Master's thesis

NTNU
Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

Sunniva Westermann

The potential of cardiac troponins I and T, CK-MB and NT-proANP as biomarkers of cardiac stress in farmed Atlantic salmon (*Salmo salar*)

Master's thesis in MSBIO
May 2022

NTNU
Kunnskap for en bedre verden

Sunniva Westermann

The potential of cardiac troponins I and T, CK-MB and NT-proANP as biomarkers of cardiac stress in farmed Atlantic salmon (*Salmo salar*)

Master's thesis in MSBIO
May 2022

Norwegian University of Science and Technology
Faculty of Natural Sciences
Department of Biology

NTNU
Kunnskap for en bedre verden

Abstract

This thesis compared cardiac biomarker levels in Atlantic salmon (*Salmo salar)* from a net pen at Frøya before and after transportation to a processing plant at Averøy. The objective was to determine if stress due to crowding, pumping and transportation would lead to heart damage and subsequent elevation of levels of cardiac markers in blood. Levels of cardiac troponin T, cardiac troponin I, CK-MB and NT-proANP in salmon plasma were measured using Enzyme-Linked Immunosorbent Assay (ELISA). Additionally, histopathological analysis was done on heart samples from Averøy to assess the overall health status of the population.

Troponin T increased significantly from Frøya to Averøy. Although non-significant, levels of cardiac troponin I and CK-MB were also increased at Averøy compared to Frøya. The cardiac troponins and CK-MB in plasma samples from both Frøya and Averøy were all elevated above the human reference ranges indicating cardiac damage. Histopathological examination of heart tissue from fish sampled at Averøy revealed epicarditis, myocarditis and necrosis. Outbreaks of heart and skeletal muscle inflammation (HSMI) and pancreas disease (PD) along with possible cardiomyopathy syndrome (CMS) were confirmed in the net pen at the time of sampling. The year before sampling, salmon were subjected to mechanical delousing treatments which are known to induce stress responses. This explains why levels of cardiac markers were elevated above normal in salmon from the net pen before transport. The results from the blood analyses and histopathology point to poor cardiovascular health in the sample population.

Sammendrag

Denne oppgaven sammenlignet nivåer av hjertesviktsmarkører i Atlantisk laks (*Salmo salar*) fra en merd på Frøya før og etter transport til et slakteri på Averøy. Hensikten var å fastslå om stress i forbindelse med trenging, pumping og transport ville føre til skade på hjertet og påfølgende økning av hjertesviktsmarkører i blod. Konsentrasjon av hjertespesifikt troponin T, hjertespesifikt troponin I, CK-MB og NT-proANP i plasma fra laks ble målt ved å bruke Enzyme-Linked Immunosorbent Assay (ELISA). I tillegg ble det gjort en histopatologisk undersøkelse på hjertevev tatt fra Averøy for å undersøke den generelle helsetilstanden i populasjonen.

Troponin T økte signifikant fra Frøya til Averøy. Det var også en ikke-signifikant økning på troponin I og CK-MB i laks fra Averøy sammenlignet med Frøya. De hjertespesifikke troponinene og CK-MB i plasmaprøver både fra Frøya og Averøy var alle forhøyet i forhold til referanseverdiene for mennesker, noe som tyder på hjerteskade. Den histopatologiske undersøkelsen av hjertevevet fra laks fra Averøy viste epikarditt, myokarditt og nekroser. Sykdomsutbrudd av hjerte- og skjelettmuskelbetennelse (HSMB), pankreassykdom (PD) og mulig tilstedeværelse av kardiomyopatisyndrom (CMS) var påvist i merden før prøvetakingen. Året før ble laksen utsatt for flere mekaniske avlusninger, noe som er kjent for å utløse stressresponser. Dette forklarer hvorfor nivåer av hjertesviktsmarkører var forhøyet i merden før transport. Resultatene fra blodanalysene og histopatologien vitner om dårlig hjertehelse i populasjonen.

Preface

This project is a cooperation between Pure Norwegian Seafood, Møreforskning, NTNU and myself. The goal of this project is to help develop new diagnostic tools for the early diagnosis of myocardial injury in Atlantic salmon. I am very thankful I got to be a part of this project and all the new knowledge I have gained about fish farming and the challenges it faces, and I hope that I will be able to gain more experience in the future. I hope my thesis will provide additional useful information in the research field of cardiac biomarkers in salmon. I would like to thank my supervisor Morten Høydal, head of Group of Molecular and Cellular Cardiology at the faculty of medicine and health, dept. of circulation and medical imaging, for providing valuable feedback on my thesis. I would also like to thank my co-supervisor Svein Erik Gaustad from Møreforskning, Anders Martinussen from Pure Norwegian Seafood and Lillian Jørgensen from Åkerblå for carrying out the blood- and organ sampling at Frøya and Averøy and for providing information I needed for my thesis, and Ragnhild Røsbjørgen at St. Olav's Hospital who helped me analyse the blood samples. Lastly, I give my thanks to Barbo Klakegg from Åkerblå for answering my questions, Mona Gjessing from the Norwegian Veterinary Institute for carrying out the histopathological analysis, and Måsøval AS who provided the fish from their net pen at Frøya.

Table of contents

List of figures

List of tables

List of abbreviations

ANP	Atrial natriuretic peptide
BNP	Brain natriuretic peptide
CK	Creatine kinase
CNP	C-type natriuretic peptide
CMS	Cardiomyopathy syndrome
cTnI	Cardiac troponin I
cTnT	Cardiac troponin T
ELISA	Enzyme-Linked Immunosorbent Assay
HSMI	Heart and skeletal muscle inflammation
LDH	Lactate dehydrogenase
MI	Myocardial infarction
PD	Pancreas disease
PMCV	Piscine myocarditis virus
PRV	Piscine orthoreovirus
SAV	Salmonid alphavirus
SEM	Standard error of mean

Introduction

Health status and diseases in farmed salmon

According to the fish health report from 2021 by the Norwegian Veterinary Institute (Sommerset *et al*., 2022), health and welfare of Norwegian farmed salmon is far from optimal, and health issues is a serious problem. Mortality in salmon bigger than 3 grams in the freshwater phase in 2021 was reportedly 33.4 million, while mortality for mature salmon in the seawater phase was 54 million, which corresponds to 15.5%. This is higher than ever. The high mortality is caused by a complex interaction of several factors, including infectious diseases, poor environmental conditions, injuries, trauma and a lack of physiological adaptation. Measures seem to not be working, despite many companies deploying great resources for a more sustainable production and for battling salmon louse and diseases.

The fish in the Norwegian aquaculture industry are currently living with several diseases (Sommerset *et al*., 2022). The top 3 viral diseases in 2021, like the previous year, are cardiomyopathy syndrome (CMS), heart and skeletal muscle inflammation (HSMI) and pancreas disease (PD). All of these affect the heart of the fish. Fish health personnel and inspectors in the Norwegian Food Safety Authority ranked CMS as the most important cause of mortality in the seawater phase in 2019, 2020 and 2021, making it the most serious viral disease.

HSMI affects farmed Atlantic salmon in the seawater phase and mainly affects the heart and red skeletal muscle (Yousaf *et al.*, 2013). The disease was first discovered in 1999 and is caused by Piscine orthoreovirus (PRV) (Sommerset *et* al., 2022). Low mortality (20%) but high morbidity (100%) has been reported (Yousaf *et al.*, 2013). Histopathological finding in the hearts of HSMI-infected fish include epicarditis, mononuclear cell infiltration in spongiosum and compactum of the ventricle and necrotic myocytes. PD is a disease caused by Salmonid alphavirus (SAV) and was first described in Atlantic salmon in Norway in 1989 (Yousaf *et al.*, 2013; Sommerset *et al*., 2022). It is a major economic and welfare problem in Europe. Mortality ranges from 1-42% (Yousaf *et al.*, 2013). Infected fish develop inflammatory lesions predominantly in the heart and pancreas in the acute phase, followed by additional lesions in muscle in the sub-acute phase, then a chronic phase where lesions in muscle dominate, and lastly a recovery phase. Cardiac pathology includes cardiac myopathies

and epicarditis. CMS is a cardiac disease that mainly affects the atrium and trabecular ventricle but does not affect skeletal muscle (Yousaf *et al.*, 2013). It was first discovered in 1985 in farmed Atlantic salmon in Norway and is caused by Piscine myocarditis virus (PMCV) (Sommerset *et al*., 2022). Like HSMI, it causes myocarditis (Yousaf *et al.*, 2013). Histopathologic findings include necrosis and inflammation of trabecular myocardium of the atrium and ventricle, epicarditis and infiltration of mononuclear leukocytes. The atrium and sinus venosus have been known to rupture in terminal stages. The onset of the disease usually happens late during the production cycle, which is an economically unfavourable time. This is because most of the costs have already been spent at this stage (Sommerset *et al*., 2022) The most common findings of CMS, HSMI and PD are necrosis and inflammatory cells in the heart (Yousaf *et al.*, 2013).

Effect of stress on infectious diseases

Repeated stressors or prolonged exposure to stress have detrimental effects on necessary life functions like growth, development, disease resistance, behaviour and reproduction, mostly due to the energetic cost of the stress response (Schreck *et al.*, 2016). A persisting stressor will normally lead to suppression of the immune response because of the lack of resources to support highly demanding processes like cell division and protein synthesis. All types of stressors may affect the immune system as long as mechanisms involving energy supply or metabolic pathways related to key immune molecules are affected. Stress can render fish vulnerable to infections and diseases. Diseases typically appear in fish and other animals after they have been subjected to stressful situations. Stressors related to husbandry practices, aggression, behavioural challenges, environmental pollution and dietary alternations are known to have immune-suppressive effects, among others (Schreck *et al.*, 2016).

Various stressful episodes are described as risk factors for outbreaks of CMS, HSMI and PD (Sommerset *et al*., 2022). Especially pumping of fish from a net pen into a live fish carrier produce large amounts of stress for the fish. Stress can make latent infections develop into clinical disease and increase mortality. Fish with CMS handle stress poorly because the large amounts of inflammatory cells in the heart, especially the atrium, makes the wall of the heart fragile (Sommerset *et al*., 2022). Handling related to transport can produce a severe stress response in salmonids and cause injuries to skin and mucus (Iversen, Finstad and Nilssen, 1998; Sommerset *et al*., 2022). Fish have to be crowded before they can be transported, which is a considerable welfare risk in itself. It can cause harmful changes in water quality like

decreases in oxygen saturation or oversaturation of other gases (Sommerset *et al*., 2022). Stress due to crowding can have deleterious effects on the disease resistance in fish. In sea bass, rates of cytotoxicity and chemiluminescence activities were lowered, leading to immunocompetence (Schreck *et al.*, 2016). Sea bream and red porgy reared under high densities had reduced levels of complement proteins and elevated levels of cortisol. Crowding in sea bream also depressed complement proteins and phagocytic activities in head-kidney leukocytes and activated migration of cells into the blood (Schreck *et al*., 2016). Iversen, Finstad and Nilssen (1998) showed that both hauling and transport caused stress in Atlantic salmon smolts. There was a significant increase of cortisol levels in the smolts 1 hour after hauling and transport, and the levels stayed elevated 48 hours after transport. Capture of smolts appeared to be the most severe stressor, more stressful than the transport process itself.

Stress and crowding can influence the oxygen consumption of the fish and lead to hypoxia which acts as an additional stressor, especially in combination with disease as it can compromise energy resources needed for the immune response (Lund *et al.*, 2017). Increased cardiac output and induced erythropoiesis are two important responses to hypoxia. Since PRV induces lesions in cardiac muscle, cardiomyocyte contractility and cardiac function might be impaired in HSMI-infected fish, rendering them vulnerable as they have a lesser ability to respond properly to hypoxia (Lund *et al*., 2017). Moreover, induced erythropoiesis may provide PRV with more erythrocytes to potentially infect in addition to possibly affecting their ability to transport oxygen. Additionally, epicarditis and inflammation of the myocardium associated with HSMI might cause further negative consequences for cardiorespiratory functions and blood transport (Lund *et al.*, 2017).

In an experiment by Lund *et al*. (2017), HSMI-infected Atlantic salmon had impaired hypoxia tolerance and cardiac performance during peak levels of PRV and HSMI lesions, but periodic exposure to hypoxia during disease development diminished these effects. At 10 weeks post infection (WPI) when heart pathology reached peak level, fish infected with HSMI were less tolerant to hypoxia compared to both the control group and HSMI-infected fish that had previously been exposed to periodic hypoxic stress. The red blood cells of HSMI-infected salmon also had a reduced blood oxygen binding affinity. The transcriptional factor HIF1α, which is upregulated under hypoxia, has been identified in moderate levels in PD-affected hearts, but in low levels in CMS- and HSMI-affected hearts of Atlantic salmon (Yousaf *et al.*, 2013). This suggests that the PD-affected hearts were hypoxic. On the contrary, a different

study concluded that constant hypoxia did not increase the severity of lesions or progress of PD (Andersen, Hodneland and Nylund, 2010).

Cardiac markers

The term "biomarker", short for "biological marker", was defined by the National Institutes of Health Biomarkers Definitions Working Group in 1998 as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention." (Strimbu and Tavel, 2010). A biomarker could be anything from pulse or blood pressure to laboratory blood tests and tissue tests. Cardiac biomarkers are important for a timely, accurate diagnosis of acute coronary syndrome as well as management and prognosis (Jacob and Khan, 2018). A good cardiac marker must be specific to myocardial damage.

Creatine kinase (CK) is an enzyme that catalyses the reversible transformation of creatine and ATP to phosphate and ADP (Aydin *et al.*, 2019). It is present in mitochondria and in the cytosol of myocytes. CK is a dimeric enzyme consisting of two subunits, M and/or B, and exist as three isoenzymes: CK-BB, CK-MM and CK-MB. CK-MB is a biomarker of myocardial necrosis. Around 20% of the total CK in the myocardium is in the form of CK-MB according to Aydin *et al*. (2019), or nearly 30% according to Jacob and Khan (2018), making it a sensitive and specific cardiac biomarker. CK-MB appears in the bloodstream 4-6 hours after onset of chest pain and peaks 10-12 hours after myocardial infarction (MI). The best time for detection is 6-48 hours, after which the blood levels return to normal (Jacob and Khan, 2018). A rise of 5% or more of the total CK-activity indicates myocardial damage, but the mass of CK is found to be of better diagnostic value than the enzyme activity of CK or CK-MB. MI is unlikely if CK is not elevated in patients with chest pain and failure of elevated CK levels to fall indicates that there is an extension of the infarct.

The cardiac troponins are also biomarkers of myocardial necrosis (Jacob and Khan, 2018). Troponins are contractile proteins in muscle cells. Cardiac troponin I (cTnI) and cardiac troponin T (cTnT) are specific to the myocardium and are highly sensitive for myocardial damage. They are the most commonly chosen biomarkers. They are present in the circulation 3-9 hours after MI (Jacob and Khan, 2018). Cardiac troponin I increases in 4-6 hours, peaks at 12 hours and returns to normal in 3-10 days. Cardiac troponin T stays elevated for 12-48

hours and returns to normal in 10 days. MI is highly unlikely if troponins are not elevated (Jacob and Khan, 2018).

Natriuretic peptides are biomarkers of haemodynamic stress (Jacob and Khan, 2018). Natriuretic peptides are a group of proteins that are structurally similar but genetically different. They are involved in regulating blood pressure by sodium and water excretion and also inhibit cardiac hypertrophy and remodelling. This family includes α-atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP). Increased blood flow into the ventricle creates pressure and stretching of the ventricular wall, which induces transcription of natriuretic peptides. Natriuretic peptides help reduce blood pressure by modulating diuresis, natriuresis and vasodilation, and by inhibition of renin and aldosterone.

ATP is synthesized and secreted from the heart in response to atrial stretch. The 28-amino acid peptide α-ANP is the major molecular form of circulating ANP in humans (Nakagawa, Nishikimi and Kuwahara, 2019). The precursor for ANP is the 151-amino acid preproANP. The 25-amino acid signal peptide is removed to form the 126-amino acid proANP (γ-ANP) which is the tissue form of the hormone. ProANP is rapidly cleaved upon secretion, giving rise to the biologically active C-terminal 28-amino acid peptide and the biologically inactive 98-amino acid N-terminal proANP (NT-proANP), which both circulate in equimolar amounts (Volpe, Carnovali and Mastromarino, 2015). NT-proANP is preferred to ANP as a cardiac biomarker due to its longer half-life (60-120 minutes) in the circulation (Yandle *et al.*, 1986). ANP has a half-life of 3-4 minutes and is rapidly cleared. ANP is expressed in both the atrium and ventricle, but levels of ANP in the atrium is 250-1000 times than in the ventricle (Nakagawa, Nishikimi and Kuwahara, 2019).

Previous research on cardiac markers in fish

Research on fish cardiac biomarkers is limited, but CK, lactate dehydrogenase (LDH), troponins and natriuretic peptides like salmon cardiac peptide cSP have been highlighted as potential candidates. Special relevance has been placed on CK and LDH in salmon, although LDH is not specific to heart damage (Smith, Walter and Walker, 2013; Costa *et al.*, 2021). One study by Yousaf and Powell (2012) found significantly higher levels of CK and LDH in HSMI-sick fish compared to healthy fish, and the enzyme levels also correlated significantly with necrosis and inflammation scores. The diseased fish showed the histopathological

changes in heart and skeletal muscle characteristic for HSMI. The highest mean LDH values were present at the earlier stage of the disease, while the highest levels of CK were present in the acute phase. On the other hand, in fish chronically sick with CMS, the levels of enzymes were equal to or lower than those of non-diseased fish, and there was no correlation between histopathology scores and levels of enzymes. This was despite CMS-sick fish showing high inflammation scores compared to fish that were not diagnosed with CMS, and despite histopathology showing that more lesions were present in the heart than the skeletal muscle. This indicate that the elevated levels in HSMI-sick fish were due to myopathy.

CK levels are elevated in PD in salmon (Rodger *et al.*, 1992). This was supported by Braceland *et al.* (2013), who found a correlation between serum CK levels and lesions from PD, but the CK could have come from both muscle lesions and heart lesions. Neither Rodger *et al.* (1992), Yousaf and Powell (2012) or Braceland *et al.* (2013) specified what kind of isoenzyme they measured. Costa *et al*. (2021) compared differences in protein expression in serum pools between clinically healthy fish and CMS-sick fish and identified a creatine kinase M-type protein in CMS sera but not in sera from clinically healthy fish. They stated that since CMS does not cause skeletal muscle lesions, it is likely that this protein was of cardiac origin. However, this study did not do a histopathological examination of skeletal muscle, so skeletal muscle damage cannot be ruled out. Research is lacking regarding the CK-MB heterodimer in salmon.

It has been documented that ANP is released from the hearts of Atlantic salmon infected with HSMI (Takle *et al.*, 2006; Takle and Andersen, 2007), and from perfused Atlantic salmon hearts subjected to increased mechanical load (Tervonen *et al.*, 2001). A study by Frisk *et al.* (2020) found evidence of intensive smolt production with fast growth rates inducing maladaptive cardiac remodelling and pathophysiological hypertrophy along with molecular indications of pathology. Fast smolt had a significantly higher abundance of ANP mRNA in ventricular tissue compared to slow smolt. BNP mRNA levels were also elevated, although not significantly. Upregulation of genes associated with mammalian pathological hypertrophy are also found in hypertrophic fish hearts following chronic cold or stress (Keen *et al.*, 2016). Pathological and physiological hypertrophy are differentiated by markers of myocardial stretch, including ANP and BNP.

Costa *et al.* (2021) stated that they did not identify troponins in their proteome study and attribute this to serum being a complex biological sample with proteins being present over a vast dynamic range, and the sensitivity of the technique that was used (liquid chromatography–electrospray ionization–tandem mass spectrometry). Info about cardiac troponins in salmon appears to be scarce, as most research seems to focus on troponins released from skeletal muscle.

Hypothesis

Fish that are transported from the net pen to the processing plant will be subjected to several procedures that are known to be stressful, like crowding and pumping, in addition to hypoxia. These factors can induce heart damage, especially if the salmon are already weakened from diseases. The main hypothesis for this study is that fish that have been transported to the processing plant have higher levels of cardiac markers than fish from the net pen.

Materials and methods

Ethics

All procedures on salmon were performed under general anaesthesia in compliance with ethical guidelines and legislation, by qualified veterinarians and fish biologists. This was a non-recovery experiment, meaning that the fish were euthanized after being anesthetised.

Funding

The project covered all costs, including costs related to sampling of biological material at Frøya and Averøy, as well as analyses performed at NTNU laboratory and at the Norwegian Veterinary Institute.

Health status of the population

During the time of sampling, there were confirmed outbreaks of HSMI and PD with clinical disease in net pen number 208 where we took the samples. There were also some cases of bacterial sores. Right after sampling, CMS was discovered in net pen number 207, the “sister group” of net pen number 208. Original net pen number 107 was split into 2 net pens in week 48 of 2020, namely net pen 207 and 208. There is reason to believe that there was CMS also in net pen 208. Mortality was moderate to high during the period of sampling. During the

transition to the seawater phase the fish were treated with Slice vet, a type of louse treatment in feed form containing the drug emamectin benzoate. Additionally, fish were treated 3 times with FLS delouser, a type of mechanical delousing treatment, in week 35, 42 and 53 of 2020.

Transport process from Frøya to Averøy

Fish are not fed 2-5 days prior to transportation from net pen to processing plant. Starvation is done in order to empty the bowel and reduce the metabolism and the oxygen consumption of the fish, ensuring good water quality during crowding and in the live fish carrier. The fish will also be calmer and tolerate the transport process better. Starvation is also done for quality- and hygiene reasons for when the fish arrive at the processing plant.

The net pen is raised and a sweep net is inserted into the net pen where it collects a given amount of fish, not too many as this would increase stress and damages to the fish. The sweep net is then dragged towards a hose that pumps the fish into a live fish carrier. The live fish carrier transports the fish to the processing plant under surveillance. The transportation process takes about 5-6 hours from Bukkholmen, Frøya to Averøy. The environment inside the carrier is controlled either by an open solution with intake of surrounding water, or by a closed solution where the fish are kept in the same water where factors like O_2 and CO_2 are controlled. Upon arrival, fish are pumped into a waiting net pen, where they spend a maximum of 6 days and are observed for welfare reasons. The fish in this study spent less than 24 hours in the waiting net pen. The purpose of the waiting net pen is for the fish to relax and calm down, which is important for good quality during slaughter. Fish are then pumped from the waiting net pen in the same way they were pumped from the original net pen. They are pumped into the processing plant where they are sedated by an electric shock to the head.

Sampling protocol

Figure 1: Blood sampling from the caudal vein. Picture taken at Pure Norwegian Seafood's processing plant at Averøy February 23, 2021.

20 fish were taken from net pen number 208 belonging to Måsøval AS at Bukkholmen, Frøya. Samples were taken February 22, 2021. The fish were lured with food into a large dip-net that was submerged in the net pen. Fish were then netted into an anaesthesia bath with Benzoak vet. 20% containing benzocaine, dosage according to instructions in the manual. Fish were kept in the anaesthesia bath until there were no signs of consciousness. Weight and length were measured (Appendix 1). Greiner bio-one 21G vacuettes were used to sample blood from the caudal vein, needle was inserted midline posterior to the anal fin. Blood was collected in Plasma K2EDTA vacutainers and CAT Sep Clot activator vacutainers. Blood samples were left to rest for a minimum of 5 minutes before they were centrifuged at 5000 rpm for 5 minutes. Plasma and serum samples were pipetted into cryotubes, 2x plasma tubes and 2x serum tubes for each fish. The heart was dissected out and a piece of the atrium and ventricle were put in separate cryotubes. Standard organ package (gills, heart, liver, pyloric caeca, spleen, kidney, skin and muscle) was taken out and organs were put on formalin.

The following day, on February 23, 2021, 14 fish were sampled at Pure Norwegian Seafood's processing plant at Averøy after they had been transported in the live fish carrier from net pen 208 at Bukkholmen, Frøya. The fish came from the same net pen for optimal research design. The original plan was to use the same sample size of 20 fish, but 6 of the fish had to be discarded as there was some trouble taking blood samples from them. Fish were sedated with an electric shock to the head which is standard procedure at the processing plant, and the sampling procedure of blood and organs was the same as of Frøya. Organs on formalin taken at Frøya and Averøy were kept cool below 4 ℃ during the first week and afterwards in temperatures of 10-15℃ up until the organs from Averøy were sent to the Norwegian Veterinary Institute for histopathology in October 2021. The organs sampled at Frøya were not used for histopathology.

After sampling was done, the cryotubes with serum, plasma and heart tissue were put in liquid nitrogen and transported to St. Olav's Hospital by car. This took around 2 and a half hours from Frøya to St. Olav's Hospital and around 4 hours from Averøy to St. Olav's Hospital. All samples were frozen to -80 degrees Celsius.

Plasma analysis

Enzyme-Linked Immunosorbent Assay (ELISA) was used for the detection of cardiac markers in blood. It is a labelled immunoassay that is used to detect and quantify antibodies, antigens, proteins and glycoproteins in a sample (Alhajj and Farhana, 2022). This method utilizes the interaction between antibodies and antigens in order to produce a measurable result. There are several types of ELISA and depending on the type the following are required: a coating antibody/antigen, analyte/antigen, a primary and/or secondary detection antibody, buffer, wash, and substrate/chromogen. The primary detection antibody is specific to and only binds to the antigen of interest, while the secondary detection antibody is an enzyme-conjugated antibody that binds to the primary antibody. There are 4 main steps in ELISA: coating with antibody or antigen, blocking, detection, and final read (Alhajj and Farhana, 2022). In between these steps the plate is washed with a buffer and a non-ionic detergent for the removal of unbound material. Blocking of unbound protein binding sites is done to prevent background noise and unspecific binding. Detection is carried out by adding a substrate that can generate colour. A serial dilution of concentrations is usually placed in the wells. The data from these are used to make a standard curve with concentration plotted in log

scale on the x-axis and absorbance in a linear scale on the y-axis. The intensity of the colour is proportional to the concentration of the specimen in the sample (Alhajj and Farhana, 2022). In this thesis all the kits used were sandwich ELISAs. In this method, capture antibodies are coated onto the wells of the plate and bind the antigens which in turn bind the detection antibodies. The antigens are "sandwiched" between two layers of antibodies, hence the name.

The "Fish high sensitivity cardiac troponin I ELISA kit" and "Fish high sensitivity cardiac troponin T ELISA kit" were ordered from Bioassay Technology Laboratory, Shanghai, China. Intraassay CV (coefficient of variation) was < 8% and interassay CV was <10% for both kits. Samples had been frozen for 2 months prior to analysis.

The "CKMB Human ELISA kit" was ordered from Thermo Fisher, Waltham, United States. According to a representative for Thermo Fisher, this human kit was expected to work on fish because of the high sequence homology (~83 %) between salmon and the antigen sequence of the antibody included in the kit. We also looked up sequence homology between human and salmon CK-MB by using Constraint-based Multiple Alignment Tool (COBALT) by the U.S. National Library of Medicine and found out they were well conserved (Appendix 6). Intraassay CV was < 10% and interassay CV was <12%. Samples had been frozen for 6 months prior to analysis.

The "NT-proANP ELISA" kit was ordered from Biomedica, Wien, Austria. The kit is listed as suitable for human, rat and mouse samples, but was also expected to work on salmon because it had already been used in research on salmon (Kristensen *et al.*, 2012). According to Cousins *et al.* (1997) and Potter *et al.* (2009), there is a high degree of homology between ANP in vertebrates. Intraassay CV was ≤ 5% and interassay CV was ≤ 9%. Samples had been frozen for 10 months prior to analysis.

Plasma samples were analysed using a DYNEX DS2 automated ELISA machine for the quantitative detection of cardiac troponin I and T, CK-MB and NT-proANP. Serum samples were not used as they were treated incorrectly, not being able to clot for long enough. Samples were taken out of the freezer and thawed completely before analysis. Plasma from each individual fish were analysed in duplicates, and samples from Frøya and Averøy were analysed in the same run. Procedures were done according to the manual for each kit.

Testing for interference of haemolysis

We used the FLUOstar Omega microplate reader from BMG LABTECH to measure the absorbance in undiluted plasma. Samples were initially diluted but this provided no signal different from the background noise, so we had to try again with undiluted samples. Plasma was measured in duplicates except for plasma from fish number 1 from Frøya, because of the limited volume. 200 µL of sample was pipetted into wells on a microplate. Absorbance was read at wavelengths 543 nm and 576 nm, the tops in the absorbance curve of oxyhaemoglobin in salmon, and 559 nm, the peak in the absorbance curve for deoxyhaemoglobin (Sone, Olsen and Heia, 2012). The values for wavelengths 559 and 576 gave the strongest signals and were nearly identical. We chose to use data from wavelength 559, which is illustrated in Appendix 5. This was done to see if there was any linear relationship between levels of cardiac markers and levels of haemoglobin.

Histology

Heart tissue and gill tissue from fish sampled at Averøy were sent to the Norwegian Veterinary Institute for histopathological analysis.

Statistics

All statistical analyses and graph plotting were done using Graphpad Prism version 9.3.1 and Microsoft Excel version 2204. Samples taken from the net pen before (Frøya) and after transportation (Averøy) were compared using Student's unpaired t-test, assuming equal variance. Standard error of the mean (SEM) was calculated. Significance level was set at $p<0.05$.

Results

Cardiac markers

We used CK-MB and cardiac troponins I and T, which are markers of myocardial necrosis, and NT-proANP which is a marker of haemodynamic stress. We found that the mean troponin T concentration was significantly increased in blood samples from Averøy compared to Frøya (84.4 ± 3.5 ng/L for Frøya and 97.2 ± 5.0 ng/L for Averøy (p=0.036)) (Figure 2). We found a slight increase in mean troponin I concentration in blood samples from Averøy compared to Frøya (41.5 ± 1.2 ng/L for Frøya and 43.7 ± 1.7 ng/L for Averøy (p=0.27)) (Figure 2). Same as for troponin I, we found a trend of increased levels of CK-MB in blood samples from Averøy compared to Frøya (22.5 ± 1.4 ng/mL for Frøya and 27.3 ± 3.6 ng/mL for Averøy (p=0.18)) (figure 3). We found that the means of NT-proANP levels were identical in blood samples from Frøya and Averøy (0.46 ± 0.1 nmol/L for Frøya and 0.46 ± 0.1 nmol/L for Averøy (P=0.96)) (figure 4). NT-proANP values for 8 fish from Frøya and 6 fish from Averøy were lower than the detection limit of the ELISA kit and are therefore missing. See Appendix 3 for individual values for all fish.

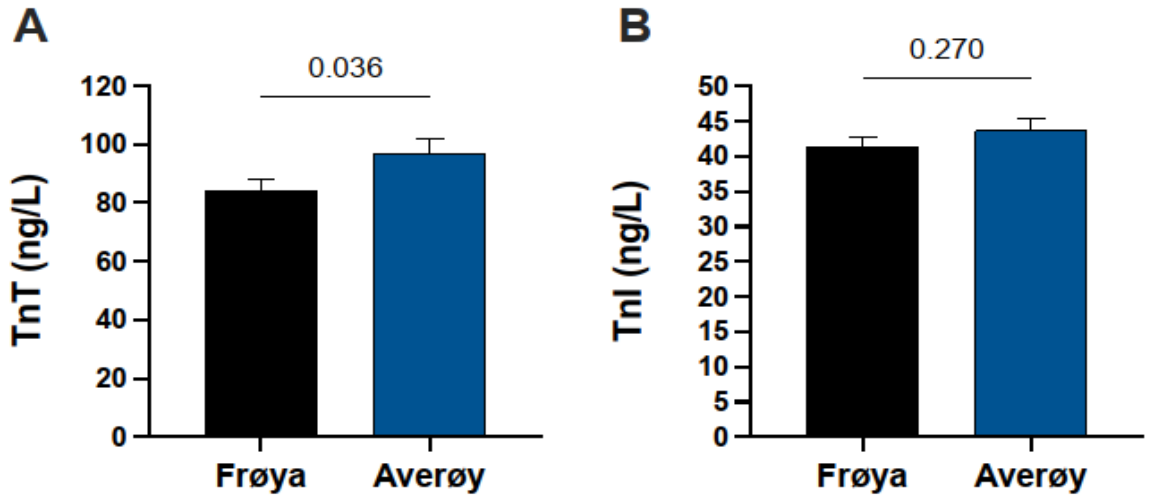


Figure 2: Levels of cardiac troponin I (TnI) and cardiac troponin T (TnT) in salmon plasma from Frøya (N=20) and Averøy (N=14). Data presented as mean and SEM. P-values indicated in figure.

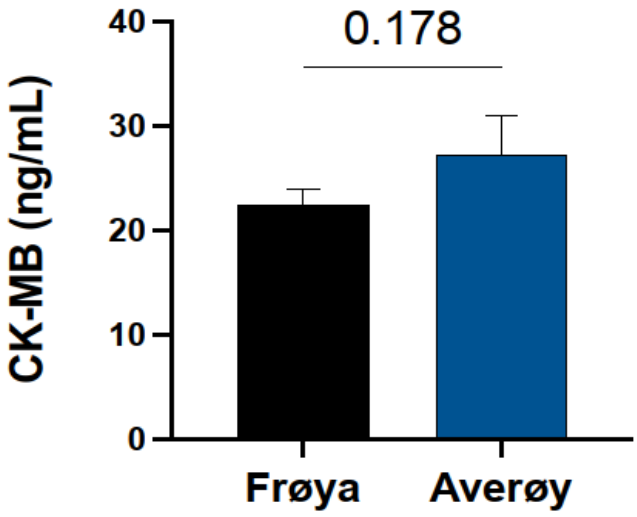


Figure 3: Levels of CK-MB in salmon plasma from Frøya (N=20) and Averøy (N=14). Data presented as mean and SEM. P-value indicated in figure.

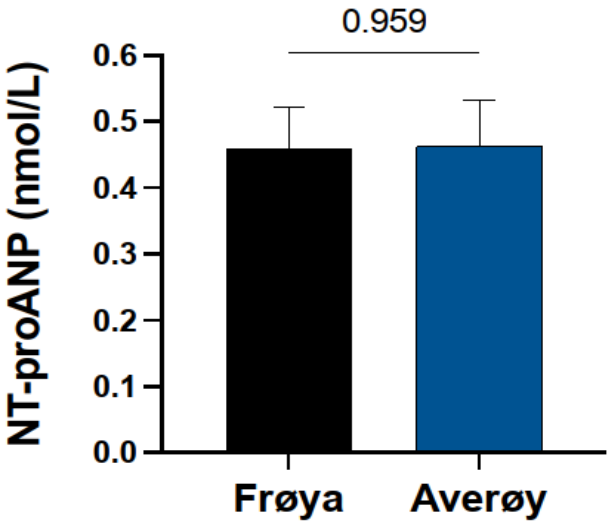


Figure 4: Levels of NT-proANP in salmon plasma from Frøya (N=12) and Averøy (N=8). Data presented as mean and SEM. P-value indicated in figure.

Histology

Both heart and gill tissue from our study population were sent to the Norwegian Veterinary Institute for histology, but since this thesis focuses on heart pathology, only the results from the heart histopathology are provided. The histopathology consists of tissue sampled from fish from Averøy and served as an additional analysis in order to assess the population's general health status in the net pen.

The histopathology revealed a high degree of heart pathology in the study population (figure 5). The most prevalent diagnosis is epicarditis, occurring in 93% of salmon whereas 14% of them had mild epicarditis and 79 % moderate to severe. Myocarditis, mostly in the spongiosum but also in the atrium and compactum, was found in 50% of salmon. 7% of these had mild myocarditis while 43% had moderate to severe myocarditis. 50% had necroses in the myocardium. For comments about each fish, see Appendix 4.

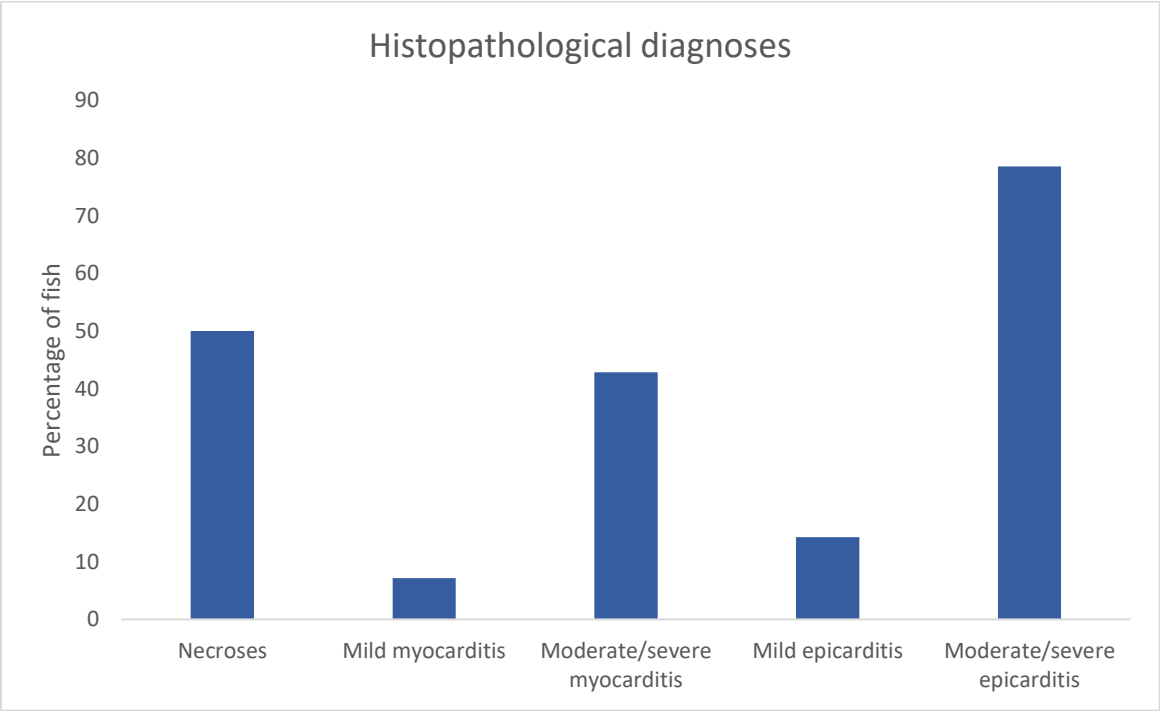


Figure 5: Histopathological diagnoses displayed as the percentage of salmon they occur in (N=14).

Discussion

Troponin T was significantly increased in fish sampled at Averøy after transportation compared to fish sampled from Frøya (p=0.036). Although not significantly different, there was a tendency of elevated levels of troponin I and CK-MB in fish from Averøy. There was no difference in NT-proANP levels between the groups. Histology showed that every fish from Averøy had heart pathology to varying degrees (Appendix 4), and that conditions like epicarditis, myocarditis and necrosis were prevalent (figure 5). The histopathological findings fit well with the confirmed disease outbreaks in the net pen at Frøya as well as the literature

on histopathologic changes related to HSMI, PD and CMS. Epicarditis, which is commonly found in all 3 diseases, was the most prevalent condition.

Standard levels in humans compared to levels in our population

Even small increases in troponins can indicate heart damage. Troponin I levels in humans above 40 ng/L indicate heart problems (Mariathas *et al.*, 2019). Troponin T levels are considered to be elevated when they are above 14 ng/L for women and 22 ng/L for men (Peacock *et al.*, 2018). According to our results troponin I levels were slightly higher than what is considered normal, with a mean of 41.5 ng/L for Frøya and 43.7 ng/L for Averøy. This means that the troponin I levels are just high enough to indicate some heart damage. However, the troponin T levels in fish were much higher than the normal levels, being 84.4 ng/L for Frøya and 97.2 ng/L for Averøy.

Normal levels of CK-MB in humans are equal to or below 5 ng/mL (Lindsey *et al.*, 2011). Every single fish from both locations had CK-MB levels higher than this reference range, with the average being 22.5 ng/mL for Frøya and 27.3 ng/mL for Averøy, which also points to heart damage.

Different standard levels of NT-proANP have been reported. One study by Loke *et al.* (2003) identified the standard levels for healthy women to be 0.42 nmol/L and for healthy men to be 0.37 nmol/L. In a different study by Büttner *et al.* (2022), the median level of NT-proANP in a healthy control group consisting of both men and women was 5.4 ng/mL, which is the same as 0.43 nmol/L according to the conversion factor in the NT-proANP ELISA kit from Biomedica. However, a limitation to the above studies is that they only included middle aged and older men and women. Out of the fish with levels high enough to be detected by the ELISA kit, the mean was 0,46 nmol/L for both Frøya and Averøy, which is a little above but still close to the reference range from the human studies. In comparison, Kristensen *et al.* (2012), who used the same ELISA kit, found mean NT-proANP levels of a little under 0.3 nmol/L in aquaculture smolts. However, since values for 8 fish from Frøya and 6 fish from Averøy were too low to be detected by the ELISA kit, the true means are lower than this and might fall into the reference range.

Levels of cardiac markers are elevated in fish in the net pen

While the mean concentration of cardiac troponin T was significantly higher in fish transported to Averøy along with a tendency of increase in cardiac troponin I and CK-MB, the difference was not as pronounced as first hypothesised. At the time of sampling and blood analysis I did not know the health status of the fish, so I believed that the fish from the net pen from Frøya would be relatively healthy and that blood levels from these fish could be used as a baseline. Compared to reference ranges for humans, levels of troponin T, troponin I and CK-MB are all elevated above normal for both Frøya and Averøy. This taken together with the disease status of the fish indicate poor health and heart damage in the net pen already before transportation. In addition to the infectious diseases, the fish were subjected to several mechanical delousing treatments which reduce welfare, increase mortality and leave the salmon stressed and weak (Sommerset *et al*., 2022). Fish that are weakened by infectious diseases will very often not tolerate the delousing treatment on top of their poor health status. Underlying or active diseases like CMS, HSMI, PD, amoebic gill disease (AGD) and poor gill health in general can give high mortality in relation to non-pharmaceutical delousing (Sommerset *et al*., 2022). When surveying disease outbreaks two weeks after non-pharmaceutical delousing treatment in 2021, 79% of Norwegian fish health personnel registered sores, 42% registered CMS and 39% registered HSMI (Sommerset *et al*., 2022). Our population was subjected to 3 mechanical delousing treatments starting from 6 months prior to sampling to 1 and a half months prior to sampling, and it is reasonable to think that these delousing treatments could have made the disease outbreaks worse or caused the infections to develop into clinical disease.

Effect of transport

Since levels of cardiac markers were elevated above normal in the net pen at Frøya, the majority of histopathological changes in fish hearts from Averøy were most likely already there before transportation. The heart pathology is assumed to be caused mainly by the infectious diseases and stressful delousing treatments. However, as troponin T was significantly increased in fish from Averøy along with a trend of increase for troponin I and CK-MB, at least some heart damage was likely caused by stress due to transport. This is supported by previous reports of crowding, pumping and transport having caused reduced disease resistance and poor health in salmon.

Only troponin T was significantly different

It was unexpected that troponin T was significantly different while the other cardiac markers were not. This is probably because of differences in the time period it takes each marker to peak, and the time it takes before they are cleared from the circulation. The time of the blood sampling plays an important role in the measured levels. As stated above, fish waited up to 24 hours in the waiting pen. Since troponin T stays elevated in blood longer than troponin I according to Jacob and Khan (2018), it might be that the fish experienced heart damage a while before the blood samples were taken, and that troponin I levels fell faster than troponin T levels. It could also be that transportation process or other previous events caused heart damage, and that the cardiac troponins (at least troponin I) had not been elevated significantly yet. It could be that these cardiac markers reach detectable levels and peak levels slower than in humans. It is possible that we would have seen higher levels if we had taken the blood samples either at an earlier time point or a later time point.

Value of the project

The results of this study can provide new useful information for this field of research and help the further development of new diagnostic tools that do not require lethal sampling of fish. Of special importance are diagnostic tools that can detect a viral infection early on before clinical symptoms emerge and losses will ensure. Blood tests can be used as an alternative diagnostic method to lethal sampling or be used in addition to it. We have demonstrated that some humane kits (CK-MB and NT-proANP) can be applied to salmon, which can be useful for other researchers. This research on cardiac markers serves as an addition to other findings and can be useful for future research, for example to compare heart pathology or stress levels between different populations.

Recommendations for further research

Future research should establish baseline levels of cardiac markers in Atlantic salmon by taking blood samples from unstressed fish, or at least fish that have experienced as little stress as possible. Since it might be difficult to find farmed salmon in net pens that are minimally stressed due to continuous disease outbreaks, louse treatments and other stressful procedures, it might provide better data to use blood samples from salmon kept in a research facility. Other cardiac markers should be tested, like ischemic markers for example. ELISA kits other than the ones used in this study could be tested, preferably ones that are listed as suitable for salmon or other fish. Since we did not screen for viruses known to cause heart disease in this

study, using PCR along with blood analysis and histopathology could be something to further research. Further comparison of histology and cardiac marker levels and testing how blood levels compare to histopathology scores is something that could provide valuable new information. Another topic of further research is establishing the time it takes for the cardiac marker to increase in fish blood, when they reach peak level and when they fall to baseline levels again. Lastly, as this study was done almost exclusively on haemolysed samples, eliminating this issue in further research would be of great use.

A similar project to this one is currently on the way, aiming to identify specific cardiac markers in fish. The participants include Cooke Aquaculture, the University of Edinburgh, Life Diagnostics, Benchmark Genetics, Moredun Research Institute and the Scottish Aquaculture Innovation Centre (SAIC).

Strengths and limitations

One of the purposes of this project was to test if humane cardiac biomarkers could be applied to salmon. As previously stated, since ANP is well conserved between vertebrates (Cousins *et al.*, 1997; Potter *et al.*, 2009) and there was a high degree of sequence homology between CK-MB in humans and salmon (Appendix 6), it is likely that the human ELISA kits from Biomedica and Thermo Fisher detected the correct peptide/enzyme.

Avoiding selection bias

It is important that the selected sample of salmon is representative for the whole population. The fish caught in the net pen at Frøya were lured towards the large dip-net with feed and the net was then tightened when enough fish had been trapped. This will usually give a representative sample of fish. The sample did not include any loser fish, which are usually close to the surface and have a lower activity level and are more susceptible to salmon lice. The large dip-net was submerged down to approximately the first 10 meters as to not catch the sickest and most lethargic fish at the top of the net pen. Besides, loser fish are netted out and euthanized consecutively for welfare reasons.

Sample size

The sample size for Averøy was smaller than that of Frøya. It would have been better statistically to have at least 20 fish from both sites, unfortunately the last 6 fish from Averøy could not be used for blood sampling. The lower sample size reduces the statistical power and increases the margin of error. It is unclear why is became difficult to take blood samples from the last 6 fish at Averøy, but possible reasons are faulty equipment and low blood pressure.

Histology

Only 5 out of 14 samples that were sent to the Norwegian Veterinary Institute for histopathology had an intact atrium, meaning that we do not have valuable information about the condition of the atrium in most of the fish. Some samples had artefacts and holes making it difficult to assess these. Only samples from Averøy were sent to the Norwegian Veterinary Institute for analysis, so there was no opportunity to compare heart histopathology between the 2 groups as we do not have any histopathological information about the group from Frøya. It would have been optimal to have histopathological results from both groups as it would have be easier to estimate how much heart damage was caused by transport-related procedures. On the other hand, as a lot of the histopathological changes seem to have been present already in the net pen, the samples taken from Averøy could very well be representative for the net pen as a whole. We did not use any methods for detection of the viruses causing HSMI, CMS and PD, but it would have been interesting to compare this with plasma analysis and histopathology.

Possible differences in standard levels between humans and fish

Although mammalian hearts and fish hearts have similar evolutionary origin and are similar in many ways, there also exist differences that can influence the levels of cardiac markers in blood. Fish generally have far smaller hearts and far lower cardiac outputs than mammals and birds of similar size (Hill, Wyse and Anderson, 2018). This is because fish have far lower metabolic rates and lower oxygen demands than mammals. Fish hearts also maintain lower arterial pressure. The lower cardiac output, metabolic rate and oxygen demand could potentially mean that healthy, unstressed fish would have lower standard levels of cardiac markers than humans as the fish hearts do not have to work as hard. The smaller hearts could mean that less proteins, enzymes, peptides etc. related to heart damage would be released from the heart relative to the size of the fish.

Fishes are ectotherm animals, having a similar body temperature to that of the environment. Ambient temperature has a major influence on physiological processes in ectothermic animals (Tervonen *et al.*, 2001), so the rate at which cardiac marker levels in blood rise and fall is assumed to be dependent on temperature in the case of fish. Blood sampling was done during winter, late February to be specific, so there is a great difference in the temperature of salmon blood in this time period compared to the temperature of human blood. This can have large implications for the clearance rate of cardiac markers in salmon compared to humans. Tervonen *et al.* (2001) reported large seasonal differences in plasma levels of salmon cardiac peptide (sCP) in Atlantic salmon, with the highest levels occurring during the warm season. The increased plasma levels at higher temperatures were due to decreased elimination, while secretion of sCP was not affected by temperature. This might also be true for other natriuretic peptides in salmon, like ANP and NT-proANP, and different cardiac markers as well.

Genome duplications have occurred in fish, giving rise to paralogous genes (Bone and Moore, 2008). The multiple copies of genes can potentially complicate blood analysis in fish, but this has not been properly looked into.

Haemolysis

In vitro haemolysis posed a serious challenge as it occurred in almost all of the plasma samples to varying degrees (figure 6). In all ELISA kits purchased, the protocol booklet stated to avoid using haemolysed samples as this could give erroneous results. Concentrations of cardiac markers were plotted against OD-values of haemoglobin in a scatter plot (Appendix 5). No linear relationship was found between the degree of haemolysis and the concentration of the cardiac markers, but this does not mean there was no effect of haemolysis. It might be that haemolysis affected the measured concentration of the proteins without there being an obvious pattern. The effect of haemolysis would be easier to assess if we did a controlled experiment where haemolysate was added to known concentrations of cardiac markers, but this was outside the scope of this thesis.

Figure 6: Cryotubes containing plasma samples from Frøya (top row) and Averøy (bottom row). Cryotubes are organized in a numbered fashion from lowest fish ID number (left) to highest (right).

According to Ozcan, Karakas and Yucel (2012), CK-MB (mass) and troponin I are not affected by haemolysis, but there have been discrepancies about the level of interference of haemolysis on troponin assays. One study showed that components other than haemoglobin released from erythrocytes interfere in the Opus cTnI immunoassay (Behring Diagnostics) (Wenk, 1998). Sodi *et al.* (2006) used the Roche E170 immunoassay analyser to measure levels of cardiac troponin T in 3 patients' serum. In all patients, concentrations of troponin T decreased with increasing haemolysis index. They also found out that troponin levels decreased significantly with increasing levels of haemoglobin. Still, the effect was greater for haemolysate.

It has been hypothesized that proteases released from erythrocytes degrade troponin T. Sodi *et al.* (2006) discovered that pepstatin A, a reversible inhibitor of aspartic proteases, cathepsins, pepsin and renin, reduced the loss of measured troponin T due to proteolysis over the entire 48-hour measurement period. Of all the proteases inhibited by pepstatin A, the authors were only aware of cathepsin E being present in erythrocytes. Troponin I has also been documented to be sensitive to proteolysis, and is rapidly degraded by proteases present in serum, especially after necrosis (Sodi *et al.*, 2006). Monneret *et al.* (2015) used electrochemiluminescence immunoassay (ECLIA) on Modular E170 (Roche Diagnostics) to measure high-sensitive troponin T (hsTnT), and Modular P800 to analyse CK. They found out that the concentration of hsTnT decreased with haemolysis, while the concentration of CK

was highly enhanced by haemolysis. Daves *et al.* (2012), measured cardiac troponin I and CK-MB using chemiluminescent immunoassays on the Access 2 analyser (Beckman Coulter), and cardiac troponin T using Modular E (Roche Diagnostics). They found no significant effect of moderate haemolysis on any of the parameters. Sodi *et al.* (2006) reported no effect of haemolysis on CK-MB on the Roche 170 platform. Al-Shidhani *et al.* (2014) used Access (Beckman Coulter) and Cobas e601 (Roche Diagnostics) analysers for cardiac troponin I and cardiac troponin T respectively. They found out that haemolysis generally caused a concentration-dependent negative interference for both assays, but the effect was more pronounced for troponin T. Significantly decreased values of troponin T due to moderate haemolysis (free haemoglobin concentration >1 g/L) have been reported and has also been confirmed for high sensitivity cTnT assays (Krintus and Panteghini, 2020). Bruneel *et al.* (2012) detected a negative interference of haemolysis on cTnI for haemoglobin concentrations above 310 μmol/L, using the Dimension Vista analyser.

The literature suggests that haemolysis can decrease measured troponin I and T concentrations. Even if this is the case, the mean levels of troponin I and especially troponin T in our sample population were still elevated above normal. In the case of CK-MB, the literature suggests that measured concentrations of CK or CK-MB might be increased due to haemolysis. Still, CK-MB levels in our samples were elevated enough for it to be unlikely that haemolysis alone was responsible for this increase. No studies about the effect of haemolysis on NT-proANP assays have been found.

No baseline levels in fish

The original plan was to compare blood levels of cardiac markers in stressed salmon versus unstressed salmon, but the salmon in the net pen from Frøya had already been exposed to several stressful delousing treatments the year before sampling, in addition to diseases. This means that their blood levels could not serve as an indicator of what the standard levels are supposed to be in fish. Currently no baseline levels for fish exist in the literature. It would have been beneficial to have baseline values to compare with to better assess to what extent the blood levels are elevated.

ELISA

The kits chosen were limited by what was available for fish and what humane kits we knew would work. For reasons unknown to us, the fish troponin kits ordered from BT Laboratory

were later removed from their website. There are not a lot of commercial fish kits available, and one company that does sell them has gotten bad reviews from other researchers, which steered us away from buying any kits from them. Although it is possible to buy humane ELISA kits, there will be limitations to what humane kits will work on fish plasma or serum. There needs to be sufficient sequence homology between the antigen sequence and the fish protein sequence for the kit to detect the analyte of interest. We initially looked into the possibility of ordering customized ELISA fish kits, but it turned out to be too costly for this project. Only 1 (NT-proANP) out of 4 kits included a positive control, which would be beneficial to have for the other 3 kits as well. Positive controls help validate that negative readouts truly are negative.

Repeated freeze-thaw cycles are something that should be avoided in ELISA and other blood assays, as they can affect the stability of constituents in plasma or serum (Hillebrand, Heijboer and Endert, 2017). For each fish there were 2 cryotubes of plasma and we ran 5 ELISAs in total because we had problems with our first CK-MB kit, meaning that each cryotube was subjected to either 2 or 3 freeze-thaw cycles. In addition, our samples were kept in room temperature for several hours each time they were thawed. However, in the protocol booklet of the NT-proANP kit it was stated that plasma samples could be subjected to a maximum of 3 freeze-thaw cycles, which means that the number of freeze-thaw cycles our samples were subjected to were within the limit. For this reason, it is assumed that the freezing and thawing of samples did not affect the NT-proANP results to a significant degree. Protocol booklets for the CK-MB kit and the cardiac troponin kits stated to avoid multiple freeze-thaw cycles, but none of them stated an upper limit. Still, it is thought that the freeze-thaw cycles our samples were exposed to were few enough to not affect the results to any significant degree also for these kits.

The NT-proANP ELISA kit was not sensitive enough to detect the lowest plasma levels, resulting in data lacking for several fish from both locations. Unfortunately, we were not aware of a more sensitive kit. ANP is considered inferior to BNP as a biomarker because its assessment is considered less reproducible (Idzikowska and Zielińska, 2018), and in recent years BNP measures have been preferred over ANP because of higher stability (Volpe, Carnovali and Mastromarino, 2015). Also, since NT-proANP can be cleaved into smaller amino acid fragments, mid-regional proANP (MR-proANP, amino acids 53-90) is the preferred detection site (Yagmur *et al.*, 2019). When it comes to CK-MB, this test is not

always optimal as it is not sensitive enough to detect minor myocardial damage (Aydin *et al.*, 2019).

Conclusion

Cardiac biomarker levels were elevated above the reference ranges in fish from the net pen at Frøya, indicating poor health and heart damage due to infectious diseases and stressful treatments. This was supported by histopathological analysis revealing inflammation and necroses in the hearts of the fish. Significantly elevated levels of cardiac troponin T from Frøya to Averøy point to additional heart damage caused by crowding, pumping and transport from the net pen to the processing plant.

References

Alhajj, M. and Farhana, A. (2022) 'Enzyme Linked Immunosorbent Assay', in *StatPearls*. Treasure Island (FL): StatPearls Publishing. Available at: http://www.ncbi.nlm.nih.gov/books/NBK555922/ (Accessed: 14 May 2022).

Al-Shidhani, M. *et al.* (2014) 'Effect of Hemolysis on Plasma Cardiac Troponin Levels at Clinically Relevant Concentrations: An Experimental Study', *Biology and Medicine*, 07(01). doi:10.4172/0974-8369.1000217.

Andersen, L., Hodneland, K. and Nylund, A. (2010) 'No influence of oxygen levels on pathogenesis and virus shedding in Salmonid alphavirus (SAV)-challenged Atlantic salmon (Salmo salar L.)', *Virology Journal*, 7(1), p. 198. doi:10.1186/1743-422X-7-198.

Aydin, Suleyman *et al.* (2019) 'Biomarkers in acute myocardial infarction: current perspectives', *Vascular Health and Risk Management*, Volume 15, pp. 1–10. doi:10.2147/VHRM.S166157.

Bone, Q. and Moore, R.H. (2008) *Biology of fishes*. 3rd ed., Thoroughly updated and rev. New York: Taylor & Francis.

Braceland, M. *et al.* (2013) 'The serum proteome of Atlantic salmon, Salmo salar, during pancreas disease (PD) following infection with salmonid alphavirus subtype 3 (SAV3)', *Journal of Proteomics*, 94, pp. 423–436. doi:10.1016/j.jprot.2013.10.016.

Bruneel, A. *et al.* (2012) 'External Evaluation of the Dimension Vista 1500® Intelligent Lab System: Dimension Vista 1500® Evaluation', *Journal of Clinical Laboratory Analysis*, 26(5), pp. 384–397. doi:10.1002/jcla.21539.

Büttner, P. *et al.* (2022) 'NT-proANP levels in peripheral and cardiac circulation', *Journal of Interventional Cardiac Electrophysiology*, 63(2), pp. 409–415. doi:10.1007/s10840-021-01020-z.

Costa, J.Z. *et al.* (2021) 'Proteomic characterization of serum proteins from Atlantic salmon (*Salmo salar* L.) from an outbreak with cardiomyopathy syndrome', *Journal of Fish Diseases*, 44(11), pp. 1697–1709. doi:10.1111/jfd.13488.

Cousins, K. *et al.* (1997) 'Release of atrial natriuretic factor prohormone peptides 1-30, 31-67 and 99-126 from freshwater- and seawater-acclimated perfused trout (Oncorhynchus mykiss) hearts', *Journal of Experimental Biology*, 200(9), pp. 1351–1362. doi:10.1242/jeb.200.9.1351.

Daves, M. *et al.* (2012) 'Influence of hemolysis on routine laboratory cardiac marker testing', *Clinical Laboratory*, 58(3–4), pp. 333–336.

Frisk, M. *et al.* (2020) 'Intensive smolt production is associated with deviating cardiac morphology in Atlantic salmon (Salmo salar L.)', *Aquaculture*, 529, p. 735615. doi:10.1016/j.aquaculture.2020.735615.

Hill, R.W., Wyse, G.A. and Anderson, M. (2018) *Animal physiology*. International 4th ed. New York: Sinauer associates.

Hillebrand, J., Heijboer, A. and Endert, E. (2017) ‘Effects of repeated freeze–thaw cycles on endocrine parameters in plasma and serum’, *Annals of Clinical Biochemistry: International Journal of Laboratory Medicine*, 54(2), pp. 289–292. doi:10.1177/0004563216657361.

Idzikowska, K. and Zielińska, M. (2018) ‘Midregional pro-atrial natriuretic peptide, an important member of the natriuretic peptide family: potential role in diagnosis and prognosis of cardiovascular disease’, *Journal of International Medical Research*, 46(8), pp. 3017–3029. doi:10.1177/0300060518786907.

Iversen, M., Finstad, B. and Nilssen, K.J. (1998) ‘Recovery from loading and transport stress in Atlantic salmon (Salmo salar L.) smolts’, *Aquaculture*, 168(1–4), pp. 387–394. doi:10.1016/S0044-8486(98)00364-0.

Jacob, R. and Khan, M. (2018) ‘Cardiac Biomarkers: What Is and What Can Be’, *Indian Journal of Cardiovascular Disease in Women WINCARS*, 03(04), pp. 240–244. doi:10.1055/s-0039-1679104.

Keen, A.N. *et al.* (2016) ‘The Dynamic Nature of Hypertrophic and Fibrotic Remodeling of the Fish Ventricle’, *Frontiers in Physiology*, 6. doi:10.3389/fphys.2015.00427.

Krintus, M. and Panteghini, M. (2020) ‘Laboratory-related issues in the measurement of cardiac troponins with highly sensitive assays’, *Clinical Chemistry and Laboratory Medicine (CCLM)*, 58(11), pp. 1773–1783. doi:10.1515/cclm-2020-0017.

Kristensen, T. *et al.* (2012) ‘Atrial natriuretic peptide levels and heart morphology in migrating Atlantic salmon (Salmo salar) smolts from 4 rivers with different environmental conditions’, *Aquaculture*, 362–363, pp. 172–176. doi:10.1016/j.aquaculture.2011.08.003.

Lindsey, J.B. *et al.* (2011) ‘Prognostic Implications of Creatine Kinase-MB Elevation After Percutaneous Coronary Intervention: Results From the Evaluation of Drug-Eluting Stents and Ischemic Events (EVENT) Registry’, *Circulation: Cardiovascular Interventions*, 4(5), pp. 474–480. doi:10.1161/CIRCINTERVENTIONS.111.962233.

Loke, I. *et al.* (2003) ‘Reference ranges for natriuretic peptides for diagnostic use are dependent on age, gender and heart rate’, *European Journal of Heart Failure*, 5(5), pp. 599–606. doi:10.1016/S1388-9842(03)00108-9.

Lund, M. *et al.* (2017) ‘Hypoxia tolerance and responses to hypoxic stress during heart and skeletal muscle inflammation in Atlantic salmon (Salmo salar)’, *PLOS ONE*. Edited by Q. Cai, 12(7), p. e0181109. doi:10.1371/journal.pone.0181109.

Mariathas, M. *et al.* (2019) ‘True 99th centile of high sensitivity cardiac troponin for hospital patients: prospective, observational cohort study’, *BMJ*, p. l729. doi:10.1136/bmj.l729.

Monneret, D. *et al.* (2015) ‘Hemolysis indexes for biochemical tests and immunoassays on Roche analyzers: Determination of allowable interference limits according to different calculation methods’, *Scandinavian Journal of Clinical and Laboratory Investigation*, 75(2), pp. 162–169. doi:10.3109/00365513.2014.993691.

Nakagawa, Y., Nishikimi, T. and Kuwahara, K. (2019) ‘Atrial and brain natriuretic peptides: Hormones secreted from the heart’, *Peptides*, 111, pp. 18–25. doi:10.1016/j.peptides.2018.05.012.

Ozcan, O., Karakas, A. and Yucel, D. (2012) 'Effects of hemolysis on the assays of serum CK, CK-MB activities and CK-MB (MASS), troponin and myoglobin measurments', *Turkish Journal of Biochemistry*, 37(4), pp. 375–385. doi:10.5505/tjb.2012.21043.

Peacock, W.F. *et al.* (2018) 'Efficacy of High-Sensitivity Troponin T in Identifying Very-Low-Risk Patients With Possible Acute Coronary Syndrome', *JAMA Cardiology*, 3(2), p. 104. doi:10.1001/jamacardio.2017.4625.

Potter, L.R. *et al.* (2009) 'Natriuretic Peptides: Their Structures, Receptors, Physiologic Functions and Therapeutic Applications', in Schmidt, H.H.H.W., Hofmann, F., and Stasch, J.-P. (eds) *cGMP: Generators, Effectors and Therapeutic Implications*. Berlin, Heidelberg: Springer Berlin Heidelberg (Handbook of Experimental Pharmacology), pp. 341–366. doi:10.1007/978-3-540-68964-5_15.

Rodger, H. *et al.* (1992) 'Acute skeletal myopathy in farmed Atlantic salmon Salmo salar', *Diseases of Aquatic Organisms*, 12, pp. 17–23. doi:10.3354/dao012017.

Schreck, C.B. *et al.* (eds) (2016) *Biology of stress in fish*. (Fish physiology / ed. by Anthony P. Farrell and Colin J. Brauner, volume 35).

Smith, G.S., Walter, G.L. and Walker, R.M. (2013) 'Clinical Pathology in Non-Clinical Toxicology Testing', in *Haschek and Rousseaux's Handbook of Toxicologic Pathology*. Elsevier, pp. 565–594. doi:10.1016/B978-0-12-415759-0.00018-2.

Sodi, R. *et al.* (2006) 'Mechanism of interference by haemolysis in the cardiac troponin T immunoassay', *Annals of Clinical Biochemistry: International Journal of Laboratory Medicine*, 43(1), pp. 49–56. doi:10.1258/000456306775141687.

Sommerset I. *et al* (2022) 'Fiskehelserapporten' 2021, the Norwegian Veterinary Institute's series of reports nr. 2a/2022, published by the Norwegian Veterinary Institute 2022

Sone, I., Olsen, R.L. and Heia, K. (2012) 'Spectral Changes of Atlantic Salmon (Salmo salar L.) Muscle during Cold Storage As Affected by the Oxidation State of Heme', *Journal of Agricultural and Food Chemistry*, 60(38), pp. 9719–9726. doi:10.1021/jf302505y.

Strimbu, K. and Tavel, J.A. (2010) 'What are biomarkers?':, *Current Opinion in HIV and AIDS*, 5(6), pp. 463–466. doi:10.1097/COH.0b013e32833ed177.

Takle, H. *et al.* (2006) 'Hyperthermia induced atrial natriuretic peptide expression and deviant heart development in Atlantic salmon Salmo salar embryos', *General and Comparative Endocrinology*, 147(2), pp. 118–125. doi:10.1016/j.ygcen.2005.12.017.

Takle, H. and Andersen, Ø. (2007) 'Caspases and apoptosis in fish', *Journal of Fish Biology*, 71(sc), pp. 326–349. doi:10.1111/j.1095-8649.2007.01665.x.

Tervonen, V. *et al.* (2001) 'Temperature has a major influence on cardiac natriuretic peptide in salmon', *The Journal of Physiology*, 536(1), pp. 199–209. doi:10.1111/j.1469-7793.2001.00199.x.

Volpe, M., Carnovali, M. and Mastromarino, V. (2015) 'The natriuretic peptides system in the pathophysiology of heart failure: from molecular basis to treatment', *Clinical Science*, 130(2), pp. 57–77. doi:10.1042/CS20150469.

Wenk, R.E. (1998) ‘Mechanism of Interference by Hemolysis in Immunoassays and Requirements for Sample Quality’, *Clinical Chemistry*, 44(12), pp. 2554–2554. doi:10.1093/clinchem/44.12.2554.

Yagmur, E. *et al.* (2019) ‘Elevated MR-proANP plasma concentrations are associated with sepsis and predict mortality in critically ill patients’, *Journal of Translational Medicine*, 17(1), p. 415. doi:10.1186/s12967-019-02165-2.

Yandle, T.G. *et al.* (1986) ‘Metabolic clearance rate and plasma half life of alpha-human atrial natriuretic peptide in man’, *Life Sciences*, 38(20), pp. 1827–1833. doi:10.1016/0024-3205(86)90137-2.

Yousaf, M.N. *et al.* (2013) ‘Comparative cardiac pathological changes of Atlantic salmon (Salmo salar L.) affected with heart and skeletal muscle inflammation (HSMI), cardiomyopathy syndrome (CMS) and pancreas disease (PD)’, *Veterinary Immunology and Immunopathology*, 151(1–2), pp. 49–62. doi:10.1016/j.vetimm.2012.10.004.

Yousaf, M.N. and Powell, M.D. (2012) ‘The Effects of Heart and Skeletal Muscle Inflammation and Cardiomyopathy Syndrome on Creatine Kinase and Lactate Dehydrogenase Levels in Atlantic Salmon (*Salmo salar* L.)’, *The Scientific World Journal*, 2012, pp. 1–9. doi:10.1100/2012/741302.

Appendix 1

Table 1: Weight and length of individual fish from Frøya, net pen number 208. Sampled 22.02.2021. The symbol "*" means that weight and length are missing.

Fish ID	Weight (kg)	Length (cm)
208-1	8.10	76
208-2	6.30	73
208-3	5.67	75
208-4	5.42	72
208-5	4.50	69
208-6	5.80	70.5
208-7	4.60	67.5
208-8	4.00	63.5
208-9	4.32	69
208-10	5.32	74
208-11	6.09	71
208-12	5.96	77
208-13	5.24	69.5
208-14	5.18	71.5
208-15	6.87	77
208-16	5.10	71
208-17	3.67	64
208-18	4.93	72
208-19	4.28	65
208-20	*	*

Table 2: Weight and length of individual fish transported from net pen number 208, Frøya to Averøy. Sampled 23.02.2021.

Fish ID	Weight (kg)	Length (cm)
208-1	4.60	70
208-2	5.40	74
208-3	2.44	56
208-4	4.60	68
208-5	4.74	69
208-6	5.06	73
208-7	5.06	70
208-8	4.40	69
208-9	5.64	74
208-10	4.36	68
208-11	4.18	68
208-12	5.66	75
208-13	6.02	75
208-14	4.44	69

Appendix 2

Table 3: Individual vales for troponin I and T, CK-MB and NT-proANP for fish from Frøya. The symbol "-" means that the value was lower than the detection limit of the ELISA kit.

Fish ID	**Troponin I (ng/L)**	**Troponin T (ng/L)**	**CK-MB (ng/mL)**	**NT-proANP (nmol/L)**
208-1	41.784	54.245	36,102	0.509
208-2	49.161	79.777	17,656	-
208-3	38.655	92.185	18,016	-
208-4	47.354	69.293	27,822	0.199
208-5	42.257	81.395	19,362	-
208-6	52.827	81.626	23,504	0.353
208-7	38.062	94.648	15,25	0.463
208-8	43.914	72.671	26,158	0.473
208-9	37.211	120.873	32,838	0.671
208-10	32.853	76.176	26,136	0.545
208-11	36.08	66.358	18,528	-
208-12	37.954	73.363	17,834	-
208-13	38.097	100.037	24,098	-
208-14	45.712	100.691	15,3	-
208-15	37.92	87.145	18,568	-
208-16	38.461	95.639	17,866	0.186
208-17	49.989	101.639	21,452	0.284
208-18	41.208	89.683	33,922	0.954
208-19	36.114	81.17	15,18	0.35
208-20	44.109	69.089	24,692	0.521

Table 4: Individual values for troponin I and T, CK-MB and NT-proANP for fish from Averøy. The symbol "-" means that the value was lower than the detection limit of the ELISA kit.

Fish ID	**Troponin I (ng/L)**	**Troponin T (ng/L)**	**CK-MB (ng/mL)**	**NT-proANP (nmol/L)**
208-1	52.042	124.992	24,748	0.515
208-2	50.056	101.976	64,564	-
208-3	37.223	65.175	31,284	-
208-4	33.38	99.694	15,064	-
208-5	42.63	106.954	18,728	-
208-6	35.955	118.781	35,762	0.086
208-7	38.762	92.303	18,202	0.575
208-8	48.969	60.656	17,148	0.413
208-9	45.088	94.77	19,85	0.681
208-10	44.353	105.533	20,888	-
208-11	43.662	89.016	13,31	-
208-12	48.579	83.404	26,734	0,652
208-13	52.391	117.25	35,932	0,335
208-14	38.9	99.88	40,076	0.454

Appendix 3

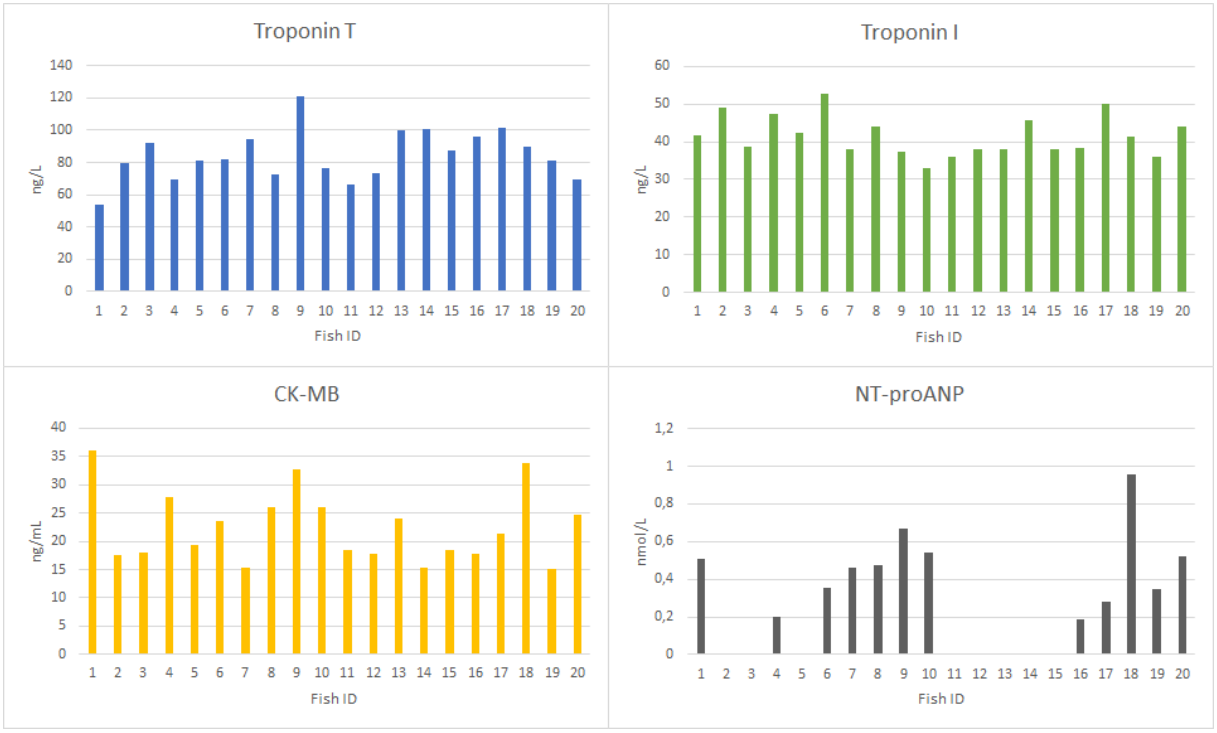


Figure 7: Individual values for each fish from Frøya. NT-proANP-values are lacking for several fish because they were below the detection limit of the ELISA kit.

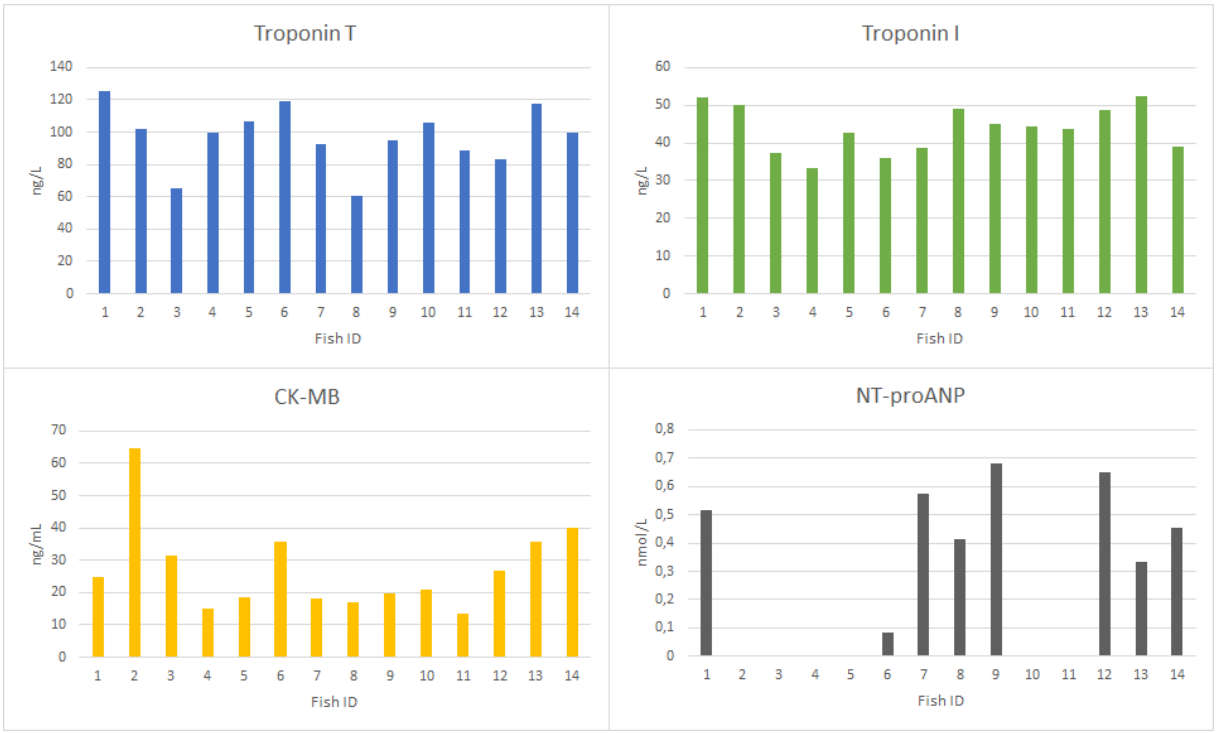


Figure 8: Individual values for each fish from Averøy. Like in figure 6, several values for NT-proANP were below the detection limit of the ELISA kit.

Appendix 4

Table 5: Results from the heart histopathology conducted by the Norwegian Veterinary Institute. Material: hearts on formalin. Cell rich: rich in inflammatory cells. No atrium: atrium not included in the cut/sample provided.

Fish ID	Comment
208-1	Atrium not included in sample. Moderate epicarditis. Cell rich between compactum and spongiosum. Moderate inflammation in spongiosum. Focal myocardial necroses.
208-2	Some artefacts (holes), no atrium. Moderate epicarditis. Some cell rich foci in spongiosum. Focal necroses.
208-3	No atrium. Moderate epicarditis. Focal inflammation in compactum with some haemosiderin.
208-4	No atrium. Moderate to severe epicarditis. Moderate to severe inflammation in spongiosum. Some cell rich foci in compactum. Multifocal muscle necroses.
208-5	No atrium. Moderate epicarditis. Light to moderate inflammation in spongiosum. Focal necroses.
208-6	A good amount of blood in the atrial lumen. Ventricle difficult to assess because of the cut, but some epicarditis and cell rich spongiosum.
208-7	Moderate epicarditis. Few cell rich foci in ventricle.
208-8	Moderate epicarditis. Cell rich in spongiosum. Cell rich in the transition between compactum and spongiosum. Multifocal inflammation in spongiosum. Moderate inflammation in atrium.
208-9	No atrium. Moderate epicarditis and few cell rich foci in spongiosum.
208-10	Some cell clusters in the atrial lumen. Moderate epicarditis. Focal necrosis.
208-11	No atrium and some holes in sample. Slightly cell rich in some foci in spongiosum.
208-12	No atrium. Moderate epicarditis. Multifocal inflammation in spongiosum. Few focal muscle necroses.
208-13	No atrium. Moderate to severe epicarditis, multifocal inflammation in spongiosum. Multifocal muscle necroses.
208-14	Slightly difficult to assess because of direction of the cut, but some epicarditis.

Appendix 5

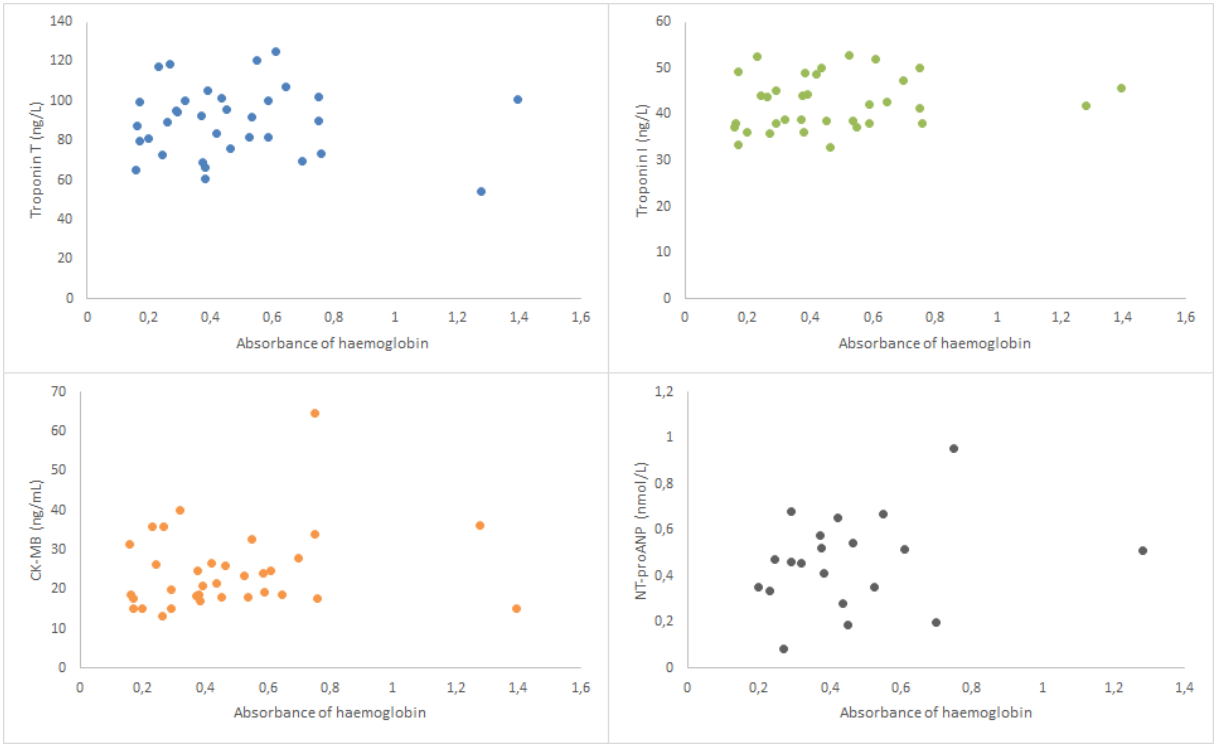


Figure 9: OD-values/absorbance of haemoglobin at 559 nm. N=34 for troponin T, troponin I and CK-MB. N=20 for NT-proANP.

Appendix 6

We aligned sequences of the following 3 proteins using Constraint-based Multiple Alignment Tool (COBALT) by the National Center for Biotechnology Information:

creatine kinase, muscle, partial [Homo sapiens]
Accession: EAW57337.1 GI: 119577741

creatine kinase-2 [Salmo salar]
Accession: NP_001133188.1 GI: 213512959

creatine kinase M-type [Salmo trutta]
Accession: XP_029555924.1 GI: 1696111506

Proteins are accessible from:
https://www.ncbi.nlm.nih.gov/protein/EAW57337.1,NP_001133188.1,XP_029555924.1
COBALT is accessible from: https://www.st-va.ncbi.nlm.nih.gov/tools/cobalt/cobalt.cgi

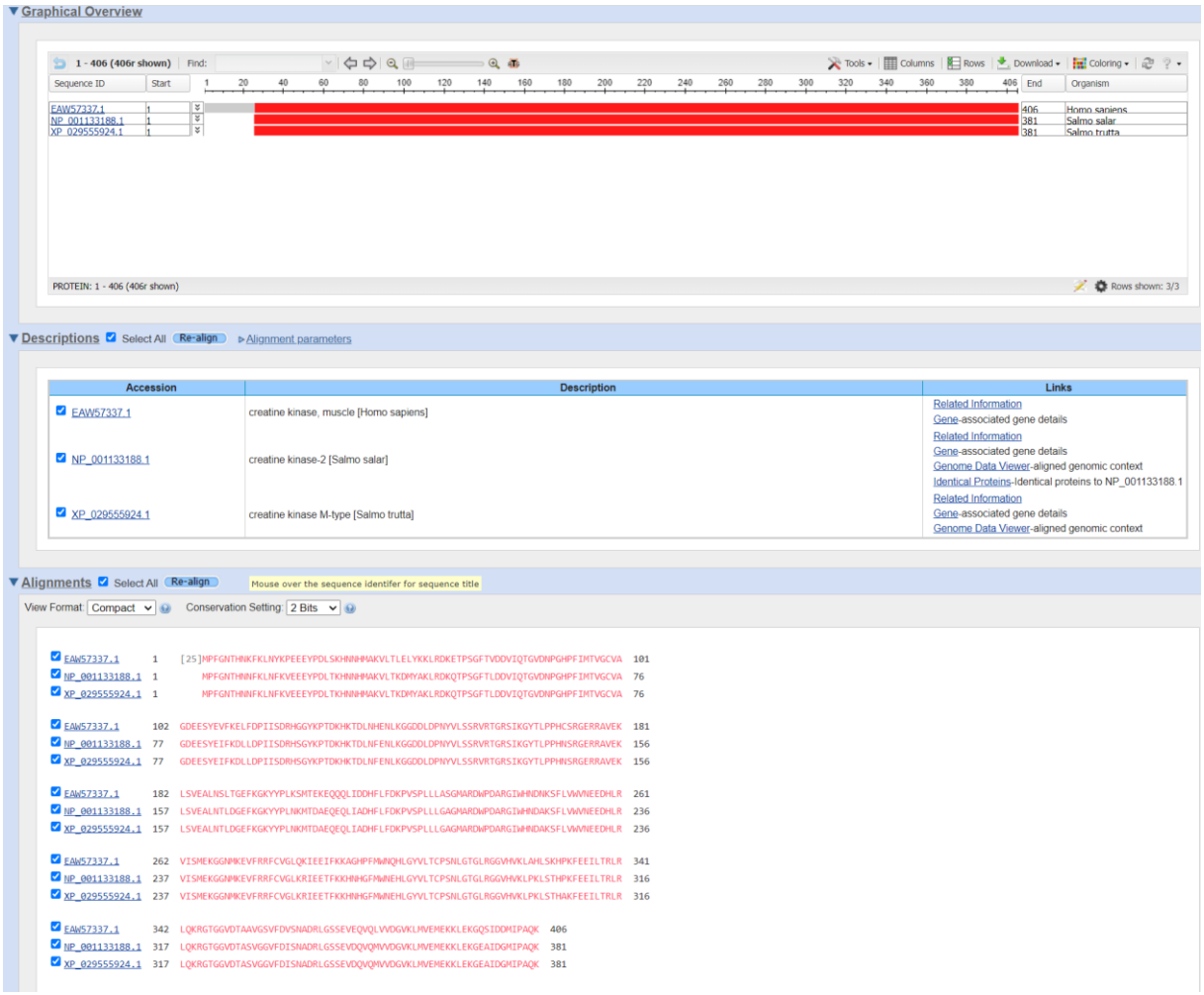
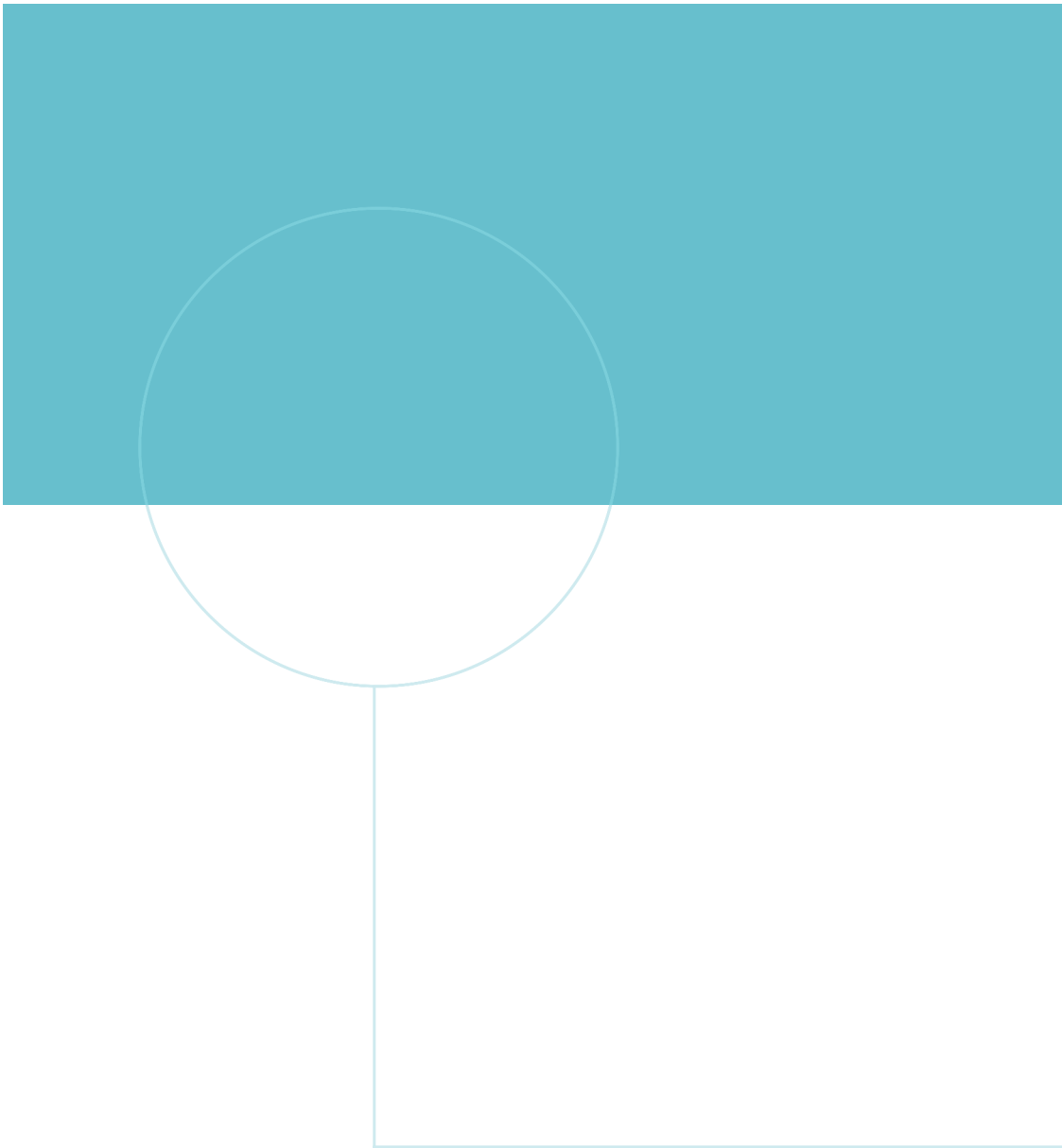


Figure 10: Sequence alignments using COBALT.



NTNU

Norwegian University of
Science and Technology