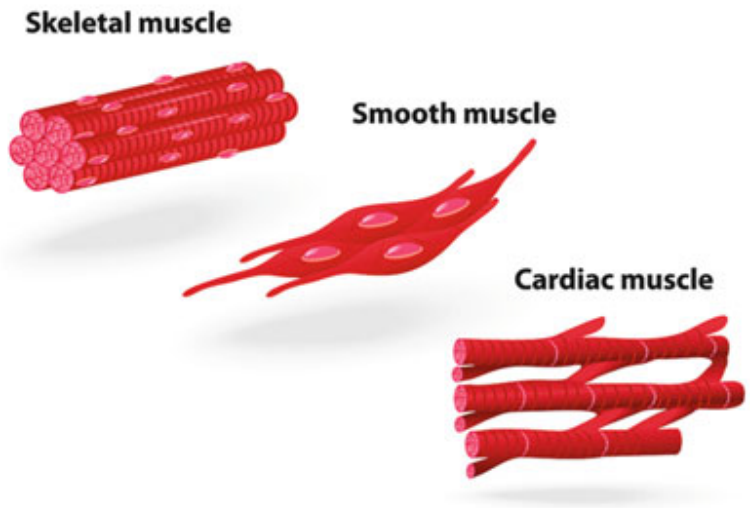

Figure 3.1.1: The three muscle types: skeletal, smooth, and cardiac. Adopted from the book Comparative Medicine, Anatomy and Physiology (Jensen-Jarolim, 2013).

Skeletal muscle principally consists of muscle fibres and connective tissue. The connective tissue is distributed on three levels of scale in the muscle: the endomysium, which surrounds each muscle fibre, the perimysium, which constitute muscle in fibre bundles, and the epimysium, which is the muscle's external envelope connecting it to tendons. The contractile unit of the muscle fibre is the sarcomere, which is the smallest contraction unit (Listrat et al., 2016).

In the sarcomeres myofibrils (filaments), actin and myosin are arranged in a typical manner. Myosin filaments are connected in the Z-line; in between the M-line is a dominant characteristic. This gives the skeletal muscle across-striated appearance (Fig. 3.1.2.) (Jensen-Jarolim, 2013).

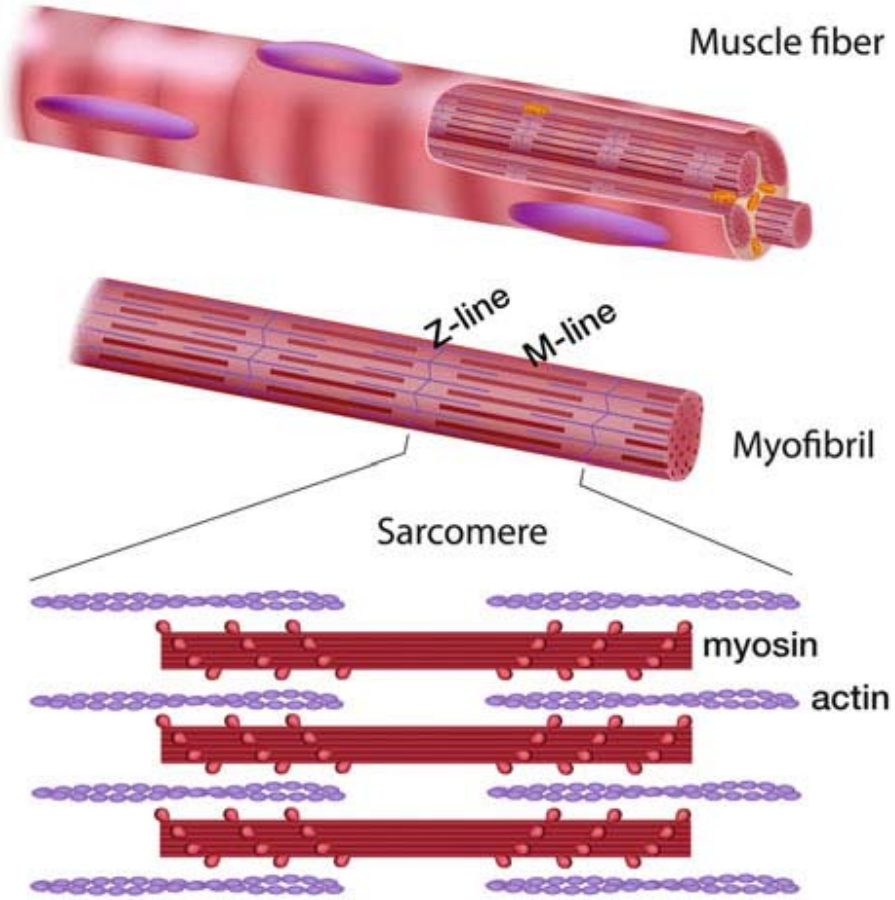

Figure 3.1.2: A muscle fibre. Adopted from book Comparative Medicine, Anatomy and Physiology (Jensen-Jarolim, 2013).

Fish skeletal muscles differ from those of mammals and birds. The main function of fish musculature is movement, in mammals musculature also gives support to the skeleton (Kiessling et al., 2006). In most fish species, skeletal muscles are arranged in very long bundles of fibres. Fish muscles are divided into myotomes (myomeres) separated from each other by thin sheets of connective tissues (myosepta, myocommata). Within each myotome, the muscle fibres are approximately parallel to each other, but at varying angles to the myocommatal sheets to provide the necessary moment for swimming during contraction. The muscle cells are arranged roughly parallel to the longitudinal axis of the fish (Hultmann, 2003). The length of muscle fibres decreases towards the tail end of the fillet and the muscle fibre cross sectional area is smaller at the tail and head than in between (Kiessling et al., 2006).

There are two main types of muscle fibres and several minor types. Red (slow) and white fibres (fast) are the principal divisions, which can be easily distinguished in fish. These two fibre types differ greatly in their physiology, biochemistry and organisation (Kiessling et al., 2006).

In many fish species, red muscle is located as a wedge laterally along the lateral line or may be associated with opercula or with fins where they are used predominantly for locomotion (Ferguson, 2006). Red muscle fibres usually constitute less than 10 % of the myotomal musculature and are small in diameter (25-45 μm). The red muscle fibres are also called slow fibres and are used mainly for sustained energy-efficient swimming. The characteristics of this muscle type are good capillary supply, high amount of mitochondria, lipid droplets and glycogen stores. The concentrations of myoglobin and cytochromes are high. The energy metabolism in red muscle is almost entirely aerobic, based mainly on lipid as fuel complemented by carbohydrates (Kiessling et al., 2006).

White muscle fibres compose the major part of the skeletal muscle in fish and never constitute less than 70 %. The white fibres show the largest cell diameter, ranging between 50 and 100 μm or even more. Normally, the white muscle type is used at high swimming speeds (Kiessling et al., 2006). The white muscles are not as well vascularised as red fibres and have a lower number of mitochondria, with less-developed cristae (internal membranous divisions), although these features may be affected by nutritional and toxic factors (Ferguson, 2006).

Separating the red and white fibres is the intermediate or “pink” fibre. In some species, for instance in salmonids, these fast aerobic fibres comprise a thin scattering situated between the red and white muscle, whereas in other such as carp, “pink” fibres are much more extensive (Ferguson, 2006). Pink fibres are characterised by fast contraction with intermediate resistance to fatigue and a speed of shortening intermediate between red and white muscles (Kiessling et al., 2006).

3.2 Muscle pathology

Pathology is the study (*logos*) of disease (*pathos*). It is devoted to the study of the structural, biochemical, and functional changes in cells, tissues, and organs that underlie disease.

According to *Stedman's Medical Dictionary,* the definition of pathology is *"the medical science, and specialty practice, concerned with all aspects of disease, but with special reference to the essential nature, causes, and development of abnormal conditions, as well as the structural and functional changes that result from the disease processes*".

Understanding of disease begins with understanding processes that occur inside the cell, cell life-cycle and death processes. There are four fundamental aspects of disease processes that form the core of pathology: its cause (*aetiology*), the mechanisms of its development (*pathogenesis*), the biochemical and structural alterations induced in the cells and organs of the body (*molecular and morphologic changes*), and the functional consequences of these changes (*clinical manifestation*) (Kumar et al., 2010).

The causes of cell injury can be grouped broadly into the following categories: oxygen deprivation (*hypoxia*), physical agents (mechanical trauma, temperature, radiation, atmospheric pressure, electric shock), chemical agents and drugs, infectious agents (viruses, bacteria, fungi and parasites), immunologic reactions, genetic derangements, nutritional imbalances (Kumar et al., 2010).

There are several levels of diagnosis. A clinical diagnosis is based on the data obtained from the case history, clinical signs, and physical examination of the patient. The differential diagnosis uses a list of diseases that could account for the evidence or lesions of the case. A clinical pathologic diagnosis is based on changes observed in the chemistry of fluids and hematology, structure and function of cell collected from the living patient. A morphologic diagnosis or lesion diagnosis is based on the predominant lesion(s) in the tissue(s). It can be macroscopic or

microscopic, and describes the severity, duration, distribution, location, and nature (Zachary and McGavin, 2012).

The basic tool for the study of pathology is a variety of techniques of histopathology, histochemistry and immunohistochemistry, which allow the evaluation the macroscopic and microscopic changes in cells, tissues, and organs. Likewise, a variety of techniques of molecular genetic tools are also widely used now (Zachary and McGavin, 2012).

3.3 Muscle degeneration and necrosis

Common causes of degenerative processes include ischemia, nutritional, toxic, exertional, traumatic, and infective. Degenerative myopathies may result in segmental or global myofibre necrosis followed by inflammaiton damage (Zachary and McGavin, 2012).

Early degenerative changes in the muscle include loss of cross-striations, swelling, increased eosinophilia, vacuolation, and pallor. Degenerative changes in the muscle can be divided into hyaline degeneration and granular degeneration (Gopinath and Mowat, 2014).

In the **hyaline** type, sections of affected fibres appear swollen and eosinophilic, with loss of cross-striations. The sarcoplasm may next undergo coagulative or hyaline necrosis, forming eosinophilic fragments or discs that remain within the intact lamina of the muscle cell (Gopinath and Mowat, 2014).

In **granular degeneration**, the damaged sarcoplasm appears granular due to the presence of mineralised mitochondria. In both types, recruitment of inflammatory cells (generally macrophages with some lymphocytes) can appear as the lesions progresses (Gopinath and Mowat, 2014).

Degenerative processes in fish skeletal- and heart muscle (myocardium) can cause different types of diseases with different aetiologies. Diseases such as heart and skeletal muscle inflammation,

pancreas disease and cardiomyopathy syndrome can affect muscle structure. The lesions are typically peroxidative in nature, namely hyaline degeneration with swollen eosinophilic fragmented sarcoplasm, with subsequent invasion of the muscle cell by macrophages. However, in most cases lesions may be more severe with involvement of few inflammatory cells. Degeneration and necrosis are predominantly seen in red musculature and myocardium, but may also be observed in white musculature (Kongtorp et al., 2004, Ferguson, 2006, Rodger et al., 2014).

*"**Necrosis** (from the Greek νέκρωσις "death, the stage of dying, the act of killing" from νεκρός "dead") is the death of living cells or tissues"*. It occurs when e.g. too little blood flows to the tissue. This can be from injury, radiation, or chemicals. Necrosis cannot be reversed.

The common causes of necrosis are several, and similar to degeneration: ischemia, nutritional, toxic, exertional, traumatic, viruses, bacteria, and protozoa can induce necrosis (Kumar et al., 2010)

In muscle tissue, necrotic myofibres have several different histological manifestations. Affected fibres are often vacuolated and fragmented, with pyknotic nuclei. Myofibres under necrosis are swollen, deeply eosinophilic, homogeneous and lack cross-striations (hyalinisation) (Manor and Sadeh, 1989, Gopinath and Mowat, 2014). The earliest change is often segmental hypercontraction, resulting in segments of slightly larger diameter that are slightly darker staining ("large dark fibre") that are best seen on transverse sections. On longitudinal sections, "twisting" or "curling" of stricken fibres is usually observed. Increased intracellular calcium is a common trigger of necrosis in all cells. Moreover, myofibres contain a high level of calcium ions stored in the sarcoplasmic reticulum (Zachary and McGavin, 2012).

In addition, the diagnoses of necrosis and degeneration can be difficult because necrosis and degeneration both show similar morphologic features, histologic manifestation and aetiological causes.

If degeneration occurs a long time in muscle cell, it can lead to activation of irreversible process and can result in myofibre necrosis (Ikeda et al., 2016). Muscle necrosis is followed by the activation of muscle regeneration process and depend on satellite cell activation. Muscle satellite cells are stable to damaging factors which can result in myofibre necrosis. Muscle satellite cells are beginning to divide in preparation for regeneration (Zachary and McGavin, 2012).

Morphologically, degenerate and necrotic muscle fibres are often accompanied by an inflammatory cell infiltrate.

Fish species have a large range of infections and non-infections pathological processes that affect skeletal muscle structure and can lead to necrosis. Bacterial diseases, such as classical vibriosis and cold-water vibriosis, result in multifocal liquefactive necrosis of muscle, especially in the subacute or chronic stage of the diseases. Bacterial kidney disease leads to cavitation of muscle. In the subcutis stage, lesions usually contain straw-coloured flocculent contents that create large blisters visible on gross external examination. In addition, lesions can be so large as to grossly distort the whole fish. Other viral diseases and nutritional misbalances can affect the muscle and lead to such diseases as pancreas disease, heart and skeletal muscle inflammation and deficiency of selenium and vitamin E, as well as fungi (*Saprolegnia spp*.) (Zachary and McGavin, 2012, Bruno et al., 2013).

3.4 Granulomatous inflammation

Granulomatous inflammation characterized by the presence of macrophages, from which may also form multinucleate giant cells. They are commonly chronic. Granuloma denotes a well-demarcated focus of granulomatous inflammation and represents a chronic stage (Zachary and McGavin, 2012).

Granuloma is a cellular attempt to contain an offending agent that is difficult to eradicate. Granulomatous inflammation is a distinctive pattern of chronic inflammation that is encountered in a limited number of infectious and some non-infectious conditions (Kumar et al., 2010). The granulomatous inflammatory response may be a manifestation of many infective, toxic, allergic,

autoimmune and neoplastic diseases and also conditions of unknown aetiology. Schistosomiasis, tuberculosis and leprosy are examples of granulomatous diseases (Williams and Williams, 1983). The granulomatous inflammatory response is a special type of chronic inflammation often characterised by focal collections of macrophages, epithelioid cells and multinucleated giant cells. In most instances these cells are aggregated into well-demarcated focal lesions called granulomas, although a looser, more diffuse arrangement may be found. Moreover, there is an impurity of other cells, especially lymphocytes, plasma cells and fibroblasts (Williams and Williams, 1983).

Granuloma is a focus of chronic inflammation consisting of a microscopic aggregation of macrophages that are transformed into epithelium-like cells, surrounded by a collar of mononuclear leukocytes, principally lymphocytes and occasionally plasma cells (Kumar et al., 2010).

Histologically, haematoxylin and eosin-stained tissue sections label epithelioid cells with a pale pink granular cytoplasm with indistinct cell boundaries. The nucleus is less dense than that of lymphocytes, it is oval or elongate, and can show folding of the nuclear membrane. Older granulomas develop an enclosing rim of fibroblast and connective tissue. The *giant cell* can form from merged epithelioid cells, either in the periphery or centre of granulomas (Kumar et al., 2010).

There are two types of granulomas**.** ***Foreign body*** *granulomas* are incited by relatively inert foreign bodies and ***immune granulomas*** are caused by a variety of agents that are capable of inducing a cell-mediated immune response (Kumar et al., 2010).

There are also forms of muscular granulomas. Granulomatous myositis is applied to a myopathic syndrome associated with non-specific epithelioid granulomas in striated muscle. Granulomatous myositis is an infrequent process and is most often related to sarcoidosis (Roux et al., 2007). However, other unusual causes and abnormalities have been registered, including infectious diseases (tuberculosis, syphilis, *Pneumocystis jirovecci*, brucellosis, HTLV-1, *Cryptococcus spp*. and foreign-body reaction) (Prieto-González and Grau, 2014).

There are different causes of granulomatous myositis, with both infectious and non-infectious aetiology. Clinical cases usually exhibit symmetrical proximal or distal muscle weakness. The presence of granulomas in muscle biopsy is required for the diagnosis of granulomatous myositis. Focal aggregates of epithelioid histiocytic cells, macrophages, inflammatory lymphocytes, and occasional giant cells of the Langerhans type within the muscle parenchyma and typically located between the muscle cells could be observed in samples. A mononuclear perivascular infiltrate may also be found. In chronic stages, muscle fibres may not be invaded by granulomas, instead showing principally atrophy or degeneration (Prieto-González and Grau, 2014).

Granulomatous inflammation in the muscle of fish can be the result of several diseases or agents. *Ichthyophnus hoferi* is one example of a causal agent of granulomatous myositis. Fungal infection "Epizootic Ulcerative Syndrome" is also known as "red spot disease" or "mycotic granulomatosis". The causative agent is *Aphanomyces invadens*. The histopathological picture of early lesions includes haemorrhagic dermatitis, while severe stages lead to degeneration of muscle and necrotising granulomatous dermatitis. Moreover, microsporidian and myxozoan infections of muscle are responsible for formation of granulomas in muscle tissue. In addition, granulomatous myositis is also seen in severe cases of "proliferative kidney disease" (Ferguson, 2006).

4 Intention of the present study

The goal of the present study was to analyse melanised changes in the dorsal musculature and compare these to muscle changes in the abdominal area. The main hypothesis is "*Melanised changes in the dorsal musculature are of the same nature as changes in the abdomen*".

Sub-hypotheses

1. Diffuse melanised dorsal changes are of a different nature compared with focal melanised changes in dorsal musculature.

2. Melanised changes are invariably related to presence of PRV in muscle tissue.

5 Material and methods

5.1 Fish

Two fish populations from two production locations, Frøya and Hjelmeland, in Mid- and Western Norway, respectively, were included in this investigation.

5.1.1 Frøya population

Sampling from the Frøya group was performed 22th January 2016 at the slaughterhouse of SalMar ASA, Frøya, in Sør-Trøndelag. The fish originated from a hatchery in Belsvik and the breeding strain was Stofnfiskur culture, fish groups 14001, produced by Stofnfiskur, Iceland. The fish was transferred from freshwater to seawater at the age of 8 months post hatching. Average weight at transfer was 93.1gr. Before transfer to seawater the fish was routinely vaccinated against *Aeromonas salmonicida* (furunculosis), *Listonella* (*Vibrio) anguillarum* (vibriosis), *Vibrio salmonicida* (cold water vibriosis), IPN (infectious pancreatic necrosis virus) and *Moritella viscosa* (winter vibriosis) (Pentium Forte Plus, Novartis Animal Vaccines Ltd, United Kingdom) according to standard procedures (Sommerset et al., 2005). Overall average weight of the fish at sampling was 5.9kg.

5.1.2 Hjelmeland population

Sampling from the Hjelmeland group was performed 17th March 2016 at the slaughterhouse of Marine Harvest ASA, Hjelmeland, Rogaland. The fish originated from a hatchery in Vindsvik and the breeding strain was produced by BLU-Tveitevåg, Norway. The fish was transferred to seawater at the age of 10 months post hatching. Average weight at transfer was 78.3gr. To prevent losses due to pancreatic disease, the fish was immunized before transfer to seawater with Compact PD vaccine (Intervet International B.V., the Netherlands). In addition, vaccination against *Aeromonas salmonicida*, *Vibrio salmonicida*, *Listonella* (*Vibrio) anguillarum*, *Moritella viscosa* and IPNV was performed using Alphaject Micro 6, PHARMAQ AS, Oslo, Norway, and

was carried out according to standard procedures (Sommerset et al., 2005). Overall average weight of the fish in the population at sampling was 5.158kg

5.2 Sampling

The general data of fish including average weight, vaccination status, and analysis for infectious agents was provided by the management at the slaughter house of SalMar ASA and Marine Harvest ASA. About 500 fillets were visually examined at the production lines at Frøya and Hjelmeland during the sampling. Collection of fillet samples took approximately 2 hours for each sampling.

5.2.1 Frøya sampling

All individuals collected on Frøya were assembled into Groups A and B, respectively. Group A included 10 fish fillets affected with pigmented changes. Four fillets had only focal melanised changes in the dorsal musculature and six individuals had focal melanised changes in the dorsal and abdominal musculatures. Group B included 10 control individuals without any macroscopically detectable changes in dorsal musculature. However, two fish fillets in Group B displayed focal melanised changes in the abdominal area. Sampling was carried out in accordance with company rules for hygiene in the production line and personal hygiene. Hygienic measures also included a change of clothes and use of disposable gloves in addition to disposable scalpels.

Before sampling, the anatomical localization for fillet investigation was defined (Fig. 5.2.1), and the study area was divided into five regions (I to V) (Fig. 5.2.2). Regions I to III were investigated for pathological changes, and control tissue was obtained from region IV. The control region (IV) has the lowest risk of muscle tissue changes (Mørkøre et al., 2015). Region V was included as an additional sampling in abdominal location.

Sampling for histology

Each fillet from both groups went through the following procedure: From regions I to III, two white muscle tissue samples for histology with indication “a” and “b”, as illustrated in Fig. 5.2.2 were collected. Sample “a” was a transverse section (cross-section of myocytes) and “b” a longitudinal section (longitudinal section of myocytes). From region IV, only a transverse section was collected. The region V additional sample was collected from fillets with focal melanised changes in the abdominal area using a transverse section. The size of the samples was approximately 1x1x0.5cm. Samples were immediately fixed in 10% neutral phosphate buffered formalin for no longer than 48 h and subsequently processed for morphological analysis.

Sampling for genetic analysis

Muscle

For genetic analysis, two additional transversally-sectioned muscle samples “c” (1x1x1mm) from regions II and IV were collected from each individual in both groups (Fig. 5.2.2). These samples were immediately transferred to tubes with RNA*later*. This material was subsequently sent to a commercial laboratory and analysed for presence of genetic material from PRV, SAV, ISAV, IPNV and *Renibacterium salmoninarum* (PatoGen Analyse AS, Ålesund, Norway).

Spleen

As the fish was processed at slaughterhouse, the organs were removed prior to fillet production. Therefore, it was impossible to collect organs from the identical individual from which muscle specimens were collected. To enable testing for a range of infectious agents on a population scale, spleen samples (1x1x1 mm) were collected from 18 sets of organs and transferred to RNA*later*. This material was also sent for PRV, SAV, ISAV, IPNV and *R. salmoninarum* genetic material (PatoGen Analyse AS,).

Figure 5.2.1: Fish fillet with area of investigation. Frøya sampling (Group A, fish 1). Scale bar = 10cm.

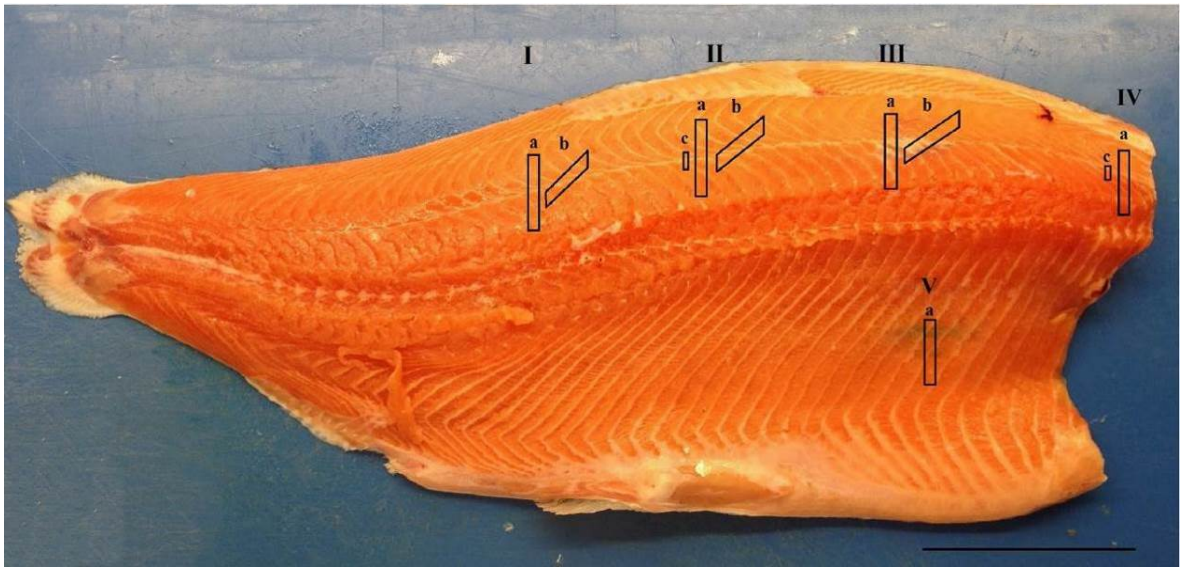

Figure 5.2.2: Fish fillet with regions of sampling. Frøya sampling (Group A, fish 5). I, II and III – investigation regions. IV – control region. V – abdominal region, "additional sample". Sample "a" – a transverse section and sample "b" – a longitudinal section for morphological analysis (1x1x0,5 cm). Sample "c" – for analysis of genetic material from pathogens (1x1x1 mm). Scale bar = 10cm.

Table 5.2.1: Sampling overview from Frøya includes fillets with focal melanised changes (Group A) and fillets without macroscopically detectable changes (Group B). Fillet regions I, II and III, two samples were collected on formalin – a longitudinal and a transverse section. From regions IV and V, one sample was collected in formalin – a transverse section. From regions II and IV, samples for genetic analysis for pathogens were collected in RNA*later*.

Group A	Region I, histology sample, a and b	Region II, histology sample, a and b	Region III, histology sample, a and b	Region IV, histology sample, a	Region V, histology sample, a	Region II, genetic sample, c	Region IV, genetic sample, c
Fish 1	1a	1 b	1 c	1 d	1	1	1
Fish 2	2a	2 b	2 c	2 d	2	2	2
Fish 3	3a	3 b	3 c	3 d	3	3	3
Fish 4	4a	4 b	4 c	4 d	4	4	4
Fish 5	5a	5 b	5 c	5 d	5	5	5
Fish 6	6a	6 b	6 c	6 d	6	6	6
Fish 7	7a	7 b	7 c	7 d	7	7	7
Fish 8	8a	8 b	8 c	8 d	8	8	8
Fish 9	9a	9 b	9 c	9 d	9	9	9
Fish 10	10a	10 b	10 c	10 d	10	10	10
Group B							
Fish 1	1a	1 b	1 c	1 d	1	1	1
Fish 2	2a	2 b	2 c	2 d	2	2	2
Fish 3	3a	3 b	3 c	3 d	3	3	3
Fish 4	4a	4 b	4 c	4 d	4	4	4
Fish 5	5a	5 b	5 c	5 d	5	5	5
Fish 6	6a	6 b	6 c	6 d	6	6	6
Fish 7	7a	7 b	7 c	7 d	7	7	7
Fish 8	8a	8 b	8 c	8 d	8	8	8
Fish 9	9a	9 b	9 c	9 d	9	9	9
Fish 10	10a	10 b	10 c	10 d	10	10	10

Fillets were separately and stockpiled in production plastic boxes. After collection, boxes with materials were taken to the local laboratory at SalMar ASA for further investigation and sampling.

Following sampling, all material was taken to the Faculty of Veterinary Medicine, Norwegian University of Life Sciences, Oslo, Norway.

5.2.2 Hjelmeland sampling

The main focus of this sampling was to identify fillets with muscle changes in the entire length of the dorsal musculature (Fig. 5.2.3) and to compare such changes with focal melanised changes. Three fillets with no macroscopical pathological changes (Group C) and three fillets with pigmented changes in the entire length of the dorsal muscle (Group D) were collected on the production line, stockpiled in a plastic box and taken to the local laboratory for further processing.

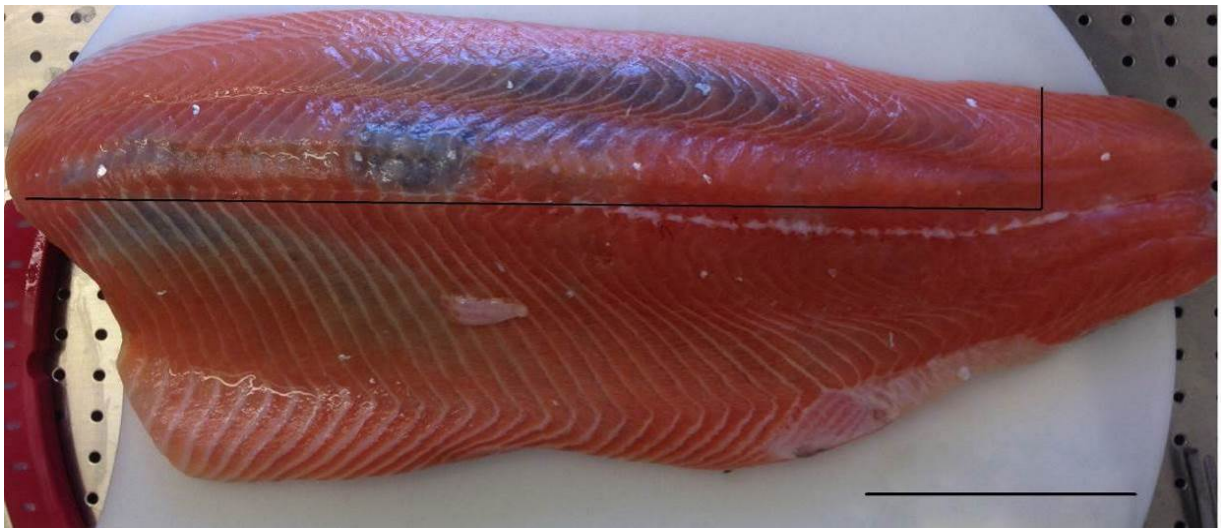

Figure 5.2.3: Fish fillet with area of investigation. Hjelmeland sampling (Group D, fish 1). Scale bar = 10cm.

Material for histology and genetic analysis were selected as follows: Region for sampling was defined at the middle of the dorsal muscle (Fig. 5.2.4). Transverse (sample "a") and longitudinal (sample "b") sections of white muscle myocytes were collected from region I. Control tissue was collected from region II (transverse section), as this was defined as macroscopically non-affected area. Size of samples was approximately 1x1x0.5 cm. The samples were immediately fixed in 10% neutral buffer formalin for no longer than 48 h and subsequently processed for histology and immunohistochemistry. From each individual in Group D, two additional samples "c" (1x1x1mm) including dorsal muscle (region I) and control region (II) were collected. These samples were immediately transferred into tubes with RNA*later*. In this sampling, no internal organs were collected.

The samples were treated as described for the “Frøya” sampling. Hygienic measures during this sampling were also identical. Following sampling, all material was taken to the School of Veterinary Medicine, Norwegian University of Life Sciences, Oslo, Norway.

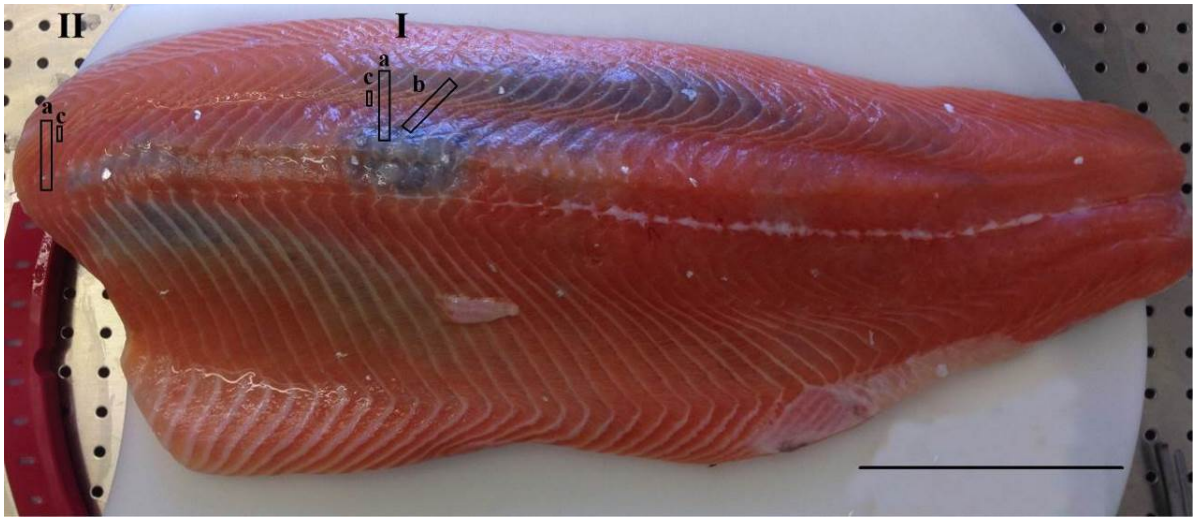

Figure 5.2.4: Fish fillet with regions of sampling. Hjelmeland sampling (Group D, fish 1). I – investigation region. II – control region. “a” – sample for histological and immunohistochemical analysis (transverse section), “b” – sample for histology and immunohistochemical analysis (longitudinal section), “c” – sample for genetic analysis for pathogens. Scale bar = 10cm.

5.3 Histology Immunohistochemistry and Genetic

5.3.1 Histology

Tissue samples for histology were fixed in 10% buffered formalin for 24-48 h. Subsequently, samples were dehydrated in graded alcohol baths and embedded in paraffin (Medite TES 99, Germany). Histological sections were produced using a Thermo Scientific™ HM 450 Sliding Microtome (3µm) and placed on glass slides (Superfrost©, Mentzel, Braunschweig, Germany). Sections were subsequently incubated for 24 h at 37 °C and stained following standard procedures for haematoxylin and eosin (H&E), van Gieson’s method (vanG), Gram, Periodic acid-Schiff (PAS), Ziehl-Neelsen (ZN) and Martius, Scarlet and Blue (MSB). Positive histological control material for Gram, PAS and ZN were provided by the laboratory at the Section for anatomy and pathology at the Faculty of Veterinary Medicine, Norwegian University of Life Sciences.

5.3.2 Immunohistochemistry

Immunohistochemistry was performed on paraffin-embedded material listed above. Sections were cut (4µm) and placed on glass slides (Superfrost©; Mentzel, Braunshweig, Germany). Subsequently, material was incubated for 24 h at 37˚C. Thereafter, sections were de-waxed in xylene and rehydrated in graded alcohol baths, and transferred to distilled water. Antigen retrieval was obtained by autoclaving in 0.01 citrate buffer at 120˚C for 15 min at pH 6.0. Endogenous peroxidase was inhibited using phenylhydrazine 0.05% (Sigma-Aldrich. St. Louis, MO, USA) for 40 min at 37˚C. The slides were then rinsed three times in phosphate buffer saline (PBS). Non-specific binding sites were blocked by adding PBS with 2% normal goat serum plus 5% bovine serum albumin (BSA) for 20 min. PRV was identified using anti-PRV rabbit sera (Finstad et al., 2012) in dilution 1:700. To identify proliferating cells, a monoclonal antibody recognizing proliferative cell nuclear antigen (PCNA) was used in dilution 1:5000 (clone PC 10, Dako, Glostrup, Denmark). Primary antibody - in tris-buffered saline (TBS) with 1% BSA was added at a quantity of approximately 150µl for each slide and incubated for 30 min. Sections were then rinsed three times in TBS. Secondary antibody to both analyses PRV and PCNA - sodium chloride in concentration ≥1% - <3 % was then added (EnVision© System kit; Dako, Glosrup, Denmark) with approximately 100µl for each slide and incubated for 30 min. The slides were then rinsed three times in TBS. For visualization approximately 100µl, 3-amino- 9-ethylcarbazole (AEC-Plus Substrate-Chromogen, EnVision© System kit) was added for each slide and incubated for 14 min to provide red colour. Slides were subsequently washed in distilled water, and counter-stained with haematoxylin for 40 sec. Mounting was performed with polyvinyl alcohol media at pH 8.0. All incubations were carried out at room temperature in a humidity chamber.

A heart sample diagnosed for HSMI in Atlantic salmon was used as positive control for PRV immunohistochemical detection. Negative controls used 1% BSA instead of primary antibody from the same material for the identification of PRV and PCNA.

5.3.3 Genetic analysis

Materials for genetic analysis were collected as described above and sent to PatoGen Analyse AS in Ålesund for further analysis. This laboratory analysed the material for the identification of genetic material originating from PRV, SAV, ISAV, IPNV and *R. salmoninarum*, respectively. Genetic investigations were performed according to company's procedures by using real-time reverse transcription polymerase chain reaction (RT-PCR).

6 Results

The result chapter is divided into three main sections. The first section includes gross pathology descriptions of white muscle dorsal changes in the different groups studied. The second section describes morphological evaluation of muscle tissue including histological examination and immunohistochemical results for PRV and PCNA. The third section describes results of genetic material from PRV, SAV, ISAV, IPNV and *R. salmoninarum* analysis.

6.1 Gross pathology

The visual examination of fillets in both study populations Frøya and Hjelmeland showed that focal melanised changes in the abdominal musculature affected approximately 20% of the individuals on the production line. Approximately 3-4% of focal melanised changes in dorsal musculature were detected in the dorsal musculature in both groups. On incision, some focal melanised changes appeared to be superficial, while others extended deep into the musculature including the intermyotomal septa. On palpation, the melanised muscle changes were often indurated with an increased texture, especially in Group D from Hjelmeland.

6.1.1 Frøya sampling

The Frøya sampling included 20 fillets; 10 of them had focal melanised changes in dorsal musculature (Fig. 6.1.1). In addition, six individuals also had focal melanised changes in the abdominal musculature (Fig. 6.1.2). The sampling also included 10 fillets without any apparent

changes in the dorsal musculature (Fig. 6.1.3). However, two individuals (Fish 1 and 8) in Group B had focal melanised changes in abdominal musculature. The approximate size of focal melanised changes in dorsal and abdominal musculature was usually 1-3 cm in Group A, typically including 2-6 myotomes. Incision through foci revealed that discoloration extended deep into the muscle tissue.

Figure 6.1.1: Fillet sample with focal melanised changes in Frøya sampling. (Group A, fish 1). Scale bar = 10cm.

Figure 6.1.2: Fillet with focal melanised changes (circle) in the abdominal region. Frøya sampling (Group A, fish 5). Scale bar = 10cm.

Figure 6.1.3: Fillet without pathological changes in muscle. Frøya sampling (Group B, fish 1). Scale bar = 10cm.

6.1.2 Hjelmeland sampling

The Hjelmeland group consisted of six fillets, three of which displayed substantial diffuse dorsal changes affecting the entire length of the dorsal musculature and also parts of the abdominal area (Group D) (Fig. 6.1.4). Three fillets without any apparent pathological changes were included as controls (Group C) (Fig. 6.1.5). The approximate size of the pathological changes in the dorsal musculature was 10-15 cm in Group D, involving 15-20 myotomes.

Figure 6.1.4: Fillet with pathological changes in the dorsal and abdominal musculature Hjelmeland sampling (Group D, fish 1). Scale bar = 10cm.

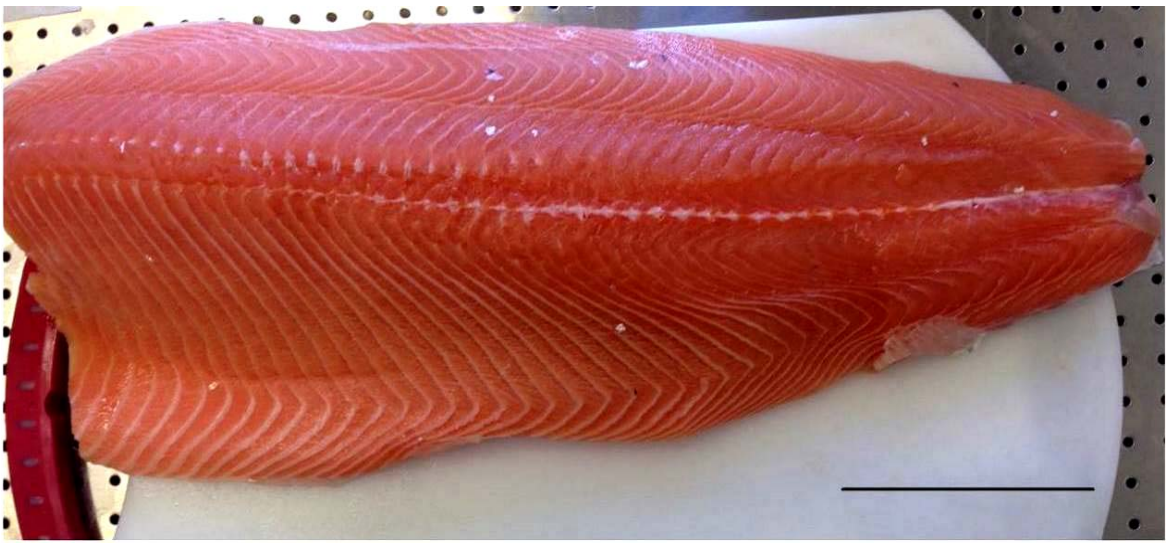

Figure 6.1.5: Fillet without apparent pathological changes in the musculature. Hjelmeland sampling (Group C, fish 3). Scale bar = 10cm.

Results of visual examination of all groups presented in Table 6.1.1 describe the level of tissue damage based on the numbers of involved myotome and location of lesions.

Table 6.1.1: Fillets with gross anatomical localization of tissue changes indicated. Level of tissue damage: 0 – none, 1– slight, 2 – moderate, and severe – 3. Locations: according to region with changes (I to V) Frøya sampling and (I to II) Hjelmeland sampling.

Group A Frøya	level of lesions (0.1.2.3.)	Location in dorsal region	Location in abdominal region
Fish 1	2	III	V
Fish 2	2	II	V
Fish 3	1	II	
Fish 4	2	II	
Fish 5	2	II	V
Fish 6	2	III	V
Fish 7	2	II	V
Fish 8	2	II	
Fish 9	1	II	V
Fish 10	2	II	
Group B Frøya	level of lesions (0.1.2.3.)	Location in dorsal region	Location in abdominal region
Fish 1	0		V
Fish 2	0		
Fish 3	0		
Fish 4	0		
Fish 5	0		
Fish 6	0		
Fish 7	0		
Fish 8	0		V
Fish 9	0		
Fish 10	0		

Group C Hjelmeland	level of lesions (0.1.2.3.)	Location in dorsal region	Location in abdominal region
Fish 1	0		
Fish 2	0		
Fish 3	0		
Group D Hjelmeland	level of lesions (0.1.2.3.)	Location in dorsal region	Location in abdominal region
Fish 1	3	I	
Fish 2	2	I	
Fish 3	3	I	

6.2 Histology

6.2.1 Frøya sampling

Focal melanised changes in dorsal musculature in Group A, Frøya sampling, were characterized with myocyte degeneration, loss of striation and invasion of macrophages (Fig. 6.2.1A). In addition, severe fibrosis, presence of melano-macrophages and inflammatory cells of a mononuclear nature were also observed (Fig. 6.2.1A and B). Macrophage-like cells within necrotic myocytes were observed (Fig. 6.2.1B). Acute myocyte degeneration and necrosis were observed (Fig. 6.2.1A). Simultaneous regeneration of myocytes, necrosis and extensive fibrosis were observed within the same sections (Fig. 6.2.1C). Haemorrhages were not observed. Immunohistochemical investigation of sections from Group A identified PRV-positive macrophage-like cells and melano-macrophages scattered throughout the chronically inflamed area (Fig. 6.2.1C). PCNA immune-reactive macrophage-like cells and melano-macrophages were detected in the changes (Fig. 6.2.1D). Widespread vacuolisation and vacuoles were frequently found in affected areas encircled by melano-macrophages (Fig. 6.2.1E). Staining for bacteria and fungi by Gram, ZN and PAS did not provide any positive reactions in the changes (Fig, 6.2.1F, G, H). Blood clots (thrombes) leading to impaired blood supply to the muscle fibres were not identified using MSB staining (Fig. 6.2.1I). Positive controls for bacterial and fungal analysis are presented in Fig. 6.2.6.

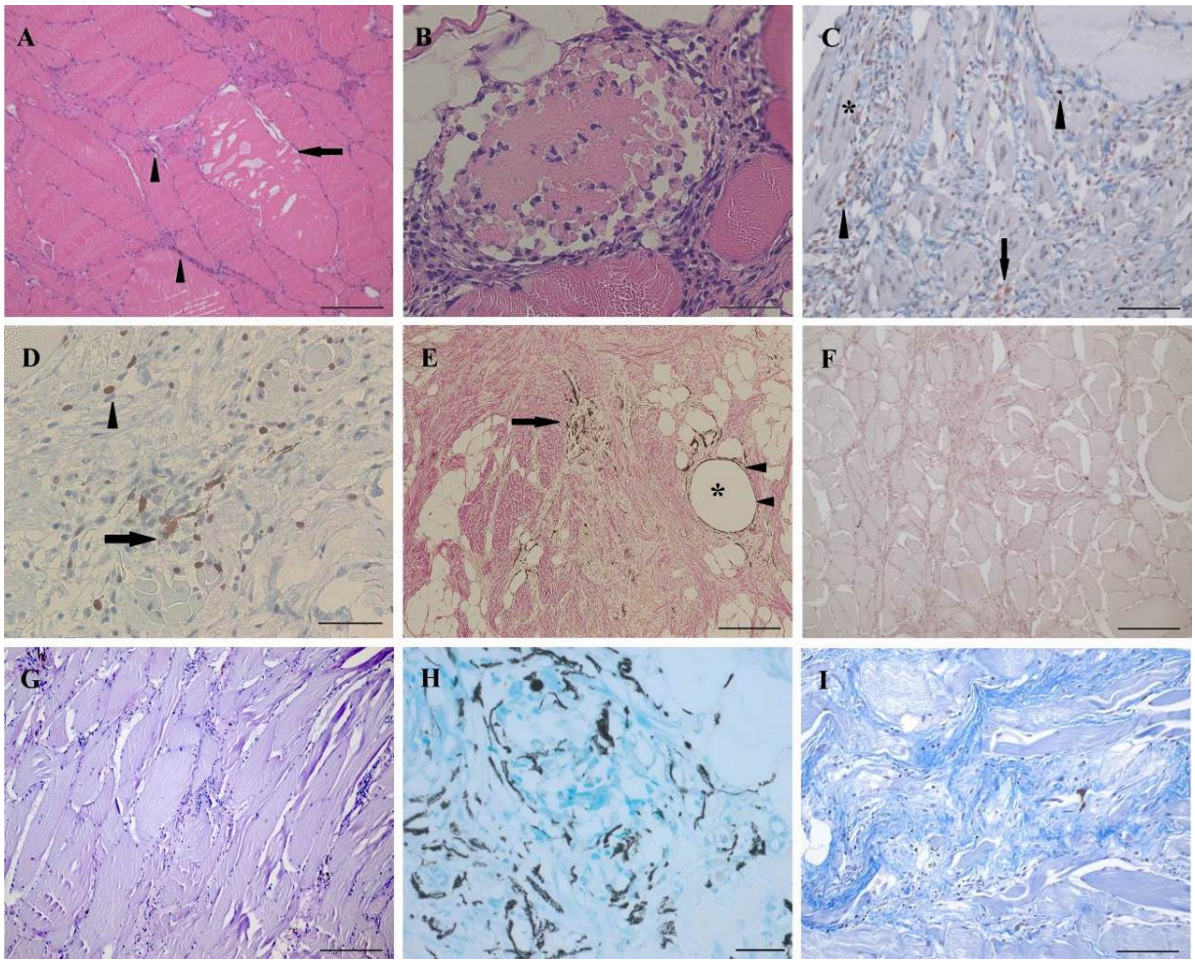

Figure 6.2.1: Histological investigations of focal melanised changes in dorsal musculature of Frøya samples (Group A). **(A)** Transverse section with single muscle degeneration (arrow) and inflammatory cell infiltrate with melano-macrophages (arrowheads) between myocytes (H&E staining). **(B)** Transverse section of necrotic myocyte with surrounding and infiltrating macrophages. (H&E staining). **(C)** A high level of viral infection in affected tissue, PRV positive cells (red staining) located between and around myocytes. Necrotic myocytes with regeneration (asterisk) (area with repair, formation of myotubes with an internal chain of nuclei), severe fibrosis and melano-macrophages (arrowheads), macrophage-like cells (arrow) positive for PRV (PRV immunostain). **(D)** Proliferating cells include melano-macrophages (arrow) and macrophage-like cells (arrowhead) (PCNA immunostain). **(E)** Vacuole (asterisk) in an area with severe fibrosis. Melano-macrophages (arrowheads) are located in the periphery of the vacuole, strong melanisation in melano-macrophages (arrow) (van Gieson staining). **(F)** Transverse section, necrotic area with melano-macrophages displays no presence of bacteria (Gram staining). **(G)** No fungi were detected in the changes (PAS staining). **(H)** No Ziehl–Neelsen- positive microorganisms were detected. **(I)** Thrombes were not identified (MSB staining). (A, E, F, G,) scale bar = 100 µm, (C, I) scale bar = 50 µm, (B, H, D) scale bar =20 µm.

Focal melanised changes in abdominal muscle samples in the Frøya groups (Groups A and B) were characterised by degeneration of myocytes, loss of striation (Fig 6.2.2B), widespread vacuolisation, infiltration of macrophages in necrotic myocytes (Fig 6.2.2A) and the presence of melano-macrophages (Fig 6.2.2B). In addition, severe fibrosis, destruction of connective tissue, acute degeneration and necrotic myocytes without macrophage invasion were detected (Fig 6.2.2B and C). Haemorrhages were not observed. In addition, presence of PRV-positive macrophage-like cells and melano-macrophages were also detected (Fig 6.2.2C). Bacteria or fungi were not detected in these changes. Results are presented in Fig. 6.2.4. D, E and F. Positive controls for bacterial and fungal analysis are presented in Fig 6.2.6.

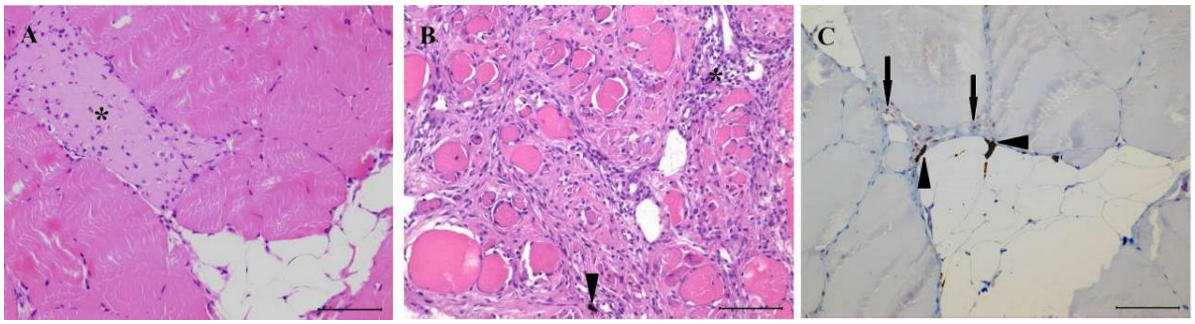

Figure 6.2.2: Histological investigations of focal melanised changes of Frøya samples in abdominal region (Group A and B). **(A)** Transverse section of necrotic myocyte with macrophages surrounding and infiltrating (asterisk) (H&E staining). Scale bar = 50 µm. **(B)** Transverse section with multiple muscle degenerations and inflammatory cell infiltrate (asterisk) with melano-macrophages (arrowheads) between myocytes (H&E staining). Scale bar = 50 µm. **(C)** Abundant amounts of PRV-positive cells were present in connective tissue. Melano-macrophage (arrowheads) and macrophage-like cells (arrows) were positive for PRV (PRV immunostain). Scale bar = 50 µm.

6.2.2 Hjelmeland sampling

Diffuse melanised changes in dorsal muscle in individuals from the Hjelmeland sampling (Group D) were characterized with severe degeneration of myocytes, necrosis with loss of striation, inflammatory cells between the muscle fibres and destruction of the connective tissue (Fig. 6.2.3A). Tissue contained melano-macrophages and macrophage-like cells in the intermyotomal septa (Fig. 6.2.3B). Haemorrhages were not observed. Macrophage-like cells and melano-macrophages positive for PRV immunostain were scattered throughout the chronically inflamed area (Fig. 6.2.3C). PCNA immuno-reactive cells in affected tissue were found in macrophage-

like cells and melano-macrophages (Fig. 6.2.3D). Severe fibrosis was detected in affected areas (Fig. 6.2.3E). Staining for bacteria and fungi by Gram, ZN and PAS did not provide any positive results in Group D (Fig. 6.2.3F, H and G). Tissue thrombes were not identified by MSB staining (Fig. 6.2.3I). Positive controls for bacterial and fungal analyses are presented in Fig 6.2.6.

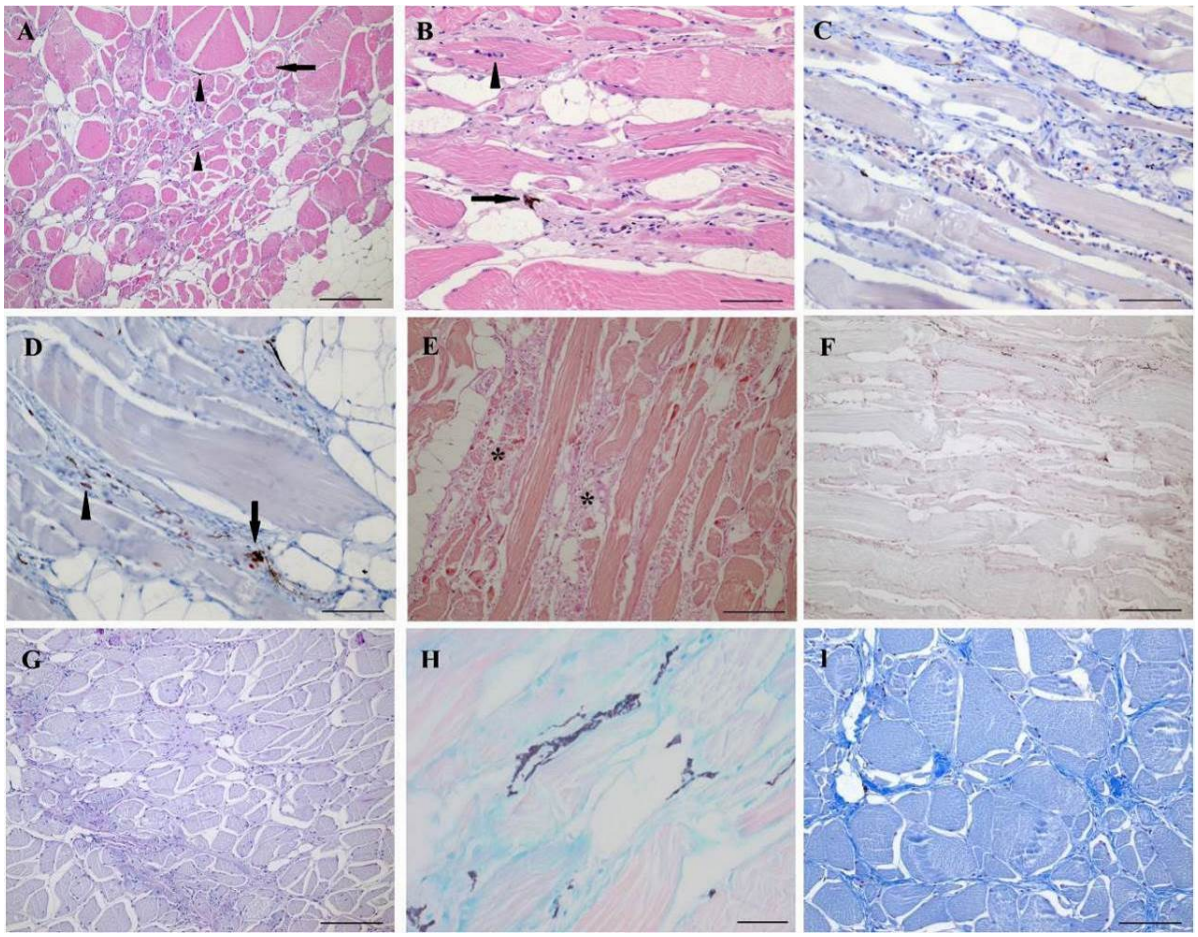

Figure 6.2.3: Histological analysis of diffuse melanised changes in dorsal musculature of Hjelmeland samples (Group D). **(A)** Transverse section of muscle sample shows extensive chronic degeneration with infiltration of macrophages in myocytes (arrow) and in the surrounding interstitium, also containing melano-macrophages (arrowheads) (H&E staining). **(B)** Longitudinal section of white skeletal muscle with high magnification shows melano-macrophages (arrow). In addition, myocyte with regeneration (arrowhead) (area with repair, formation of myotubes with an internal chain of nuclei) (H&E staining). **(C)** Abundant amounts of PRV-positive cells (red staining) were present in necrotic myocytes and connective tissue (PRV immunostain). **(D)** Proliferating cells include macrophage-like cells (arrowhead) and melano-macrophages (arrow) (PCNA immunostain). **(E)** Van Gieson staining shows extensive fibrosis and necrosis (asterisks). **(F)** Longitudinal section, necrotic area with melano-macrophages displays no presence of any bacteria (Gram staining). **(G)** Necrotic area displays no presence of any fungal infection (PAS staining). **(H)** No Ziehl–Neelsen- positive microorganisms were detected. **(I)** Tissue thrombes were not identified (MSB staining). (A, E, F, G,) scale bar = 100 µm. (B, C, D, H, I) scale bar = 50 µm.

Staining for Gram, ZN and PAS in all groups with focal melanised changes in dorsal musculature (Groups A and D) and focal melanised changes in the abdominal area of affected individuals in Groups A and B are presented in Fig. 6.2.4. Bacteria or fungi were not detected. No bacteria were identified in changes in the dorsal musculature of Group A, Frøya sampling. However, ZN staining revealed some reaction confined to melanosomes. Similar reactions were not obtained in melanosomes in changes investigated from Group A, B and D.

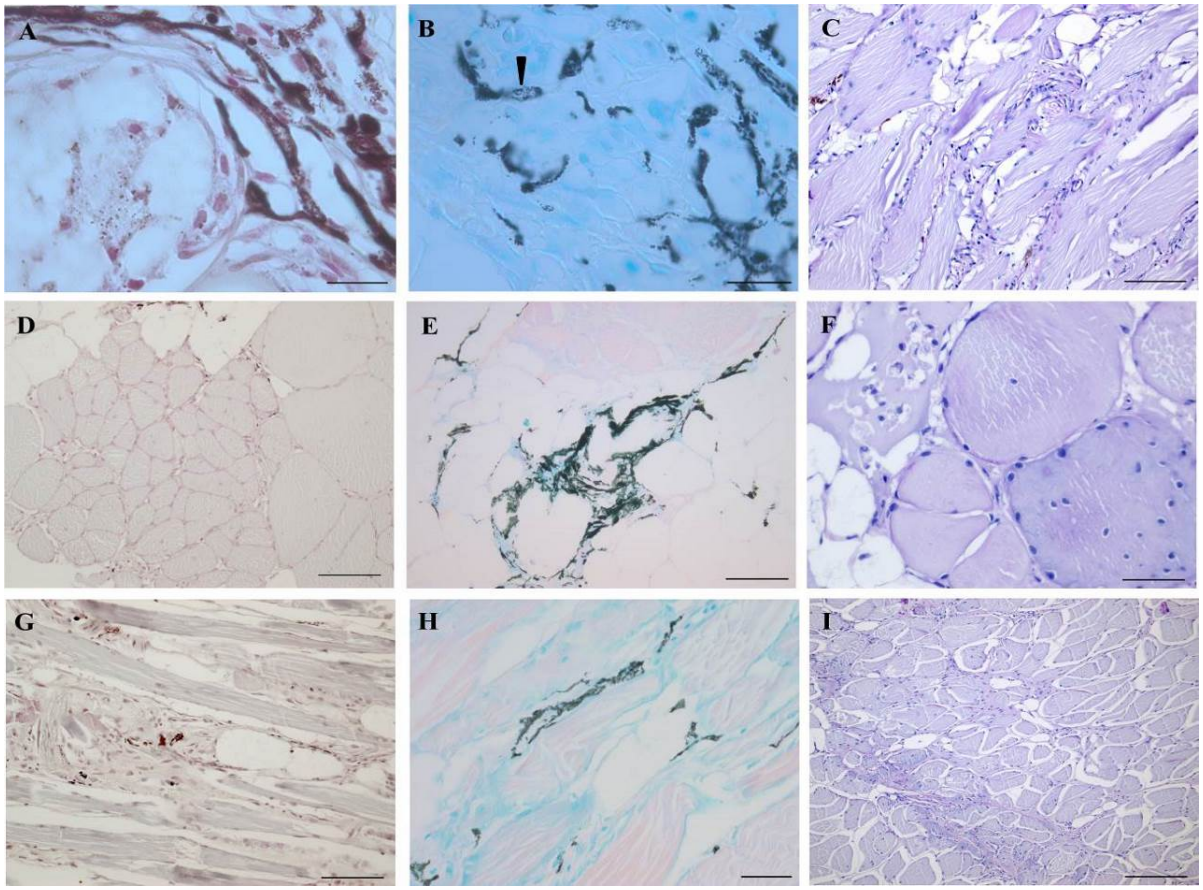

Figure 6.2.4: Histological analysis for the presence of bacteria and fungi, representative sections stained for Gram, ZN, PAS. **(A)** Section of affected dorsal musculature tissue with a necrotic process and inflammation were negative for bacteria (Group A, Gram staining). **(B)** ZN negative staining of dorsal muscle sample with some red melanosomes (arrowhead, red colour) (Group A). **(C)** No positive reaction for fungi were observed (Group A, PAS staining). **(D)** Section of affected muscle in abdominal area was negative for bacteria (Group A, Gram staining). **(E)** Section of affected muscle in the abdominal area no mycobacteria were detected (Group A, ZN staining). **(F)** Section of a focal melanised change in the abdominal area was negative for fungi (Group A, PAS staining). **(G)** Section of affected muscle was negative for bacteria (Group D, Gram staining). **(H)** Section of affected muscle in Group D was negative for bacteria (ZN staining). **(I)** Affected muscle tissue in Group D was negative for fungi. (PAS staining). (A, B) scale bar = 10 µm. (D, F, G, H,) scale bar = 100 µm. (C, E, I) scale bar = 50 µm.

Histological investigations of non-affected regions from Groups A and D were negative. Myocytes and connective tissue did not display any pathological changes. Histological investigations of samples from Frøya and Hjelmeland (Groups B and C) did also not reveal any pathological changes (Fig 6.2.5A). Slight occurrence of connective tissue was observed around muscle fibres (Fig 6.2.5B). Inflammatory cell infiltrates and melano-macrophages were not observed.

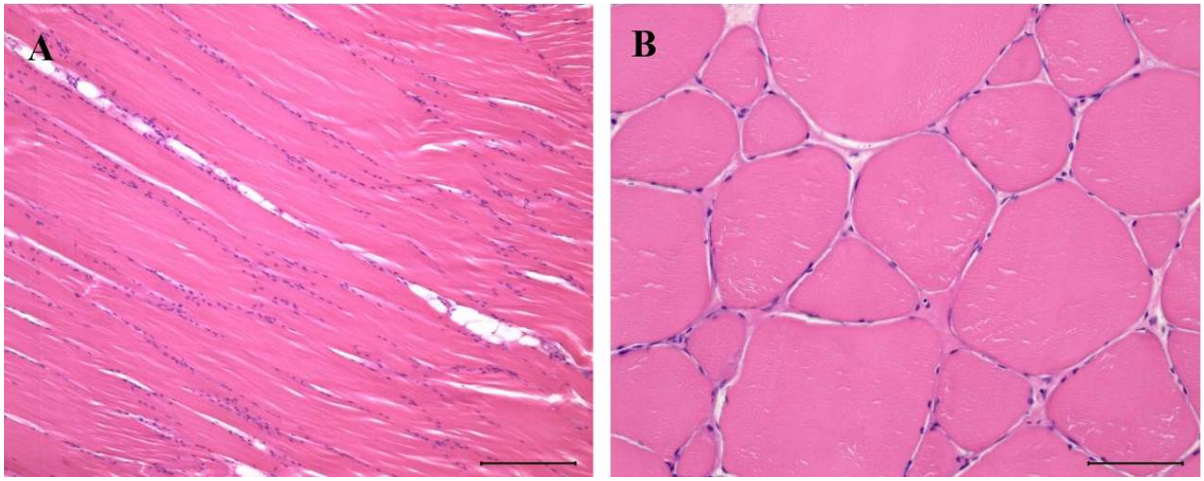

Figure 6.2.5: Histological analysis of muscle samples without any pathological changes from Frøya and Hjelmeland samplings (Groups B and C). **(A)** Longitudinal section of white skeletal muscle showed normal structure of myocytes without loss of striation. Nuclei were arranged along the periphery of myocytes. Scale bar = 100 µm. (B) Transverse section of white muscle. Myocytes with peripheral location of their nuclei and surrounded by slight amounts of connective tissue. Scale bar = 50 µm.

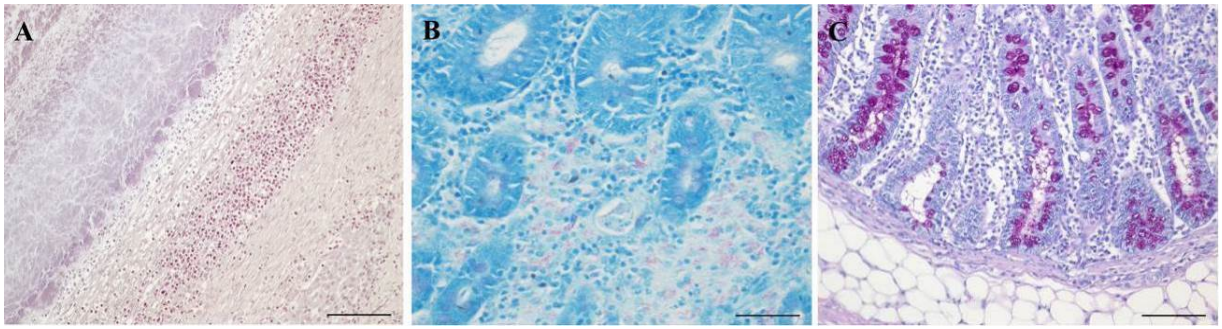

Figure 6.2.6: Positive controls for bacterial and fungi detection. **(A)** Sample of liver positive for bacteria (Gram staining). Scale bar = 100 µm. **(B)** Sample of affected intestine stained for Ziehl–Neelsen were positive. Scale bar = 20 µm. **(C)** Mucin cells (red) in salmon intestine (PAS staining). Scale bar = 50 µm.

6.3 Genetic

6.3.1 Frøya sampling

The material from Group A, the Frøya sampling, was analysed for the identification of PRV, SAV, IPNV, ISAV and *R. salmoninarum*. Samples (Group A) from focal melanised changes in regions II and none-affected region IV were screened for PRV, SAV, IPNV, ISAV and *R. salmoninarum*. PRV transcripts were identified in all individuals in Group A. Results of samples from region II, Group A, showed that individuals 4, 9, and 10 were PRV negative and the remaining samples were positive. Results from region IV showed that fish 1, 2, 6 and 8 were PRV negative and other fish 3, 4, 5, 7, 9 and 10 were PRV positive. Thus, the fish from the Group A Frøya sampling contained PRV in every single individual either in region II or control region IV. Genetic analysis for other agents including SAV, IPNV, ISAV and *R. salmoninarum* were not identified in Group A in any samples. Results are presented in Tab.6.3.1

Samples from Group B, Frøya sampling without focal melanised changes in dorsal musculature were analysed for the identification of PRV, SAV, IPNV and ISAV. Seven samples in region II and six samples in region IV in Group B, Frøya sampling were PRV positive. Other agents including SAV, IPN and ISA were not identified. Results for tests for PRV, SAV, IPNV and ISAV are presented in Tab.6.3.1. Since, Group B was sampled from the same Frøya fish population, analysis for *R. salmoninarum* was not performed for this group.

Spleen samples from the Frøya sampling were analysed for PRV, SAV, IPNV, ISAV and *R. salmoninarum*. Results showed that all of 18 spleen samples were positive for PRV and negative for SAV, IPNV, ISAV and *R. salmoninarum*. Results are presented in Tab.6.3.2.

6.3.2 Hjelmeland sampling

The material from diffuse melanised changes in dorsal musculature (region I) and not melanised dorsal musculature (region II) (Group D) Hjelmeland sampling were also analysed for PRV, SAV, IPNV, ISAV and *R. salmoninarum*. Results after screening showed presence of PRV in all samples including affected tissue region (I) and control region (II) without observable melanised

changes in dorsal musculature. Other agents including IPNV, ISAV and *R. salmoninarum* were not identified. Moreover, sample from control region (II) in the tested Group D (fish nr. 2) Hjelmeland sampling also contained SAV. Results of RT-PCR are presented in Tab.6.3.3.

Table 6.3.1: Results from RT-PCR analysis of the Frøya sampling (Group A and B). Positive – virus detected, ND – not detected. II – investigation region. IV – control region.

Group A Frøya	**PRV(II)**	**PRV(IV)**	**SAV(II, IV)**	**ISAV(II, IV)**	**IPNV(II, IV)**	***R.sal*(II, IV)**
Fish 1	positive	ND	ND	ND	ND	ND
Fish 2	positive	ND	ND	ND	ND	ND
Fish 3	positive	positive	ND	ND	ND	ND
Fish 4	ND	positive	ND	ND	ND	ND
Fish 5	positive	positive	ND	ND	ND	ND
Fish 6	positive	ND	ND	ND	ND	ND
Fish 7	positive	positive	ND	ND	ND	ND
Fish 8	positive	ND	ND	ND	ND	ND
Fish 9	ND	positive	ND	ND	ND	ND
Fish 10	ND	positive	ND	ND	ND	ND
Group B Frøya						
Fish 1	positive	positive	ND	ND	ND	
Fish 2	positive	ND	ND	ND	ND	
Fish 3	ND	ND	ND	ND	ND	
Fish 4	ND	ND	ND	ND	ND	
Fish 5	ND	ND	ND	ND	ND	
Fish 6	positive	positive	ND	ND	ND	
Fish 7	positive	positive	ND	ND	ND	
Fish 8	positive	positive	ND	ND	ND	
Fish 9	positive	positive	ND	ND	ND	
Fish 10	positive	positive	ND	ND	ND	

Table 6.3.2: Results from RT-PCR analysis of the Frøya sampling (Spleen). Positive – virus detected, ND – not detected.

Frøya sampling	**PRV**	**SAV**	**ISAV**	**IPNV**	***R.sal***
Spleen 1	positive	ND	ND	ND	ND
Spleen 2	positive	ND	ND	ND	ND
Spleen 3	positive	ND	ND	ND	ND
Spleen 4	positive	ND	ND	ND	ND
Spleen 5	positive	ND	ND	ND	ND
Spleen 6	positive	ND	ND	ND	ND
Spleen 7	positive	ND	ND	ND	ND
Spleen 8	positive	ND	ND	ND	ND
Spleen 9	positive	ND	ND	ND	ND
Spleen 10	positive	ND	ND	ND	ND
Spleen 11	positive	ND	ND	ND	ND
Spleen 12	positive	ND	ND	ND	ND
Spleen 13	positive	ND	ND	ND	ND
Spleen 14	positive	ND	ND	ND	ND
Spleen 15	positive	ND	ND	ND	ND
Spleen 16	positive	ND	ND	ND	ND
Spleen 17	positive	ND	ND	ND	ND
Spleen 18	positive	ND	ND	ND	ND

Table 6.3.3: Results from RT-PCR analysis of the Hjelmeland sampling (Group D). Positive – virus detected, ND – not detected. I – investigation region and II – control region.

Group D Hjelmeland	**PRV (II)**	**PRV(I)**	**SAV(I,II)**	**ISAV(I,II)**	**IPNV(I,II)**	***R.sal*(I,II)**
Fish 1	positive	positive	ND	ND	ND	ND
Fish 2	positive	positive	positive (I)	ND	ND	ND
Fish 3	positive	positive	ND	ND	ND	ND

7 Discussion

The present investigation of this thesis addressed three types of melanised changes in musculatures: focal melanised changes in dorsal musculature (Group A Frøya), focal melanised changes in abdominal musculature Groups A and B Frøya) and diffuse melanised changes in dorsal musculature (Group D Hjelmeland). The main hypothesis of the present study was that "*melanised changes in the dorsal musculature are of the same nature as changes in the abdomen*". Therefore, this work analysed and compared the tree types of melanised changes using different approaches.

7.1 Macroscopical investigation

Visual investigation identified both similarities and differences between the three types of melanised changes investigated.

In terms of location, focal melanised changes were located in two different places: dorsal musculature (Group A) and abdominal area (Groups A and B). However, diffuse melanised changes (Group D) were also located in dorsal musculature, similar to focal melanised changes (Group A).

The size of affected area was similar both in focal changes in dorsal (Group A) and in abdominal musculature (Group A and B). Diffuse melanised changes in dorsal musculature (Group D) were significantly larger than focal ones in both areas (Groups A and B).

On incision, all focal melanised changes both in dorsal and abdominal muscle tissue (Groups A and B) appeared to be similarly relatively superficial, while diffuse melanised changes (Group D) extended deep into the musculature.

On palpation, all the melanised muscle changes in compared groups were similar. Melanised muscle changes were indurated with an increased texture, especially in Group D from Hjelmeland.

7.2 Histological investigation

Histological investigation showed that observed changes and pathological processes in the focal melanised dorsal and abdominal muscle changes (Groups A and B) Frøya and diffuse melanised muscle changes (Group D) Hjelmeland samplings had significant similarities. The focal melanised changes dorsal musculature (Group A) in comparison with focal melanised changes in abdominal musculature (Group A and B) of Frøya sampling were identical as investigated by histology. In both cases, histological changes in melanised affected tissue were accompanied by the presence of loss of striation, inflammatory cell infiltrates, vacuolisation, and presence of melano-macrophages. Damage of myocyte structure had similarities and were observed as myocyte degeneration and necrosis in both locations of melanised changes. Both affected areas had similar changes in connective tissue. Severe fibrosis and inflammatory cell infiltrates were simultaneously present in all melanised changes of compared samples. Melano-macrophages were observed in both cases.

Histopathological differences between focal melanised changes (Group A) in dorsal musculature and focal melanised changes in abdominal musculature (Groups A and B) Frøya sampling were not detected.

Histopathological features of focal melanised changes in dorsal musculature (Group A) Frøya sampling and diffuse melanised changes in dorsal musculature (Group D) Hjelmeland sampling were also identical. Histological patterns in both Groups A and D were observed with similar changes in affected tissue. Changes in both comparable groups included loss of striation of myocytes, abundance of macrophage-like cells and melano-macrophages. Myocyte degeneration and necrosis appearing focal and multifocal changes were similar for the comparable Groups A and D. Macrophage-like cells and melano-macrophages were similarly present in these groups. In addition, severe fibrosis in connective tissue was detected, also these changes appeared similar. Vacuoles had the same structure and formation of melano-macrophages around the wall of vacuoles and were detected in both comparable Groups A and D.

No observable histological differences were detected when comparing changes in Groups A and D.

Histology of focal melanised changes in abdominal musculature (Groups A and B) Frøya sampling in comparison to diffuse melanised changes in dorsal musculature (Group D) Hjelmeland sampling also showed similarities. These were expressed as histologically not distinguishable changes in Groups A, B and D. Changes included loss of striation, degeneration and necrosis of myocytes and were similar in the different groups. Presence of macrophage-like cells and melano-macrophages were found. In addition, severe fibrosis and destruction of connective tissue with vacuolisation were also similar in comparable Groups A, B and D.

Despite focal and diffuse melanised changes in musculature macroscopically have different location and size, histological investigations did not detect any differences in affected tissues in comparable Groups A, B and D.

Histological analysis for bacteria and fungi did not reveal any presence of pathogens in Groups A, B and D.

7.3 Immunohistochemical investigation

Comparative analysis between samples of focal melanised changes in dorsal musculature Group A, focal melanised changes in abdominal Groups A and B, Frøya sampling and diffuse melanised changes in dorsal musculature Group D, Hjelmeland sampling revealed that all types of melanised changes in all groups were immunopositive for PRV. PRV – positive macrophage like cells and melano-macrophages had similar reaction in samples. PRV-positive cells were usually present in necrotic myocytes and dispersed in connective tissue. Highly PRV- positive cells in affected tissue appeared similar in all samples. In all affected samples PRV – positive cells were scattered throughout the chronically inflamed tissue.

Similarity observed by PCNA analysis. PCNA immune-reactive cells in affected tissues of all comparable Groups A, B and D were detected in the changes. The active division of

macrophage-like cells and melano-macrophages in necrotic tissue were observed that showed the presence of inflammatory processes in all comparable groups.

7.4 Genetical investigation

Results from genetical identification of PRV, SAV, IPNV, ISAV and *R. salmoninarum* of focal melanised changes in dorsal musculature (Group A) and samples without focal melanised changes in dorsal musculature (Group B) were similar. PRV virus was identified in both Groups A and B. Also, by RT-PCR, there was not identified any other agents including SAV, IPNV, ISAV and *R. salmoninarum* for Group A and SAV, IPNV and ISAV for Group B. The analysis for *R. salmoninarum* for Group B was not provided in the present study. Thus, the comparative analysis for both comparable Groups A and B is not completely accomplished. However, Group B is from the same Frøya fish population and it must be supposed that this control fish was also negative for *R. salmoninarum*.

Groups A and B have no differences in terms of RT-PCR. However, some samples with changes were PRV negative whereas control tissues from the identical individual were PRV positive. Since, the RT-PCR procedure is adapted for identifying viruses in blood and not muscle tissue. This is a possibility that the isolation has not optimal for these samples. As well acute or chronic stage of changes in tissue can lead to different results (Bjorgen et al., 2015). The melanised muscle changes in the present study were old and chronic (not acute) with a lots of connective tissue in samples.

For the identification of PRV, SAV, IPNV, ISAV and *R. salmoninarum* by RT-PCR there were also compered muscle samples from focal melanised changes in dorsal musculature in Frøya sampling and diffuse melanised changes in dorsal musculature Group D in Hjelmeland sampling. Groups A and D are positive for PRV virus. IPNV, ISAV and *R. salmoninarum* are not identified by RT-PCR in both groups.

However, the significant difference in comparable Groups A and D is that SAV agent was detected by RT-PCR in one sample of the Group D, in contrast to the Group A where SAV was not detected.

The present study was not accomplished a genetic analysis of focal melanised changes in abdominal musculature, therefore it is not possible to process a RT-PCR comparison of diffuse and focal types of changes.

High loads of PRV antigens in spleen samples were detected by RT-PCR. This parameter indicates that PRV infection spreads by blood to all tissues and organs. Since spleen samples were collected absolutely randomly and all samples were PRV-positive, it is likely that all fish in the population were positive.

8 Conclusion

The present study investigated focal and diffuse melanised changes in white musculature of Atlantic salmon. This work focused mostly on melanised changes in dorsal musculature, but focal melanised changes in abdominal musculature were also examined. The melanised changes had signs of focal and multifocal muscle degeneration and necrosis. The observations also demonstrated myocyte damage, including loss of striation, necrosis and destruction of connective tissue.

The initial hypothesis of this thesis was that "*melanised changes in the dorsal musculature are of the same nature as changes in the abdomen*". First sub-hypothesis was that "diffuse melanised dorsal changes are of a different nature compared with focal melanised changes in dorsal musculature". Results of the present study fully confirm main hypothesis and disprove the first sub-hypothesis. The histopathological research has shown that differences between focal melanised changes in dorsal musculature, diffuse melanised changes in dorsal musculature and focal melanised changes in abdominal musculature have a number of similarities. Change in connective tissue, inflammatory process within and around myocytes, loss of striations,

infiltration of macrophage-like cells, muscle degeneration, necrosis and melano-macrophages were observed in all histological samples. Connective tissue in all samples had inflammatory cell infiltrates. Myocytes showed chronic inflammation, myocytes with macrophage like cells surrounding and infiltrating were also observed. Degeneration and necrosis of myocytes were presented in all samples. Granulomatous inflammation in myotomes and myosepta were observed by histological examination. In addition, vacuolization in samples of all groups appeared similar. Vacuoles had the same structure and formation of melano-macrophages around the wall of vacuoles. Presence of melano-macrophages in all samples were also observed. All of these changes appeared identical in nature as examined with the present methods and were present in all examination groups. All these changes can be characterized as chronic polyphasic necrotizing myopathy with formation of melanin and granulomatous with degenerative process in muscle (Koppang et al., 2005, Larsen et al., 2012). Similar manifestations in all investigation groups indicates that the aetiology is common to all changes. The negative analysis to bacterial and fungi indicates that granulomatous and melanin formation have other nature of origin. The focal melanised changes in abdominal musculature were presented in the present study.

The second sub-hypothesis says that "melanised changes are invariably related to presence of PRV in muscle tissue". The present study cannot fully claim it, but the results can partially show that this is the truth. The immunohistochemical analysis of melanised changed tissue detected presence of a large number of PRV antigens. Although, some control tissue samples were collected from healthy-appearing muscle (Group B in Frøya sampling), genetic analysis RT-PCR showed that the most of them contained PRV. Moreover, 100% of all randomly selected spleen samples in the study population from the Frøya had a presence of PRV. PRV was detected in macrophage-like cells, melano-macrophages within granulomas and in less organised granulomatous tissues. The results of immunochemistry clearly tied PRV to the melanised changes. The fact that PRV agent was detected in both pigmented and not-pigmented samples generally indicates that PRV presence may not be sufficient to provoke melanised changes in the white muscle. However, status of infection, environmental and management factors may be of importance in this case.

At the same time, the results of this work indicated that the focal melanised changes arose as a consequence of chronic inflammation and necrosis. All the samples with focal melanised changes contained chronic inflammation and necrosis with presence of PRV. Granulomatous inflammation and formation of melano-macrophages indicates presence of foreign-body reaction or a chronic infection as immuno-response of organism (Koppang et al., 2005, Larsen et al., 2012). Chronic inflammation and necrosis, in turn, may be provoked by PRV virus. Thus, there is a strong connection between melanised changes and presence of PRV virus in muscle tissue in the investigated groups.

The melanised changes in the musculature were characterized as chronic inflammation with melanin production and granuloma formation. This characteristic indicates an ongoing degenerative process. In general, melanin is produced to protect against cell damage initiated by solar exposure (Wood et al., 1999, Mackintosh, 2001). Moreover, melanin production could serve as an additional defence mechanism to the innate immune system. It is proven to be an important immune response in arthropods, since it contributes to encapsulation and prevents dissemination (Cerenius and Söderhäll, 2004). These mechanisms are similar to granuloma processes (Larsen et al., 2012). Reactive oxygen species generated during melanisation are proven to be toxic to pathogens, but also to the arthropod host, causing a strict localisation of melanin to the inflammation site (Tang, 2009). In the present study, PRV antigen was detected at sites with granulomatous inflammation.

Granulomatous inflammation is dominated by cells of the monocyte-macrophage line, including macrophages, epithelioid cells and multinucleated giant cells (Tews and Pongratz, 1995). Granulomas generally serve to confine pathogens resisting destruction. In terms of the present research the well-organized granulomas with large central vacuoles and spars leukocyte response followed with a solid rim of elongated melano-macrophages. These cells can work as a seal, isolating the self from the possible foreign body and preventing repeated triggering of the immune response. In mammals, chronic inflammation with macrophage-lineage cells has been linked to non-disposable materials likely to accumulate in macrophages. Such material could consist of oil-based adjuvant (Hamilton, 2003). Additionally, chronic inflammation processes can be activated by alarm signals from damaged or stressed tissue. Thus, frequently reported

pigmented changes in fish muscle tissue can be classified as a granulomatous inflammation. (Koppang et al., 2005).

Comparative analysing the processes of chronic inflammation and melanin synthesis could show some similarities. Inflammation and melanogenesis are complementary and possibly could address the same issues (Sichel et al., 1997). They both are proven to be a reaction to external damaging and stressing factors (Sichel et al., 2002). It is possible that they could serve as a compensatory mechanism for the innate immune system in tissue with poor vacuolisation. Moreover, melanin production in granulomatous tissue could serve as protection against oxidation in chronic inflammatory conditions (Larsen et al., 2012).

In sum, the nature of the changes investigated in the present study were indistinguishable when investigated buy a series of morphological and transcriptional methods.

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