UiT Norges arktiske universitet

Faculty of Biosciences, Fisheries and Economics

Impact of temperature-dependent developmental plasticity on immune response and cardiac health to *Yersinia ruckeri* infection, in Atlantic salmon parr

Jens Herman Anthun Meland

Master's thesis in Akvamedisin BIO-3955 May 2023

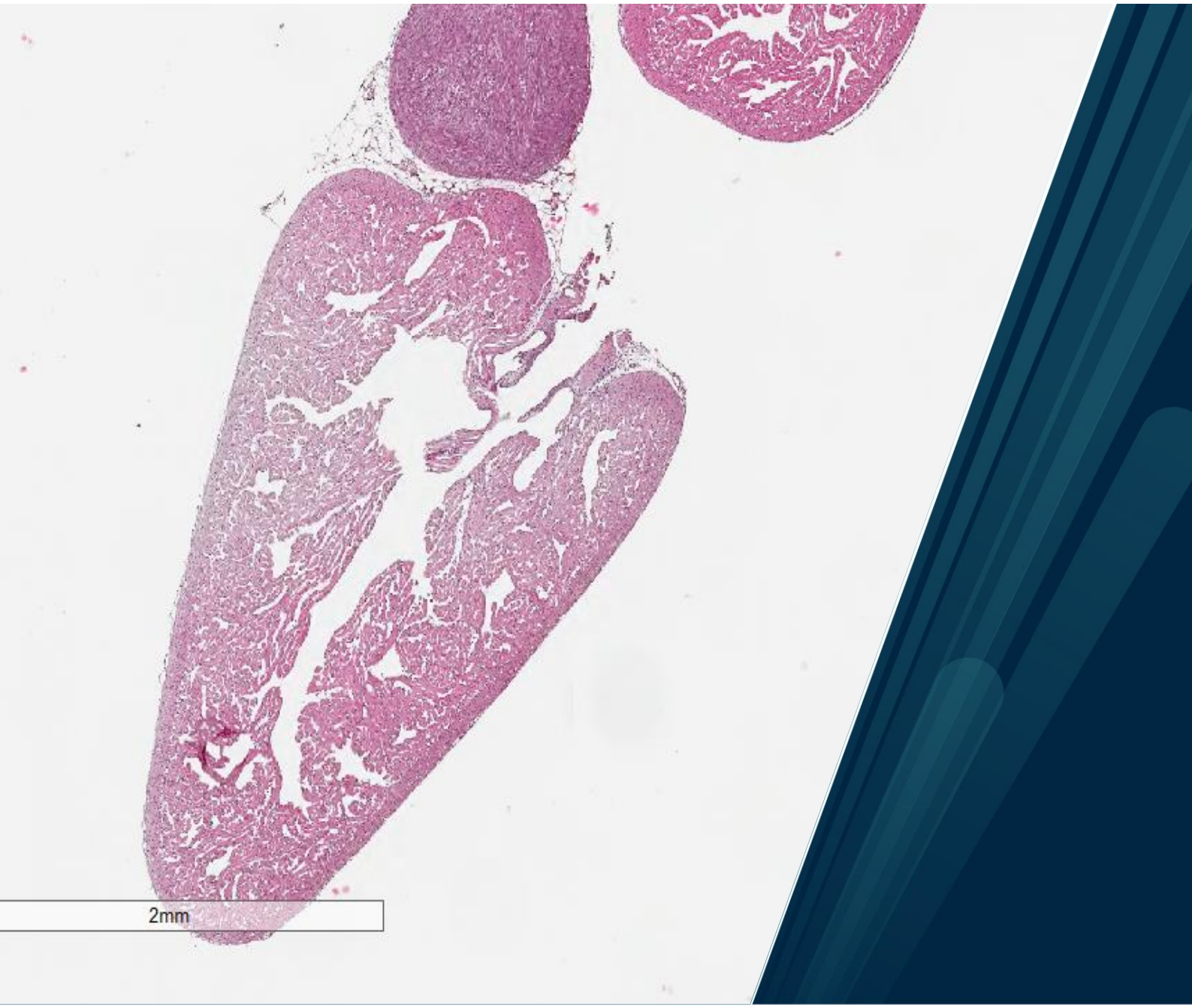

Table of Contents

List of Tables

List of Figures

Foreword

This master's thesis was part of the COOLFISH project, which is funded by the Research Council of Norway (PNO: 325571) and led by Nofima AS with Erik Burgerhout as project manager. The COOLFISH-project aims at "improving Atlantic salmon health and welfare by metabolic programming". The experiment was conducted at Nofima, Tromsø and Havbruksstasjonen i Tromsø AS, Kårvika. The master's thesis lasted from summer 2022 to May 2023.

My heartfelt gratitude goes to my **external-supervisor** and project-manager Erik Burgerhout, for offering a thesis assignment and bestowing me his knowledge and practical skills needed for the project and future research. I am also sincerely grateful to my **main supervisor** Jaya Kumari Swain for helping me write the thesis and answering my many questions. I would also like to thank researchers and lab engineers Hanne Britt Brenne, Ragnhild Stenberg Berg, Carlo C. Lazado, and Muhammad Salman Malik for teaching and helping me with the different laboratory techniques and analyses. A great thanks to my fellow office partners and friends, Samaneh Mousavi, Anne-Marja Jannok-Joma, and Mina B. Kleiv for a great and joyful time at Nofima.

Tromsø, May 2023

Jens Herman Anthun Meland

Nofima

UiT Norges arktiske universitet

Abstract

Heart injuries and diseases are one of the largest challenges in Atlantic salmon aquaculture. Presently, salmon experience intensive farming conditions and feeding regimes, and different viral and bacterial pathogens flourish and create cardiac health risks. Many have suggested how early life history plays a greater role in performance than previously assumed. The RCN-NFR funded project COOLFISH (PNO: 325571) led by Nofima has supported this thesis which aims at improving Atlantic salmon cardiac health and welfare by exploring the impact of temperature-dependent developmental plasticity. By comparing two different incubation temperature regimes, 4 °C and 8 °C at the embryonic stage, this study has provided increased knowledge on how incubation temperature affects heart morphology and immune response at the parr stage using a *Y. ruckeri* infection model, which has also increased general knowledge of bacterial infection in cardiac tissue. Gene expression analysis and observations revealed differences in immune and stress responses, as well as muscle metabolism and growth. Most of the results indicate improved performance in the hearts of 4 °C incubated fish, in contrast to observations of cardiac pathology markers in the 8 °C incubated fish.

Nomenclature and abbreviations

Zebrafish-nomenclature has been used for salmonid genes and proteins in accordance with the guidelines proposed by The zebrafish nomenclature committee (1). Different terminology and abbreviations (**Table 1**) have not necessarily been elaborated.

Table 1: Abbreviations.

Abbreviation	Description
cDNA	Complementary deoxyribonucleic acid
CFU	Colony-forming unit
DD	Degree-days ($= X$ °C $*$ Y days)
dpi	Days post infection
eyed-egg stage	The stage where salmon eggs have developed visible black spot (eyes). The term in this study refers to fully pigmented eyes (320 DD).
hpf	Hours post fertilization
PNO	Project number
qPCR	Real-time quantitative polymerase chain reaction
RNA	Ribonucleic acid

1 Introduction

1.1 Salmon farming industry and life cycle

The aquaculture industry in Norway plays an important role in employment and has a great economic impact, especially the farming of Atlantic salmon (*Salmo salar*) (2, 3). As well as being a popular fish for commercial rearing, salmon is also a highly studied organism (4, 5). Unfortunately, a lot of farmed salmon experience stress, disease, and wounds, often resulting in high mortality and low welfare. In 2022, mortality was reported to be 35.6 and 56.7 million farmed salmon (not including fish underneath 3 grams or fish sorted out) in the freshwater and seawater phases, respectively. When it comes to the heart, the Norwegian Fish Health Report 2022 points out that rapid development during early life stages probably affects heart size and shape negatively. The report also acknowledges the lack of knowledge around the subject and that the consequences in "farming conditions" are still quite unknown. At the same time, virus infections that affect the heart have been and continue to pose a challenge to farmed salmon health (6).

Farmed salmon is not the only one under pressure, the farming industry is often criticized due to increased animal welfare concerns and understanding of fish sentience. Furthermore, climate change, biodiversity loss and food security risks make it important to optimize the manpower, energy and area spent on food production. Reducing mortality and improving fish welfare are paramount if the farming industry shall continue to grow and improve, while being eco-friendly and maintaining social approval (7-13).

Atlantic salmon, *Salmo salar,* Linnaeus, 1758, is a teleost fish native to the northern Atlantic Ocean and coastal rivers. Most Atlantic salmon are anadromous, meaning they spawn, hatch, and grow up in freshwater, before migrating to the sea by river, searching for better feeding grounds to support an increased growth (**Figure 1**). Free-swimming fish that have used up their yolk-sack in the freshwater stage are called parr and are easily recognized by their "parr marks" and earth tone colored skin. Healthy fish appear quite round and have a high condition factor (K) at this stage. Towards the end of their stay in the river, they go through smoltification, which include osmoregulation adaptations and a change of color and shape. They become more streamlined (low K), and their skin develops a reflective silver color with small, dark spots, dorsally. The smolts can gradually migrate to the estuary and ocean because of increased seawater tolerance. Salmon spends around 1-4 years in the ocean, before returning in the spring and summer for spawning (14, 15).

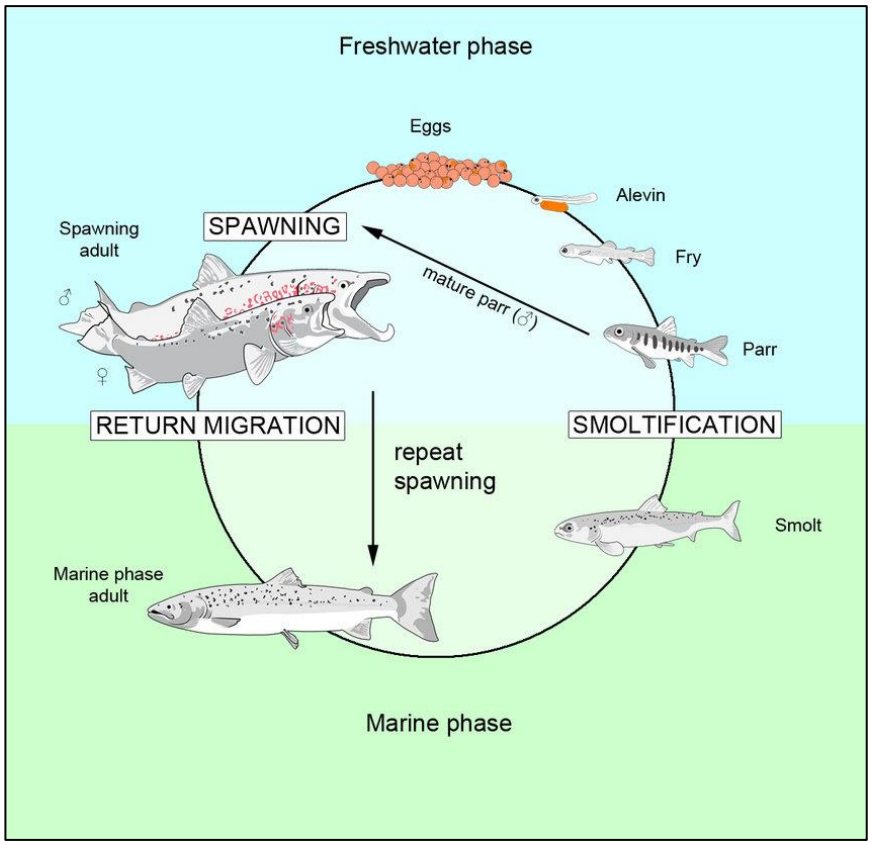

Figure 1: Atlantic salmon life cycle. Reprinted from Mobley et al. (15) (CC BY 4.0).

1.2 Heart anatomy and development

The salmon heart consists of four chambers and rests ventrally behind the gills in the pericardial cavity separated from the gut by a mesenchyme tissue wall (septum transversum). In order, from blood entry to exit, the four chambers are the sinus venosus (SV), atrium, ventricle and bulbus arteriosus (BA). Between the different chambers, valves prevent backflow of blood (**Figure 2**). Normally, the SV aids atrial filling by contraction, but SV in most teleost fishes is made of connective tissue and very little myocardium. It is therefore believed that SV "constitute a blood reservoir for atrial filling". Furthermore, the cardiac pacemaker is located at the junction between SV and atrium (16-18). The atrium is more muscular than the SV and contributes to ventricular filling, having a key role regulating cardiac output. The ventricle is the most muscular and largest heart chamber. It is the main pump driving blood flow, maintaining the blood circulation. Its myocardium is composed of two parts, an inner trabecular layer (spongiosum) and an outer layer (compactum) (19). In the end, the BA receives blood from the ventricle and equalizes the high blood pressure to maintain a smooth blood flow within safe pressure thresholds for the gills. The BA is formed of connective tissue (elastic fibers and

cartilage tissue) and smooth muscle cells, which gives it the elastic properties that are required for it to be a pressure regulating blood reservoir (20, 21).

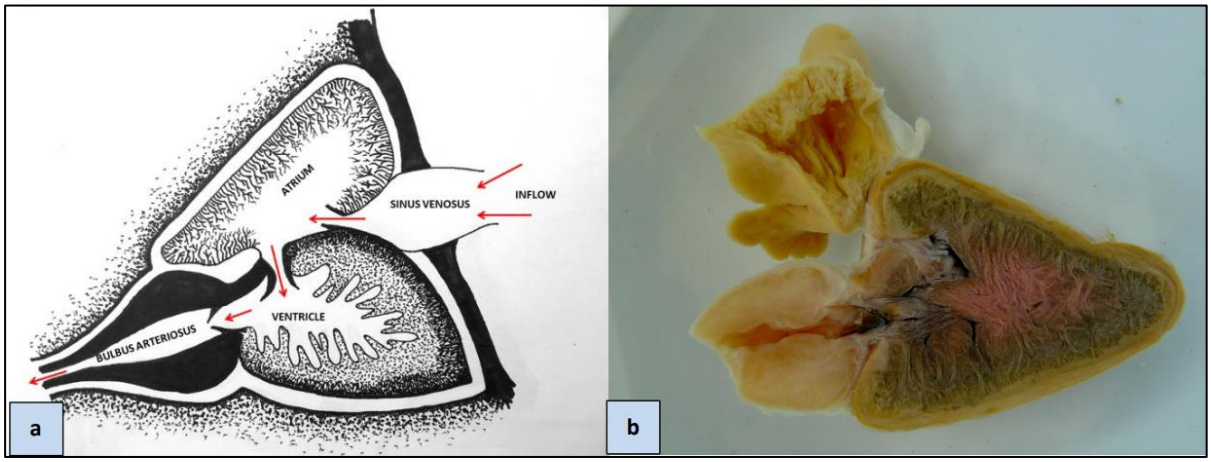

Figure 2: Normal salmon heart anatomy, mid sectioning viewed from left lateral projection. a) schematic drawing of heart chambers and blood flow. b) Picture of formalin-fixed heart. Illustrations by T.T. Poppe. Reprinted from Garseth et al. (22) (Norwegian government publication).

In contrast to adult salmon heart morphology and physiology, cardiac morphogenesis is poorly studied, but its distant relative, the zebrafish (*Danio rerio*), has received more attention and is used as a model organism for studying (vertebrae) cardiac development. It is thought that vertebrate cardiac development is highly conserved and therefore much of the information we know about zebrafish hearts can be applied to Atlantic salmon. The cardiac development process consists of roughly six parts: cardiac fate determination, migration, fusion, tube assembly, looping and remodeling (**Figure 3**). In zebrafish, cardiac fate determination happens at 5 hours post-fertilization (hpf) and embryonic heart precursors begin to develop (23). Cardiac migration starts at 16 hpf, and myocardial precursors are stimulated to differentiate and migrate towards the midline of the embryo. Cardiac fusion gradually occurs around 18 hpf. The tube assembly starts at 20.5 hpf and at 24 hpf "a tube with distinct ventricular and atrial ends" has formed. At 36 hpf, the "ventricle and the atrium exhibit distinct sequential contractions" and remodeling starts, which consists of three parts: valve formation, thickening of the ventricular wall, and formation of ventricular trabeculae (24, 25).

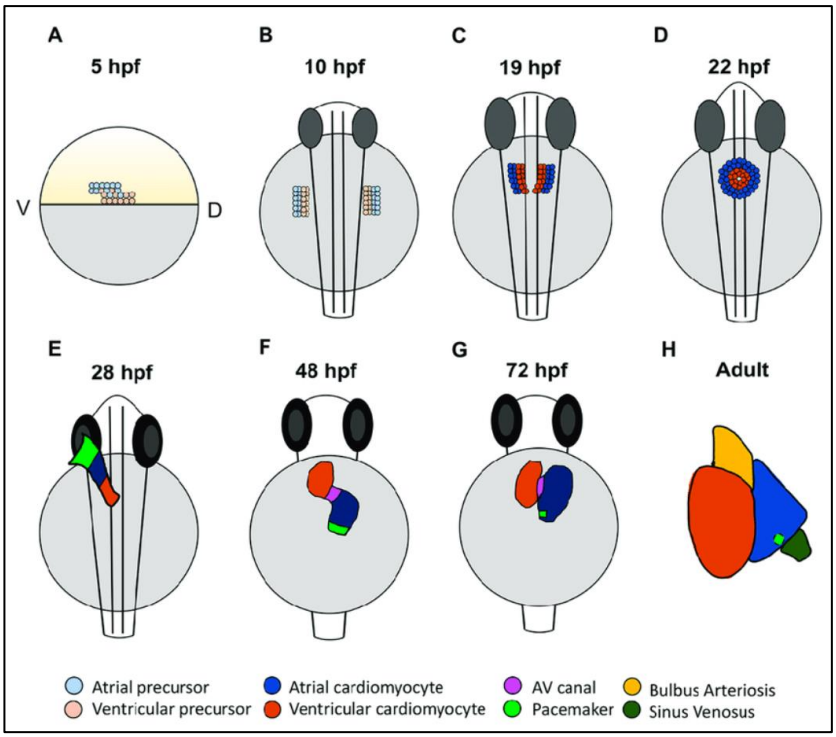

Figure 3: Different stages of zebrafish heart development. A) cardiac fate determination. B-C) Cardiac migration and cardiac fusion. D) Tube assembly. E-F) First contractions. G-H) End of development (but not growth). Reprinted from Martin and Waxman (26) (CC BY 4.0).

1.3 Embryogenetic temperature effects

The Norwegian wild salmon peak spawning occurs between mid-October to mid-January depending on latitude and local conditions. The water temperature during egg-incubation usually drops in the winter months to somewhere between 0-4 °C. When they hatch in the spring, the water temperature has risen to approximately 4.5-6.8 °C (27, 28). However, the temperature in these studies was not measured in the intra-gravel water where the eggs lay, and since water stratification or groundwater influx may occur, the actual incubation temperature may be up to a couple degrees higher than proposed (29, 30). In a study on incubation temperature which used wild salmon from the river Imsa (59° N, 6° E), Finstad and Jonsson (31) reported that both 2.6 °C and 7.2 °C incubation (main incubation before hatching) gave good performance, indicating that salmon eggs from the same population can tolerate different temperatures. On the other side, farmed salmon has been bred for many generations at higher temperatures, possibly alternating their adaptation to water temperatures, but that remains as speculation. The maximum safe and tolerable incubation temperature is reported to be 8 °C (for diploid salmon), any higher results in increased mortality, and heart- and skeleton deformities (32, 33). The tolerable temperature interval during incubation is therefore suggested to be

between 0-8 ℃. One of the industrial breeding programs recommends 1-8℃ during incubation, but lower than 8 ℃ when using triploid salmon (34). Other, more temperate salmonids like chum-, coho and chinook have an upper limit around 10 ℃ for optimal incubation (35).

One of the reasons optimal thermal conditions for incubation is of great interest, is the fact that embryonic development and growth after hatching are affected by metabolic rate and epigenetic programing, which are strongly influenced by temperature (31, 36, 37). Time of hatching, smoltifiaction, etc., are therefore often measured in degree-days (DD) and different models for predicting development at different temperatures exist today (38). In today's industry standard which uses 5+ ℃ incubation temperatures, hatching is expected at 480-520 DD (34).

In addition to changing growth, embryonic temperatures can affect other parts of salmon physiology and behavior. One study found increased larval myogenin expression and low DNA methylation levels, as well as hypertrophy in white muscle fibers in juveniles exposed to 8 ℃ compared to 4 ℃ during early development (37). Other studies have also observed increased muscle hypertrophy at high incubation temperature as well as the opposite, more hyperplasia, at low temperatures, which is measured by muscle fibers per myotome (39-45). There is still discussion around the mechanisms behind such remodeling of the muscle. Hypertrophic growth may be part of the salmon's repertoire of regulating development (e.g., hatching) to match changes in seasonal environments (as suggested by the match-mismatch hypothesis). The mechanics behind are complex, and different hypotheses have been made. For instance, one study investigated how warm-water induced hypoxia inside the eggs may contribute to changes in muscle morphology (44).

Further on, Burgerhout et al. (37) proposed that hyperplastic muscle growth shown in salmon reared at colder temperature in early life stages to have considerably better growth later in life, as well as increased ultimate size potential. The "optimal fiber number hypothesis" proposes that muscle size is limited by both maximum cell size, and muscle fiber number, which involves a tradeoff between diffusional constraints and metabolic energy cost (37, 46). Increased growth, sometimes termed "growth compensation", is also observed after fasting, which is achieved by accelerating feed intake. However, feed intake is insufficient at explaining the mechanisms underlying the rapid increased growth in temperature-manipulated fish (47).

Incubation temperatures have also been proven to affect swimming performance, timing of homing migration, developmental deformities, and the immune system, which likely is a result

of the salmon being poikilothermic and finely tuned to seasonal cycles (32, 33, 48-50). Because being poikilothermic means a less stable environment for biomolecules (e.g., enzymes), where activity levels respond to different temperatures. Some temperatures, especially during early development, may be inherently better for immune function and general health.

1.4 *Yersina ruckeri*

Yersinia ruckeri is a gram-negative rod bacteria that belongs to the family Enterobacteriaceae and is the causative agent of yersiniosis, also called enteric redmouth disease (ERM) (51). Yersiniosis is a common disease for rainbow trout (*Oncorhynchus mykiss*), but can also infect other salmonids like Atlantic salmon and it "causes significant economic losses in the salmonid farming industry" (52, 53). In response, commercial vaccines with good effect have been developed (54, 55). Infection can happen in both freshwater and saltwater, but juvenile stages in freshwater are most susceptible, while later stages will more likely have a chronic infection (52). There exist different types of strains that have different characteristics (e.g., levels of motility). Bacteria in free water can survive and colonize different substrates and surfaces, and are proven to infect horizontally (56), however, infection may also occur vertically (57). The initial route of entry is the secondary lamellae of the gill (58). Many virulence factors have been described, like toxins, pili, iron acquisition system, flagella etc. Its ability of adherence and biofilm formation seems to be especially important for its virulence and survival (59). Furthermore, it has been documented that temperature has an effect on the regulation of virulence genes (60). Infected fish can be observed swimming lethargically and close to the surface. External symptoms include hemorrhages ("point bleedings") on skin, especially fins and mouth, bilateral exophthalmos, corneal opacity, panophthalmitis, swollen abdomens and dark pigmentation. The bacteria cause systemic infection, with typical internal symptoms being hemorrhage, necrosis and inflammation in organs, septicemia, and ascites in pericardial and abdominal cavity. Significant heart pathology has been reported in rainbow trout post *Y. ruckeri* infection (61). There is evidence that peak infection in salmonid hearts happens around 7 days post infection (dpi) (58, 62). In general, little is known about the immune system in cardiac tissue during a bacterial infection, much less the specific response to *Y. ruckeri*.

1.5 Genes of interest

Genes related to muscle growth/metabolism, and immune system were the most relevant for shedding light on how incubation temperature affects heart health and heart pathology. Genes that have been used successfully in similar research were picked out. The selected genes related

to muscle growth and metabolism were *muscle atrophy f-box-β* (*mafbxb*), *b-type natriuretic peptide* (*bnp*), *atrial natriuretic peptide* (*anp*), *proliferating cell nuclear antigen* (*pcna*) and *vascular endothelial growth factor* (*vegf*). The selected (innate) immune related genes were expected to respond towards a bacterial challenge. The genes picked out were *s-adenosyl methionine decarboxylase* (*samdc*), *spermidine/spermine-N(1)-acetyltransferase* (*ssat*), *matrix metalloproteinase 9* (*mmp9*), *matrix metalloproteinase 13* (*mmp13*), *arginase 2* (*arg2*), *differentially regulated trout protein 1* (*drtp1*) and *membrane immunoglobulin M* (*mIgM*).

Additionally, other genes of interest were also targeted, but did not pass the quality controls and could not be included in this study. They were *serum amyloid A 5 protein*, *saxitoxin and tetrodotoxin binding protein 2*, *secreted IgM*, *interleukin 8*, and *cluster of differentiation 28* (*CD28*).

1.5.1 Muscle growth and metabolism related genes

Mafbxb also known as astrogin-1b, is one of two known Mafbx isoforms, and derives from the alternative splicing of muscle atrophy f-box-α gene (*mafbxa*). It is an "E3 ubiquitin ligase which plays important roles in myogenesis and muscle atrophy". The gene is expressed in most Atlantic salmon tissues, in contrast to mammals. Furthermore, it is heavily upregulated upon fasting and downregulated after refeeding, in the same manner one expects atrophy to be regulated (63).

The natriuretic peptides (NPs), Bnp and Anp, are cardiac hormones which "play important roles in the regulation of circulatory and fluid homeostasis in vertebrates" (64). *NP*s can be used as a diagnostic marker of cardiac pathology in salmonids as it signals maladaptive pathological cardiac remodeling and hypertrophic growth (65-67). In the Frisk et al. (65) study, "fast smolt" (short production time, 11 months) experienced these signs together with higher *NP* expression compared to "slow smolt" (17.7 months).

Pcna is a DNA clamp that is necessary for rapid and processive DNA synthesis (68). As the name implies, levels of Pcna increase during cell proliferation. Cardiac mRNA levels of *pcna* can therefore be used to differentiate between hypertrophic and hyperplastic muscle growth in the heart (65, 67).

Vegf is a signal protein that stimulates the formation of blood vessels by promoting proliferation and migration of endothelial cells. It can therefore be used as an angiogenesis marker. It plays an important role in vascularization and vasodilation in the fish (65, 69). In the

Frisk et al. (65) study, fast growing smolt had increased expression of *vegf* which could possibly serve to support a thicker layer of compact ventricular myocardium. Furthermore, Jørgensen et al. (69) showed in their study that salmon reared at elevated sea water temperature, also had increased expression of *vegf*. They hypothesized that high water temperatures caused more vascularization and vasodilation because of increased oxygen demand.

1.5.2 Immune related genes

Samdc is an enzyme that decarbozylates Sam, which is necessary for the synthesis of polyamines (PA) such as spermidine and spermine. PA plays an important role in regulating cell growth, proliferation, and differentiation among other things. High levels of PA are for instance thought to be beneficial during bacterial infections. Ssat is closely linked to Samdc since it regulates PA turnover by acetylating polyamines, which is energy costly for the cells. Toxins, hormones, cytokines and stress can increase *ssat* expression and an overexpression might result in pathogenesis and the formation of reactive species of oxygen (ROS). Both Samdc and Ssat have therefore a big impact on the skeletal muscle development, health and immune system of the salmon (70-72).

Mmp9 & Mmp13 are zinc-dependent endopeptidases. While Mmp13 is "very efficient in degrading type II collagen", Mmp9 can act as both collagenase (on collagen IV) and gelatinase (73). Both Mmp9 & Mmp13 are "involved in the degradation of the extracellular matrix" (ECM), "cleavage and activation of cytokines, release of cytokines and growth factors from ECM, and establishment of a gradient for migration of cells". They are quiescent in healthy salmon, but quickly upregulated after injury or disease in most tissues except liver. Fish with chronic inflammatory response, bone deformities, chronic wounds etc. typically exhibit high expressions of *mmp9* & *mmp13* in the damaged sites (74, 75).

Arg2 is a mitochondrial arginase isoform which catabolizes arginine. It is expressed in most tissues and can be used as a marker for fish M2 macrophages, which are anti-inflammatory. The alternatively-activated (polarized) M2 macrophages are developed in response to "parasite-related stimuli" and they have "increased arginase activity, associated decreased microbicidal activity, and increased production of collagen and polyamines for cell growth and healing processes" (76). They have been observed in the heart of salmon recovering from virus infection, indicating an "onset of regenerative processes" (77).

Drtp1 is part of the Ly-6/uPAR protein family. It seems that it is important for inflammation and “might function as a modulator of the nicotinic acetylcholine receptor”. Upregulation has been observed during acute phase response, stress, and bacterial infections (78). Drtp1 resembles CD59 which inhibits membrane attack complex formation and was taught to be a homologue at first (79). The whole function of the protein is not fully understood yet.

mIgM is a membrane-bound antibody presented on B-cell surfaces and plays an important part in the humoral immune system. If an antigen binds to mIgM, activation of B-cell and consequently clonal expansion and specific antibody production, can occur (80). *mIgM* seems to be one of the most expressed immunoglobulins during an infection in Atlantic salmon. There is probably a lot of other variables also affecting mIgM concentration like vaccination status and life history (81, 82).

1.5.3 Housekeeping genes

In addition to the forementioned genes, *elongation factor 1-α* (*ef1a*) and *β-actin gene* (*actb*) were picked out as reference genes. They are commonly used as endogenous standards to normalize target genes, which is necessary to compensate for variations during quantitative real-time PCR (qPCR). Ef1a is a cytoskeleton protein, while Actb is a GTPase, both housekeeping molecules. They are relatively stable, especially *ef1a*, but regulation can happen under some circumstances. The suitability of these two genes as reference genes therefore depends on the experimental conditions (83-85).

1.6 Objective

As mentioned, multiple studies have been conducted on how early life stages affect fish physiology. Especially temperature seems to have profound effects on many aspects of fish health and performance. However, there is a lack of knowledge regarding how incubation temperature affects heart physiology and immunocompetence. An unpublished master's thesis by Rønningen (86) has explored some aspects of cardiac health and incubation temperatures, but lacked histological relevance according to Frisk et al. (65) paper, and immunocompetence analysis. Furthermore, little is known about the cardiac response against *Y. ruckeri*, even though it is a growing problem in the industry. To reduce these knowledge gaps and confirm previous observations, this thesis will explore following questions:

1. To outline histological changes in cardiac tissue post *Y. ruckeri* infection

2. How incubation temperature may induce morphological changes in the parr heart.

3. How incubation temperature affects immune response in cardiac tissue of parr during a *Y. ruckeri* infection.

This thesis will broaden the understanding of how early life environment affects farmed salmon cardiac health and provide the farmer with knowledge on how to improve fish welfare and performance.

2 Material and methods

2.1 Ethical statement

All fish handling and sampling were done in compliance with Norwegian laws and regulations (9). The experiment was approved by the local animal research authorities (Norwegian Food Safety Authority, FOTS ID: 29685) according to Norwegian legislation. The fish were euthanized and autopsied during selected timepoints during the trial period. Inspection was limited to twice a day excluding sampling. Fish were fed continuously but fasted before and after translocating to minimize the potential stress induced upon the fish. Fish used for sampling were immediately transferred to a euthanistic bath to minimize stress and pain.

2.2 Husbandry

The fish were gradually moved to bigger tanks during the project to accommodate their growth. The fish stayed in circular tanks equipped with flow-adjustable freshwater inlets, sensors for temperature and dissolved O2 (DO) and emergency oxygenation equipment, enabling an optimal environment. Water parameters were registered during the whole experiment and had stable and expected levels. Husbandry was carried out by professional on-site personnel.

2.3 Experimental setup

Experimental fish from a high breeding value strain (SalmoBreed, Benchmark® Genetics) were used. The fish stemmed from 10 separate male-female pairings from a full-siblings family. The fish started as 18000 fertilized salmon eggs (eggs and milt bought from Benchmark® Genetics) which were randomly mixed and distributed between tanks. *NB*: most of the fish were used in other parts of the COOLFISH-project. The eggs were subjected to two different incubation temperature regimes, 4 ℃ and 8 ℃, creating two groups which were divided into triplicates. After the 4 ℃ group reached the eye-stage (320 DD), temperature was increased to 8 ℃, equalizing water temperatures and creating similar environmental conditions for both groups. after reaching ca. 6 g, fish of the respective triplicate groups were combined again, and temperature increased to 10-11 ℃. Each group was transported separately to a new tank set up at ~2530 DD (post fertilization), which resulted in the 8 ℃ group being transferred 36 days before the 4 ℃ group. 600 fish from each group were distributed equally between 6 tanks, amounting to 12 tanks and 1200 fish in total. Per group, three of the tanks were used for infection, while the other three were uses as controls, creating four triplicate groups in total: Control 4 ℃ (CON4), Control 8 ℃ (CON8), Infected 4 ℃ (INF4) and Infected 8 ℃ (INF8).

Infection took place after 10-11 days of acclimation. Sampling and measurements took place 0-, 2-, 7- and 13-days post infection (dpi) (0 dpi = day before infection) (**Figure 4**).

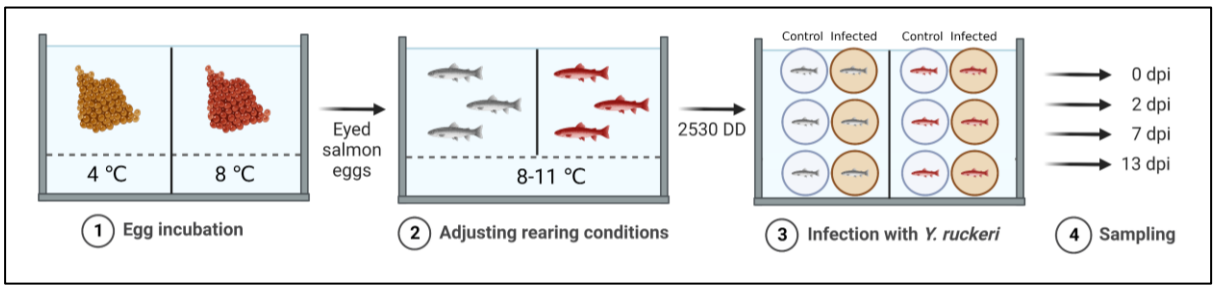

Figure 4: Experimental setup. 1) Egg incubation from fertilization to the eye-stage (320 DD). Eggs were divided into two temperature groups and received either 4 °C (yellow eggs) or 8 °C (red eggs) heated water regimes. 2) Rearing conditions during eye-stage, hatching and juvenile stages up to 2530 DD. Both the 4 °C (gray fish) and 8 °C (red fish) temperature groups received 8 °C water in the beginning before temperature was increased to 10 °C. Towards the end, temperature was increased to 11 °C. 3) Both temperature groups were divided into triplicates and moved to new separate tanks. Half of the fish were infected while the other half was not, creating four triplicate groups: CON4, CON8, INF4 and INF8. All fish received the same water (11 °C) from one common source. 4) Sampling was done the day before infection and certain days after infection (2, 7 and 13 dpi).

2.4 Experimental procedure of infection

2.4.1 Bacterial strain

Yersinia ruckeri was chosen as the infectious pathogen in the COOLFISH-project and therefore also used in this experiment. The bacteria can be used as a model organism when studying immune response. Furthermore, yersiniosis is an upcoming problem in salmon aquaculture and there is a need to learn more about the bacteria and the disease. The strain used in this experiment was *Y. ruckeri* 2014-70 F646 (serotype O1), which was isolated from the head kidney of infected Atlantic salmon and preserved in glycerol at -80 °C. Motility was observed in the microscope. Bacteria stock was originally received from the Veterinary Institute in Harstad on 20.02.15.

2.4.2 Preparations and infection

Frozen bacteria cultures from stock were put on blood agar and incubated at 12 °C until colonies were big enough for transfer. Six bottles of 25 ml liquid pre-culture were then made by transferring 3 single colonies from the blood agar in to 25 ml marine broth and incubated while shaken moderately for 20 hours at 12 °C. From the pre-cultures, 14 ml were transferred in new bottles together with 200 ml marine broth. A total of eight bottles were made in total and incubated for 20 hours at 12 °C. On the day of infection, optical density 520 nm (OD520) was measured and 6-8 of the bottles were mixed depending on the result (OD520 1:10 = 0.82). To make the infected solution, the mixed solution (distributed between centrifuge bottles) was

centrifuged to form bacterial pellets, and the liquid emptied and replaced with Gibco™ 1x Dulbecco's phosphate buffered saline (dPBS) in which the pellets were dissolved. OD was measured and confirmed to be acceptable (OD520 1:10 = 1.0). An OD520 value of 0.5 equals ca. 10^8 bacteria/ml. Pure 1x dPBS was used as control.

100 fish from each tank (V = 500 liter) were netted and moved to their respective buckets containing 40 liters of 11 ℃ still water. The temperature was maintained using water flowing around the buckets, and oxygen was provided via an air stone to keep dissolved oxygen levels above 85 %. 400 ml of dPBS was given to each control group, while the infected groups received 400 ml infected solution. After 4 hours, fish were transferred back to their respective tanks (with clean, circulating water). A ten-fold dilution series (titration) with bacterial streaks on blood agar were made from the infected and control solutions, as well as tank waters ~30 seconds after infection.

2.4.3 Sampling

For the histology, twelve fish from both temperature groups were sampled at 0, 7 and 13 dpi (72 fish in total). For the gene analysis, eighteen fish from both temperature groups were sampled at 0 dpi, while twenty-four fish from each temperature group were sampled at 4, 7 and 13 dpi (180 in total). The fish were caught by net and euthanized by benzocaine overdose (1 ml benzocaine to 1 liter water). An open weight scale with a built-in metric ruler was used to gather both weight (body mass) and length (fork to snout) measurements quickly. Tools such as scalpel, tweezers, and scissors were used to dissect and collect tissues. The main parts of the heart, ventricle and bulbus arteriosus, were sampled by grabbing the outermost part of bulbus arteriosus with a pair of tweezers and pulling it out with the ventricle still attached. This method proved to be fast, accurate and gentle when atrium and sinus venosus were not needed. For the histology analysis, ventricle and bulbus (henceforth termed "heart") were blotted for blood in PBS for a couple of minutes before being put in BiopSafe® Biopsy Container, 60 ml. Hearts for qPCR analysis were immediately put in 2 ml micro tubes containing 900 µl RNA*later*™ Stabilization Solution (cat.nr: AM7021) after sampling. Histology and qPCR samples were stored at 4 ℃ and -20 ℃, respectively.

2.5 Histology

2.5.1 Preparations and measurements

Heart samples in formalin were transferred to 70% ethanol. The hearts were taken out of the containers and photographed from the ventral projection (heart laying upside down, with BA at

the highest position) on the lab bench with a Motorola Moto G7. The mobile phone rested at a fixed height and position, while the hearts were placed on the same spot underneath, on a plastic cutting board with a metric ruler for scaling. Ventricular height and width from the ventral projection (**Figure 5**) was measured with ImageJ® computer tool (87). A scalpel was then used to cut open the hearts (laying in the same position), from the BA to the apex (tip). The cut was made slightly to the right from the middle, resulting in one piece having more of the BA and ventricle. The heart was not cut completely through, letting the two pieces stay attached to each other and making further operations easier. The cut hearts were placed in cassettes and put back in 70 % ethanol.

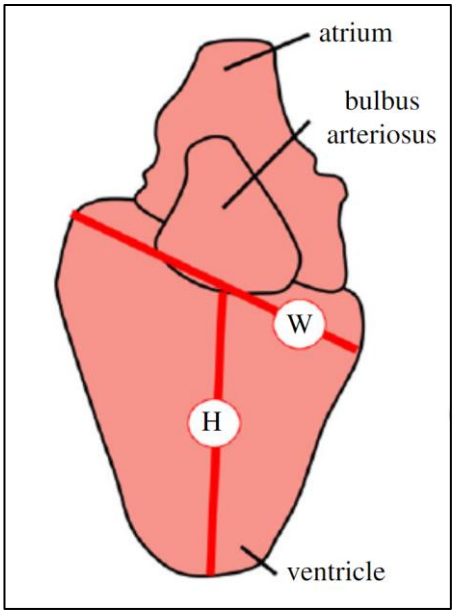

Figure 5: Schematic illustration of salmon heart and two measurements of length. Line H represents height, and line W represents width. Reprinted from Perry et al. (88) (CC BY 4.0).

2.5.2 H & E histology slides

The samples were subjected to a series of dehydration, clearing and deparaffinization processes using the Leica® TP1020 Automatic Benchtop Tissue Processor (cat.nr: 14 0422 80101). (**Appendix table 1**). Leica® EG1150 H Modular Tissue Embedding Center and Leica® EG1150 C Cold Plate were used to make paraffin embedded tissue blocks. A Leica® RM2165 Rotary Microtome was used to make 5 µm tissue sections from the blocks. The sections were put in Leica® HI1210 Water Bath (cat.nr: 14 0415 80101) at 40 °C before collecting them onto Surgipath® pearl™ Snowcoat® Precleaned Micro Slides, white (cat.nr: 3809299). Slides were allowed to dry on Adamas Instruments® slidewarmer SW 85 at 45 °C before transferring them

to an oven set at 37 ℃ overnight (15 h). Next day, slides were Haematoxylin & Eosin stained using Leica® ST5010 Autostainer XL (cat.nr: 14 0456 80101) (**Appendix table 2**) and a coverslip was mounted using Leica® CV5030 Fully Automated Glass Coverslipper (cat.nr: 14 0478 80101). Slides were allowed to dry overnight.

2.5.3 Detection of *Y. ruckeri* by immunohistochemistry (IHC)

A selected number of samples were subjected to immunohistochemical detection of *Y. ruckeri*: 2 control samples and 1 infection sample from each temperature group and two timepoints7 and 13 dpi) (12 in total + 1 negative control). The sections were mounted onto Leica® BOND™ Plus Slides (cat.nr: S21.2113.A), warmed on the slide warmer, and incubated at 60 ℃ (1 h). They were deparaffinized using Leica® TP1020 (**Appendix table 1**), before putting them in a 1% PBS bath. Meanwhile, solutions were prepared. The primary antibody used was 1:2000 Anti-*Y. ruckeri*, Clone 4B12/F8; Lot 0Y7381MS, ref FM-050AW; Ango, CA, USA, and the secondary antibody was Envision+ System- HRP, labelled polymer, anti-mouse (Ref K4001, Lot 11462074; Dako, Denmark). EnVision FLEX, HRP Magenta Substrate (Dako Omnis) was used for color production while contrast staining was done with hematoxylin. For the 12+1 IHC slides, 0.75 µl primary AB was mixed with 1462.5 µl TBS and 37.5 µl BSA to make 1:2000 dilution. The following steps (and more details) were done according to Nofima's internal protocol (**Appendix table 3**). The next day, slides were mounted manually with glue and glass. Both the normal and IHC slides were scanned with Leica® Aperio CS2 and processed with Leica® Aperio Image Scope – Pathology Slide Viewing Software version 12.4.6.

2.5.4 Histology data analysis

The scanned slides files were transferred and converted into Qupath version 0.4.3 (89). The total ventricle area was marked by using the "wand tool". The marked area included ventricular lumen, epicardium (if intact, non-inflamed, and connected to compactum) and excluded fat and large outer blood vessels. The bulbar and atrioventricular valve that were sometimes visible as an area void of tissue, were also included by marking the area between the two opposite edges of tissue. Compactum thickness (diameter) were measured at nine points, preferably equidistant from each other, with the "line annotation" function and the average was calculated. Ventricle length and width were measured from the pictures by using "line annotation" in ImageJ®. Ventricular height : length ratio was calculated by dividing height with width. Relative compact ventricular myocardium thickness (rel. compact thickness) was calculated by dividing the average compactum diameter with ventricular area (65). Ventricular height : width ratio would

reveal roundness and elongation/shortening of the hearts, while rel. compact thickness could show hypertrophic growth in the compactum, both used as markers for maladaptive cardiac remodeling.

2.6 Gene expression

2.6.1 qPCR of heart samples

First, each sampled heart had their BA and atrium remains removed. ~10 mg of the ventricle was then extracted. For big hearts, a portion would be cut off starting from the bulbus to the apex (tip) to achieve the necessary weight. The samples were then placed in VWR Empty FastPrep® 2ml Lysing Matrix tubes (cat.nr: MPB 115076200) together with Thermofisher 1.4 mm Zirconium oxide beads (cat.nr: KT03961-1.103.BK) and lysis buffer from the Applied Biosystems™ MagMAX™ mirVana™ Total RNA Isolation Kit (cat.nr: A27828), and homogenized in a MP Biomedicals™ FastPrep-96™ High-throughput Bead Beating Grinder and Lysis System (cat.nr: 116010500) for 2x 60 sec at 18000 rpm. 100 µl lysate from each sample were used for RNA isolation, which was done with the MagMAX™ mirVana™ Kit following manufacturer's protocol (thermofisher.com). RNA quality and concentration were examined with NanoDrop™ 8000 Spectrophotometer (cat.nr: ND-8000-GL) and Agilent 2100 Bioanalyzer (cat.nr: G2939BA). Each well was diluted separately to achieve 22.2 ng/µl RNA concentration. 9 µl (200 ng RNA) from each well was then used for cDNA-synthesis using the Applied Biosystems™ High-Capacity RNA-to-cDNA™ Kit (cat.nr: 4387406) and Applied Biosystems™ 2720 Thermal Cycler (cat.nr: 4359659) according to manufacturer's protocol (thermofisher.com). cDNA was diluted 1:40 and stored at -20 ℃ before further use. For qPCR, 7 µl cDNA from each well were pipetted over to an Applied Biosystems™ MicroAmp™ Optical 384-Well Reaction Plate with Barcode (cat.nr: 4309849), in duplicates. For controls, 7 µl of no reverse transcriptase control (NRT), no template control (NTC) and positive controls were used (**Appendix table 4**). The plate was either used immediately or stored at -26 ℃. 13 µl of a reaction mix consisting of 10 µl Applied Biosystems™ Fast SYBR™ Green Master Mix (cat.nr: 4385612), 1.2 µl forward primer (5 µM), 1.2 µl reverse primer (5 µM) (**Table 2**) and 0.6 µl Invitrogen™ Nuclease-Free Water (cat.nr: AM9932) (NUC) were added to each well. If the controls showed a primer dimer problem later, both primer volumes would be reduced to 0.6 µl, and NUC increased to 1,8 µl. The plate was sealed with Sarstedt Inc PCR film (cat.nr: 95.1994), centrifuged shortly ("short" button: 2000 rpm for ~1 second), and cycle threshold (Ct) values measured with QuantStudio™ 5 Real-Time PCR System (cat.nr: A34322); the recommended settings for Fast SYBR™ Green were used.

Table 2: List of primers successfully used in this experiment. FW = forwards primer, RW = reverse primer. Primers were partially designed by Erik Burgerhout and by using primers from stated references as templates. *ef1a* and *actb* were used as reference genes to normalize the expression of the other target genes.

Primer	**Gene sequence (5-3')**	**Reference**
ef1a FW *ef1a* RW	CGCCAACATGGGCTGG TCACACCATTGGCGTTACCA	Julin et al. (90)
actb FW *actb* RW	CAGCCCTCCTTCCTCGGTAT CGTCACACTTCATGATGGAGTTG	Robinson et al. (91)
mafbxb FW *mafbxb* RW	GGTCAGAGCTGGGTTAAAACTG TCTCTTTGTGGAAATCTCACG	Bower et al. (63)
bnp FW *bnp* RW	GCCGCCATAAGAGAGTTCCT CATCTTGTCTTTGCATTGTGTTTGC	Frisk et al. (65)
anp FW *anp* RW	GGCCCAATTGAAGAATCTACTG GCACCCGGACATAGCTTTAC	Frisk et al. (65)
pcna FW *pcna* RW	TGAGCTCGTCGGGTATCTCT CTCGAAGACTAGGGCGAGTG	Frisk et al. (65)
vegf FW *vegf* RW	AAACCACTGTGAGCCTTGCT CTCCTTGGTTTGTCACATCTGC	Frisk et al. (65)
samdc FW *samdc* RW	CTGTCCAGCCTTGCCCCGTG GGGCCCTGGACTAGTGGGCA	Andersen et al. (92)
ssat FW *ssat* RW	TCG TGG CGG AAG TCC CCA GT GCC GAT GCC AAA CCC CCT GT	Andersen et al. (92)
mmp9 FW *mmp9* RW	ATACGGAGCTGGCAGAGAGCTA AAGGTCTGGTAGGAGCGTACAT	Krasnov et al. (93)
mmp13 FW *mmp13* RW	AGTGTCCAGCACAAATGACCT CTCAACTGCTGATCCACTGGT	Robinson et al. (91)
arg2 FW *arg2* RW	GACAGAAAAGGAGAGGGGTGGA GAAGCTCAGGTCTCCAAAATCAT	Krasnov et al. (93)
drtp1 FW *drtp1* RW	ACCTAAAGGAAGTACAATCAGATCA ACAACGGTTGCATTTCAGACCC	Robinson et al. (91)
mIgM FW *mIgM* RW	GGTCCTTGGTAAAGAAACCCTACAA CTGCATGGACAGTCAGTCAACAC	Krasnov et al. (93)

2.6.2 Gene expression analysis

Based on previous research and Nofima's internal controls, primer efficiency was 90-105%. Relative method of quantification was calculated in excel by using the $2^{-\Delta\Delta Ct}$ method (94). The two reference genes, *ef1a* and *actb*, were used to calculate geometric mean and normalize all Ct-values of target genes. The arithmetic mean of all 0 dpi ΔCt values (uninfected, both temperature groups) of the target gene was used as the calibrator.

2.7 Statistical analysis

All processed data ($2^{-\Delta\Delta Ct}$, general measurements and histology data) was organized and processed in Excel (95). GraphPad Prism version 9.3.1 (2023) was used for statistical post-hoc analyses and graphing (96). First, all data sets were checked for outliers using Grubbs' method, and outliers removed if it purposeful. An impossible value such as a negative fork length would be an example of a non-desired outlier. When it comes to qPCR, it is difficult to decide whether an extreme value, low or high, is due to a source of error or an actual extreme transcription in the tissue. There was a high variability of clinical symptoms in the fish, which could result in large differences in transcription. It was therefore decided that outliers would not necessarily be removed. Data was analyzed for skewness and kurtosis and if values were not approximately in the interval $[-1, 1]$ and $[-2, 2]$, respectively, data would be recognized as not being from a normal distribution. Data was also analyzed with Shapiro-Wilk ($n < 50$) and Kolmogorov-Smirnov ($n > 50$) normality tests. If skewness, kurtosis, or the normality test indicated a non-normal distribution, a log transformation ($X = \log(X)$) would be used to see if data could be normalized. For normal distributed data, a parametric unpaired t test would be used to compare two groups at a time for each timepoint to look for significant differences. For a non-normal distribution, the non-parametric Mann-Whitney test would be used. Both tests were done with the Holm-Šídák method. Comparison between timepoints for each group were done using a mixed-effects model (REML) analysis and Tukey's multiple comparisons test. Significance level (alpha) was set to 0.05 for all analyses. The groups compared were CON4 vs. INF4, CON8 vs. INF8, CON4 vs. CON8, and INF4 vs. INF8. Data was presented as boxplots with 25th percentile, the median (the 50th percentile), the 75th percentile, and whiskers set to minimum and maximum values.

3 Results

3.1 General observational findings post *Y. ruckeri* infection

Before performing infection, we observed that during netting and transfer of fish to new tanks/buckets, the fish in all groups appeared to be quite stressed, based on high activity. No immediate change happened while pouring the infected and control solutions in their respective tanks, however, it was noted that after a couple of minutes, the fish receiving the bacteria became even more stressed judging by visual symptoms. They swam erratically, were easily spooked, and had a higher ventilation rate. Afterwards, the infected fish slowly became more relaxed, but still appeared more stressed than the control fish at the end of the 4-hour infection period. The results showed increased amounts of bacteria in the water after infection and many colonies appeared to be *Y. ruckeri* (colony morphology) when streaked on blood agar plate, indicating successful presence of live pathogens in the waters. Blood agar also showed quite similar amounts of colony-forming unit (CFU) from the infected liquid and in the tank waters (~30 sec. after infection) between the two temperature groups (**Table 3**). During the following weeks, clinical symptoms started to appear in some of the infected fish, such as lethargic swimming, slow reaction-time, darker skin pigmentation, exophthalmos, and hemorrhages on the snout, eyes, area behind the maxilla, internally in the mouth and on the fin-bases. Obduction showed some organs with hemorrhages, discoloration, and swellings (**Figure 6**). These signs match previous observations in infected rainbow trout (61). Control fish showed no obvious clinical sign of yersiniosis, but some had exophthalmos (or eye damage), also 1 pre-infection the 4 ℃ group. This could stem from mechanical damage or a previous infection. However, it was noted that infected fish with exophthalmos often had quite a lot of other symptoms such has dark pigmentation and lethargic swimming. It is unknown if fish with ongoing exophthalmos condition were more susceptible to an infection, or if infected fish developed exophthalmos as a symptom (or both). Short operculum, a non-pathological, chronic condition was observed in both temperature groups. Spine, head, and heart deformity were observed in different fish and only in the 8 ℃ groups. Mortality was reported to be 1.0%, 17.2%, 1.0% and 33.8% in CON4, INF4, CON8 and INF8, respectively, during the trial period (0-13 dpi) (**Table 4**). *NB*: Nofima did a PCR-test with *Y. ruckeri* specific primers on four head kidney samples from the infected groups, which detected the bacteria successfully.

Table 3: CFU measured in blood agar plates. Streaks were done from the infected solution and in tank waters ~30 sec. after infection. CFU from the infected solution is a mean value calculated from duplicate plates. CFU from infected tank water is a mean value calculated from all three infected buckets which were done on duplicate plates (6 in total per temperature group). 10^5 and 10^6 are the dilution factors. *NB*: the bacterial streak from the 8 ℃ groups was not done perfectly (too much and hard swiping), as seen by colonies growing on the side of the plates, which implies that the CFU was lower than the actual bacteria number. The streak quality gradually improved during the project and was much better when doing streaks for the 4 ℃ groups.

Temperature groups	CFU infected liquid (10^6)	CFU infected tank water (10^5)
4 ℃	100	1212
8 ℃	62	480

Table 4: Deformities and symptoms of chronic conditions or infection. Data is from all fish (528 individuals) sampled during the trial period, including fish used in other COOLFISH-projects. Some values are probably an underestimation since some abnormalities are easily overlooked during extensive sampling. The hearts had different degrees of deformities and only the most obvious cases were noted.

Group	Eye damage (e.g., exophthalmos)	Mortality (%)	Short operculum	Heart deformity	Spine or head deformity
CON4	4	1.0	11	0	0
INF4	2	17.2			
CON8	0	1.0	5	2+	3
INF8	1	33.8			

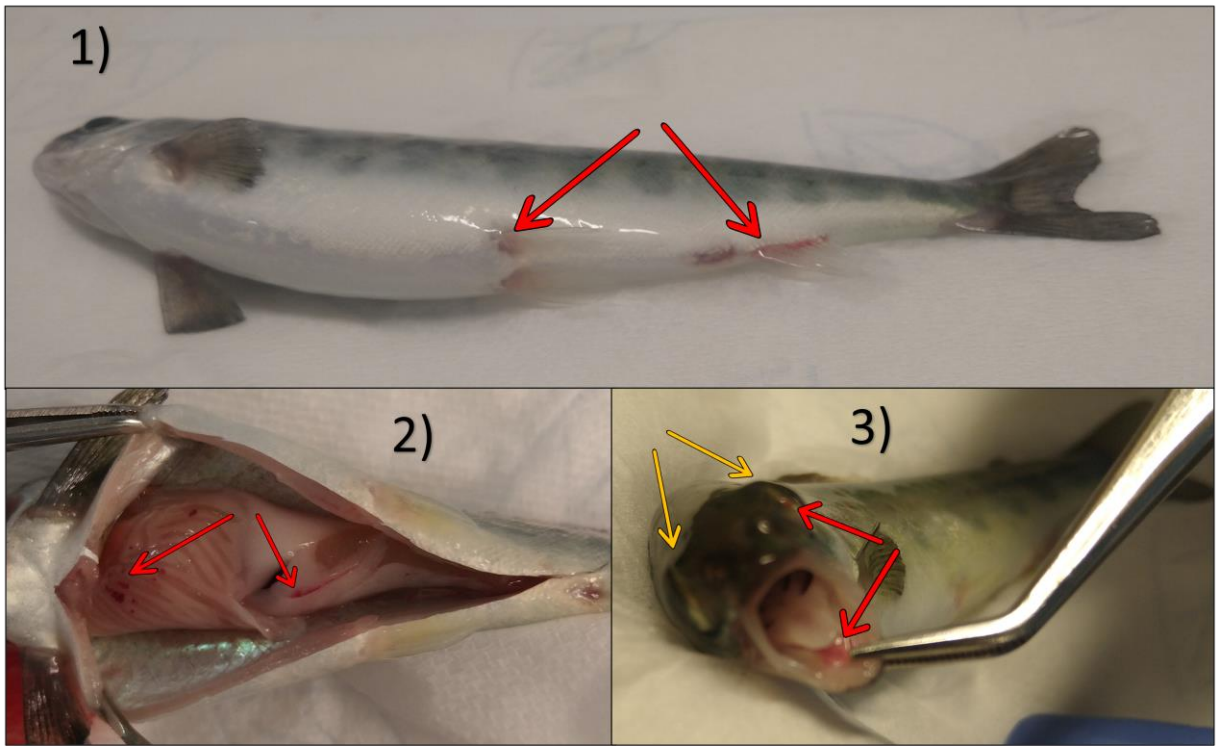

Figure 6: Infected parr. 1) Hemorrhages on anal and pelvic fin-bases. Signs of beginning smoltification (i.e., light skin, weak parr marks, blackening of fin-tips). 2) Abdominal cavity. Hemorrhages in pyloric caeca and adipose tissue and a slightly enlarged spleen (black organ behind arrow tip). 3) Hemorrhages in mouth area and eyes, and bilateral exophthalmos (yellow arrow). Notice in picture 2) and 3) a slight darkening of skin, which has a slightly rough texture and lacking in mucus compared to the fish in picture 1). For all pictures: red arrow points to hemorrhage.

Body mass and **Fork length**: using Mann-Whitney test; no significant differences ($P > 0.05$) were found between the groups for both body mass and fork length. Through time, only CON4 seemed to grow significantly ($P \leq 0.05$) in length and weight, though all groups had the same growth curve trend. Taking into consideration both mean body mass and fork length during multiple timepoints, growth from highest to lowest during the trial period in order seemed to be CON4 > CON8 > INF4 > INF8 (not significant). **Condition factor**: using Mann-Whitney test; CON4 tended to have higher K than INF4 at 7 dpi, although not significant ($P = 0.05$). CON8 had significantly higher K than INF8 at 13 dpi. CON4 had significantly higher K than CON8 at 0 and 7 dpi. INF4 had significantly higher K than INF8 at 4 dpi. No pattern over time was observed, though the groups' K seemed to move towards the same pattern as body mass and length at 13 dpi (CON4 > CON8 > INF4 > INF8). Overall, the control groups and 4 ℃ groups seemed to have higher K than the infected groups and 8 ℃ groups, respectively. (**Figure 7**).

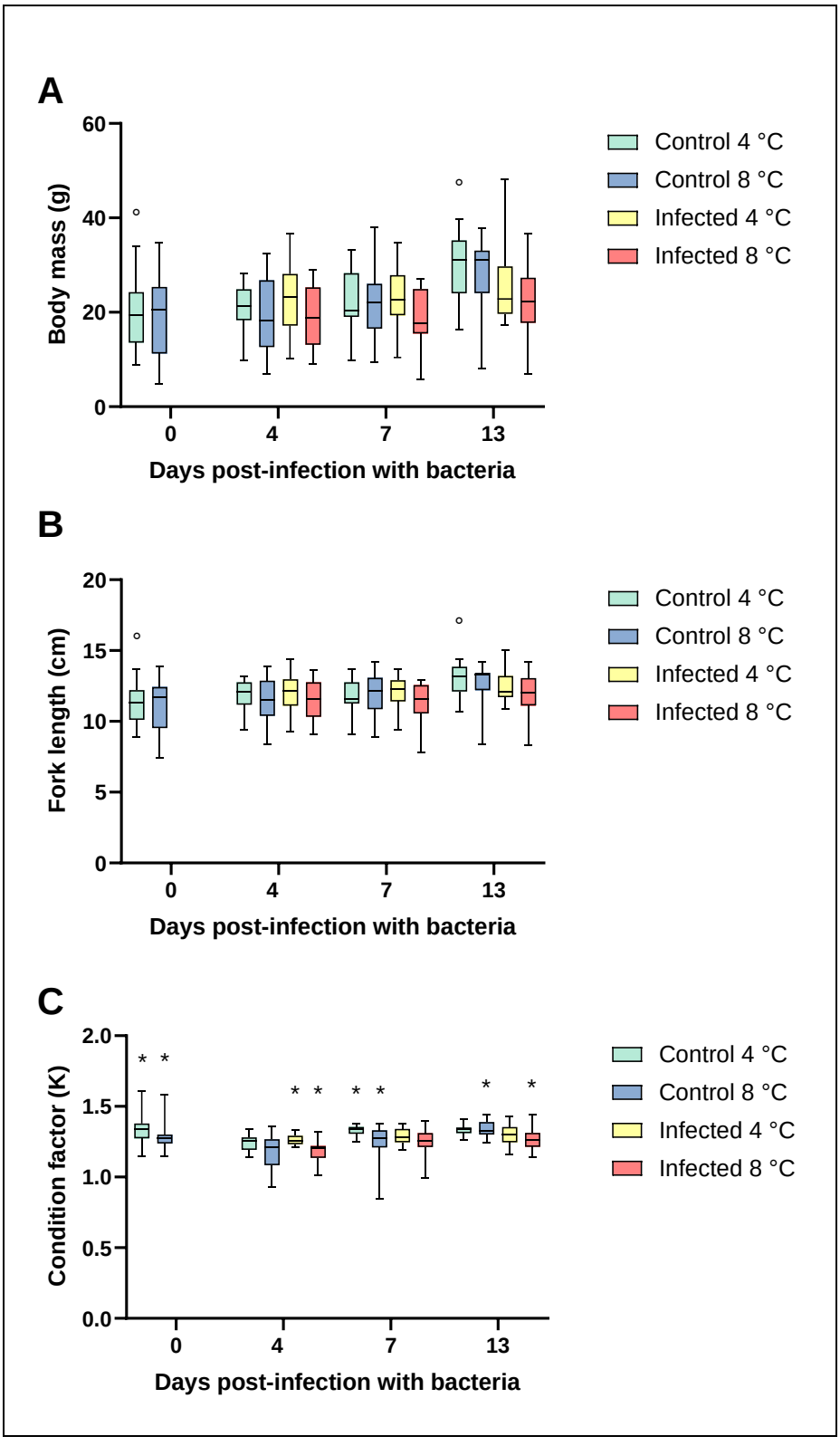

Figure 7: General measurements. A) body mass (n = 244). B) length (n = 243). C) Condition factor (n = 243). The degree sign and asterisk mark represent significant difference between timepoints and groups, respectively.

3.2 Histology

A lot of variation in heart shapes between the fishes were found. The majority had normal triangular-shaped ventricles, while some were more rounded, others elongated, and some had bent or other unusual shapes. Bulbus were observed having different attachment angles on the ventricle and had also different shapes, but to a lesser degree. Heart shape could be affected by contraction at sampling, or be a result of fish size, life history and genetics. The sectioning angles also varied slightly because of heart shape and placement during embedding. The histological slides of the ventricle showed varying pathological signs in the infected fish. The signs included multifocal, hypertrophic, and hyperplastic epithelial cells, separation of the epicardium from the underlying muscle, diffuse focal necrosis in spongiosum, and increased abundance of melanin-colored leukocytes. The most affected samples were observed from infected fish at 7 and 13 dpi and possibly stronger in the 8 ℃ groups. (**Figure 8**).

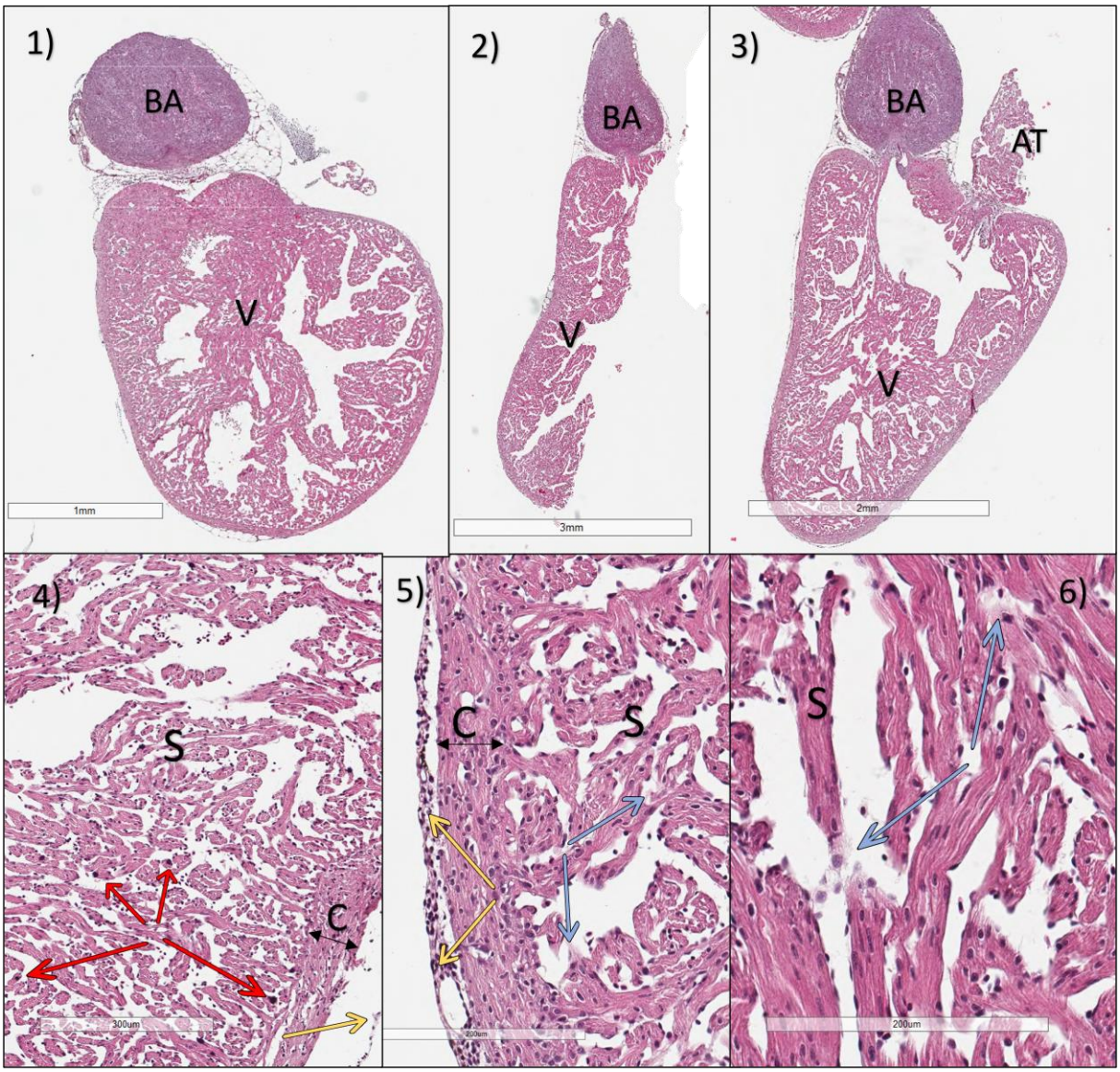

Figure 8: Histological slides of hearts. 1) Rounded ventricle from CON4, 0 dpi. Scale bar: 1 mm. 2) Elongated ventricle from CON8, 7 dpi. Part of the right-side misses, but it was clearly elongated before sectioning (see BA size in comparison). Scale bar: 3 mm. 3) Normal heart from CON8, 0 dpi. Scale bar: 2 mm 4) Infected ventricle from INF8, 7 dpi. Scale bar: 300 µm. 5) Infected ventricle from INF8, 13 dpi. Scale bar: 200 µm. 6) Infected ventricle from INF8, 13 dpi. Scale bar: 200 µm. For all pictures: BA = bulbus arteriosus, V = ventricle, AT = atrium, S = spongiosum, C = compactum. Red arrows points to melanin-colored leucocytes, yellow arrow points to separated epicardium (hypertrophic and hyperplastic in picture 5), and blue arrow points to focal necrosis.

The IHC slides had a little too strong contrast staining than preferred. Positive signals were seen in some of the BA and ventricles of infected fish, but in an extremely low amount. In one of the IHC slides from INF8 at 13 dpi, a part of the ventricle did not catch much staining, but multiple positive signals were observed in this area (**Figure 9**). Whether the positive signal was seen due to weak staining or a source of error in the IHC method is unknown.

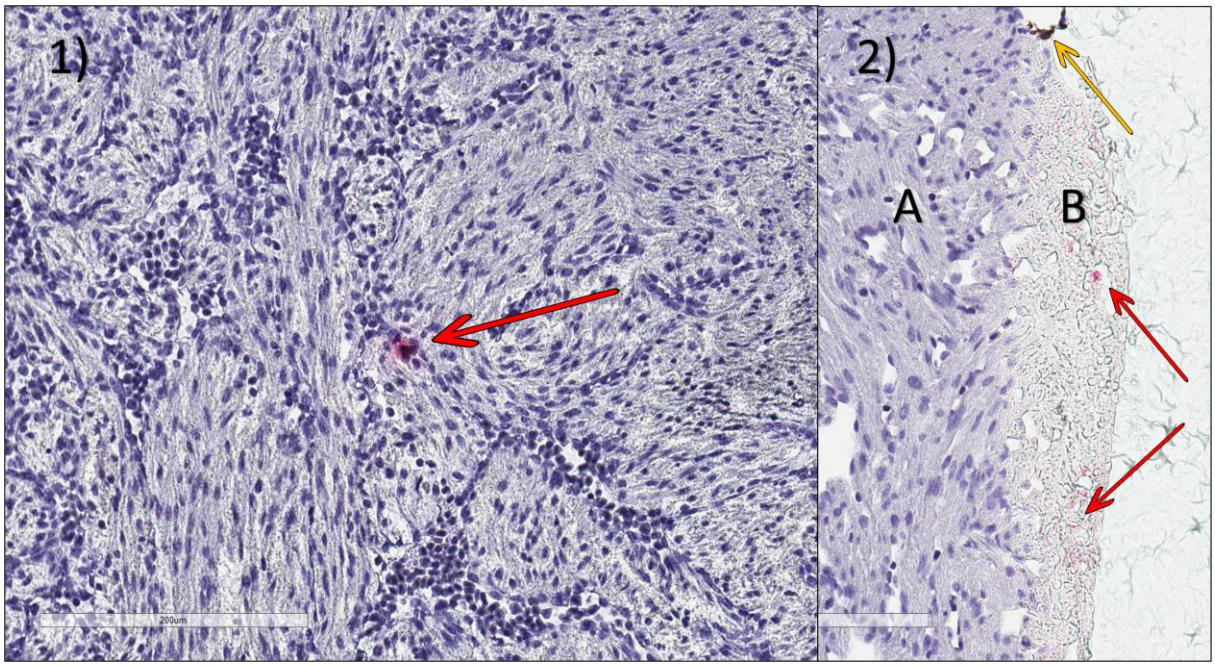

Figure 9: IHC slide from INF8, 13 dpi. 1) Bulbus arteriosus. Scale bare: 200 µm. 2) Ventricle. A = relative weak stained area, B = very weak stained area. Yellow arrow points to a melanin spot possibly consisting of leucocytes and bacteria. Scale bar ≈ 100 µm. For all pictures: red arrow points to positive IHC signals, specific for *Y. ruckeri*.

Ventricular height : width ratio: One outlier with a value of 2.2 coming from a deformed heart in INF8, was removed. Using Mann-Whitney test; CON8 had significantly higher ratio compared to CON4 at 13 dpi. CON4 seemed also much lower at 13 dpi compared to earlier timepoints. No other differences were observed. **Rel. compact thickness**: No differences or trends were observed. Sample size was quite low (n = 49 of 68 individuals) compared to other parameters due to insufficient slide quality and made it not possible to perform some comparisons (**Figure 10**).

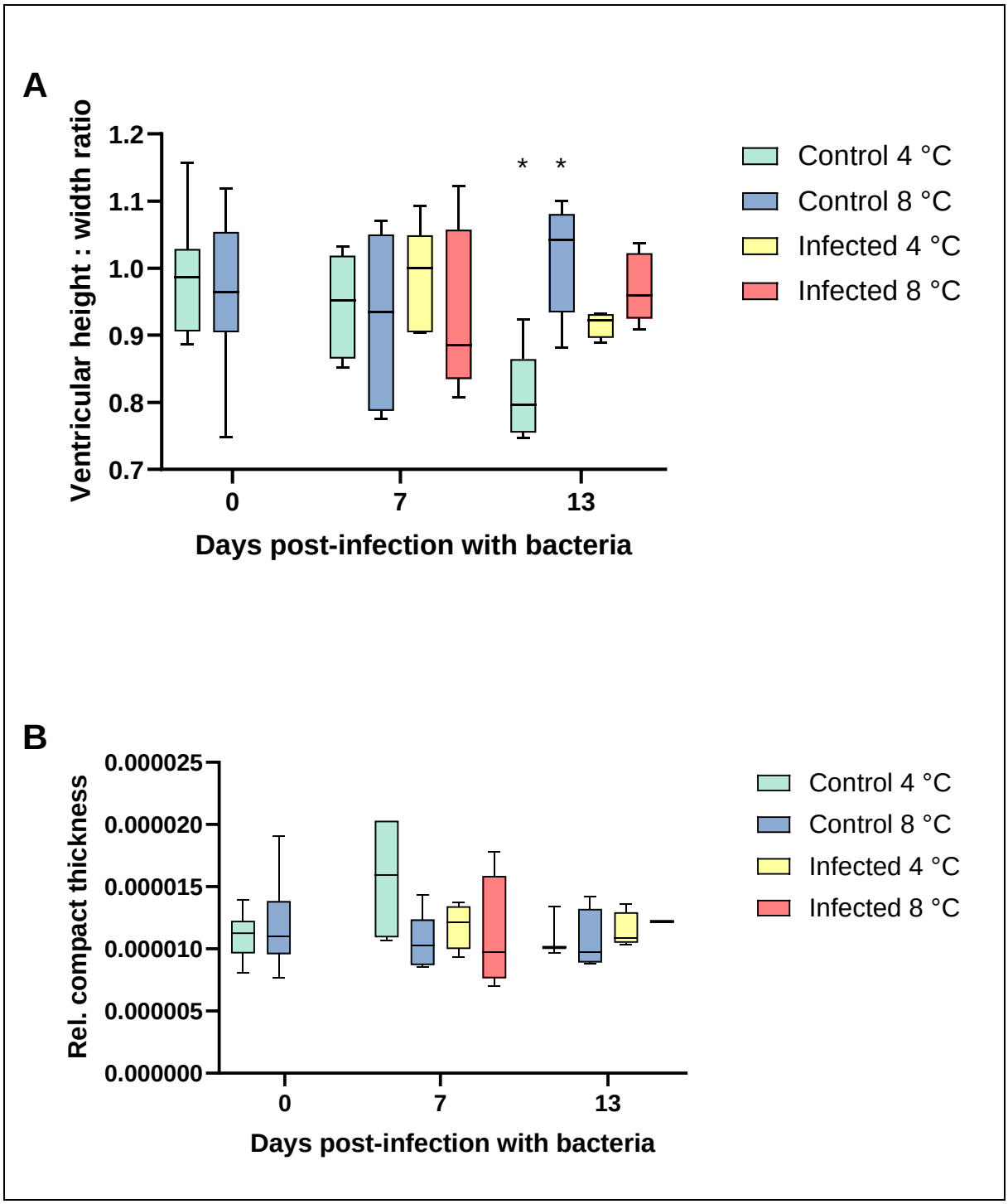

Figure 10: Histological measurements. A) Ventricular height : width ratio (n = 64). B) Relative compact thickness (n = 49). The asterisk mark represents significant difference between groups.

3.3 qPCR results

The isolated RNA samples had an acceptable 260/280 ratio, around ~2.1, and was regarded as sufficiently pure. The bioanalyzer-results showed acceptable electropherogram results and a RIN (RNA Integrity Number) score of 8.3. The reference genes were relatively stable and showed similar expression overall. Geometric mean of the two reference genes was around ~20 Ct. Due to multiple reasons (reagent supply, time, etc.), only some genes were compared at all timepoints (**Figure 11-13**).

mafbxb: Using log transformed data; CON4 had significantly higher expression of *mafbxb* than INF4 at 7 dpi and tended to be higher at 13 dpi, although not significant (P = 0.11). CON8 had significantly higher expression than INF8 at 7 dpi and tended to be higher at 13 dpi, although not significant (P = 0.05). CON8 had significantly higher expression than CON4 at 0 and 7 dpi. INF8 had significantly higher expression than INF4 at 7 dpi. Through time, CON4 seemed unchanging, while the other groups had a tendency towards decreased expression although not significant. Overall, the control groups and 8 ℃ groups had higher expression of *mafbxb* than the infected and 4 ℃ groups, respectively. ***bnp***: using log transformed data; no significant differences in gene expression of *bnp* between CON4 and CON8 at 0 dpi were found. Other timepoints were not checked. ***anp***: CON4 had significantly higher expression of *anp* than CON8 at 0 dpi. Other timepoints were not checked. ***pcna***: using Mann-Whitney test; CON4 had significantly higher expression of *pcna* than CON8 at 0 dpi (other timepoints were not checked). ***vegf***: CON4 had significantly higher expression of *vegf* than CON8 at 0 dpi. Other timepoints were not checked (**Figure 11**).

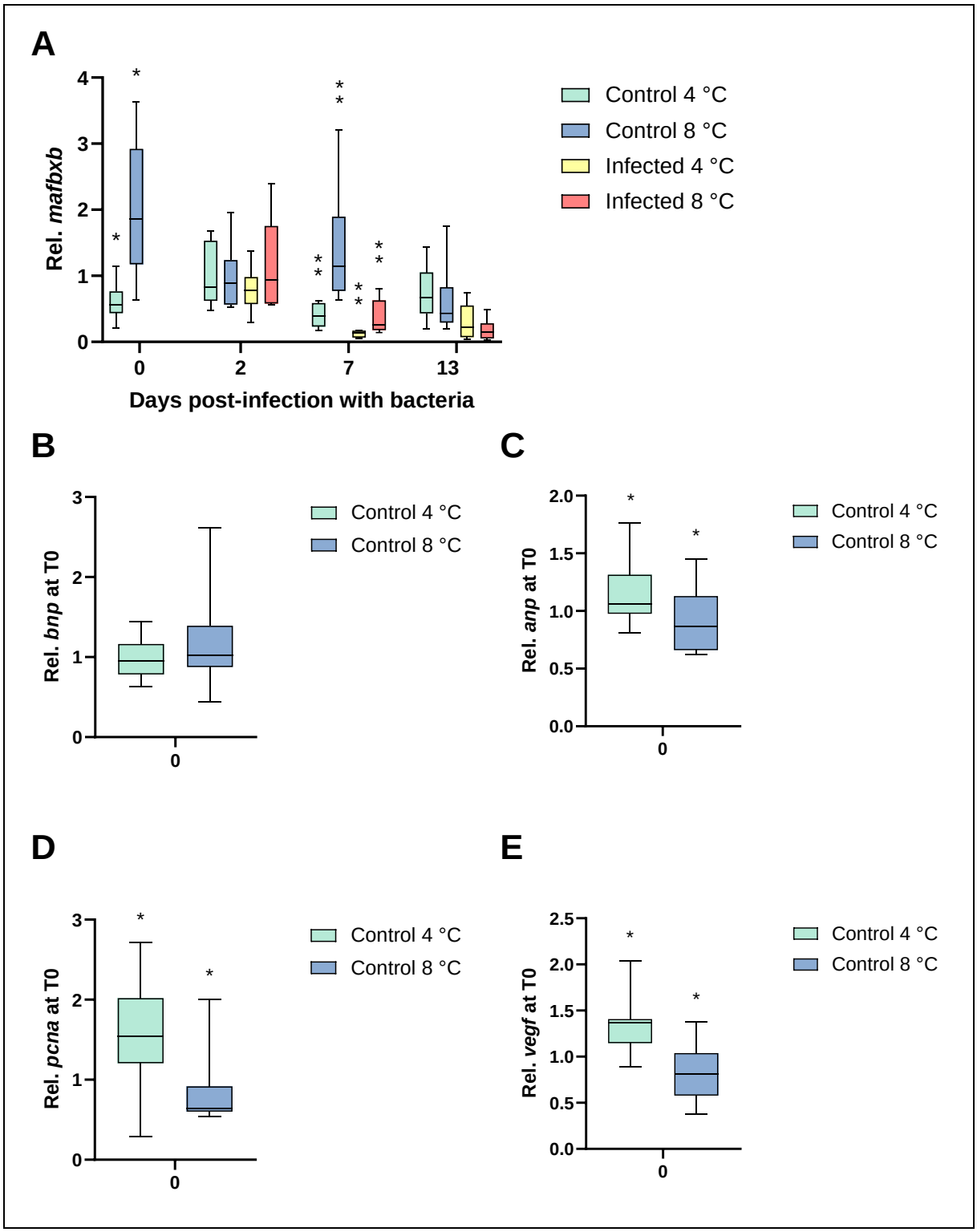

Figure 11: Muscle metabolism and growth-related genes. Relative (rel.) transcript (mRNA) levels (relative to the control genes. A) *mafbxb* (n = 96). B) *bnp* (n = 24). C) *anp* (n = 24). D) *pcna* (n = 24). E) *vegf* (n = 24). Only *mafbxb* has samples from all timepoints, the other genes have only from 0 dpi. The asterisk mark represents significant difference between groups. Notice that all comparisons were significant at 7 dpi. Significant difference between timepoints is not shown.

samdc: using Mann-Whitney test; CON4 had significantly higher expression of *samdc* than INF4 at 7 dpi. CON8 had significantly higher expression than INF8 at 7 and 13 dpi. CON4 had significantly higher expression than CON8 at 0 dpi. No differences between the infected groups were found. Through time, control groups seemed to fluctuate synchronously (significant), while the infected groups seemed unchanging. Overall, control groups had higher expression of *samdc* than the infected groups. ***ssat***: using Mann-Whitney test; CON4 had significantly higher expression of *ssat* than INF4 at 7 dpi and tended to be higher at 13 dpi, although not significant (P = 0.07). CON8 had significantly higher expression than INF8 at 7 and 13 dpi. No differences between CON4 and CON8, nor between INF4 and INF8 were found. Through time, the control groups seemed unchanging, while the infected groups had a significantly decreased expression. Overall, control groups had higher expression of *ssat* than the infected groups. ***drtp1***: using Mann-Whitney test; INF4 had significantly higher expression of *drtp1* than CON4 at 7 dpi. INF8 had significantly higher expression than CON8 at 13 dpi. CON8 had significantly higher expression than CON4 at 7 dpi. No differences between the infected groups were found, but INF8 had the individuals with the highest expression of *drtp1* at all timepoints. Through time, no significant changes in the groups were observed, but the infected groups had some individuals with a strongly increased expression at 7 dpi. Overall, the infected groups and 8 ℃ groups had higher expression of *drtp1* than the control groups and 4 ℃ groups, respectively. Furthermore, some infected individuals showed up to 600-fold higher expression than the median (**Figure 12**). ***arg2***: using Mann-Whitney test; INF4 had significantly higher expression of *arg2* than CON4 at 7 dpi. INF8 tended to be higher than CON8 at 2, 7 and 13 dpi, although not significant (P = 0.12-0.13). CON4 had significantly higher expression than CON8 at 0 dpi, but the position switched at 13 dpi (also significant). INF8 had significantly higher expression than INF4 at 13 dpi. Through time, the control groups had significantly decreased expression at 2 dpi, while the other timepoints had quite similar expressions. Both infected groups had a significantly increased expression from 2 to 13 dpi, however INF4 seemed to have a decreasing trend from 7 to 13 dpi, although not significant (P = 0.08). Overall, the infected groups had higher expression than the control groups, especially INF8. ***mmp13***: Mann-Whitney test; no significant differences in *mmp13* expression were found. However, while most individuals had a fold expression of ~ 0.0-1.0, some individuals had up to 4000-fold higher expression, including both control and infected groups. Furthermore, INF8 tended to have higher expression than INF4 at 7 dpi, although not significant (P = 0.12). In addition, the 8 ℃ groups had the individuals with the highest expression (top three). In other words, the infected groups and 8 ℃ groups may have had a slightly higher expression than the control groups and 4 ℃

groups, receptively, although not significantly. Through time, no obvious changes occurred. ***mmp9*** and ***mIgM***: using Mann-Whitney test; no significant differences between CON4 and CON8 at 0 dpi were found for neither *mmp9* nor *mIgM* expression. Other timepoints were not checked (**Figure 13**).

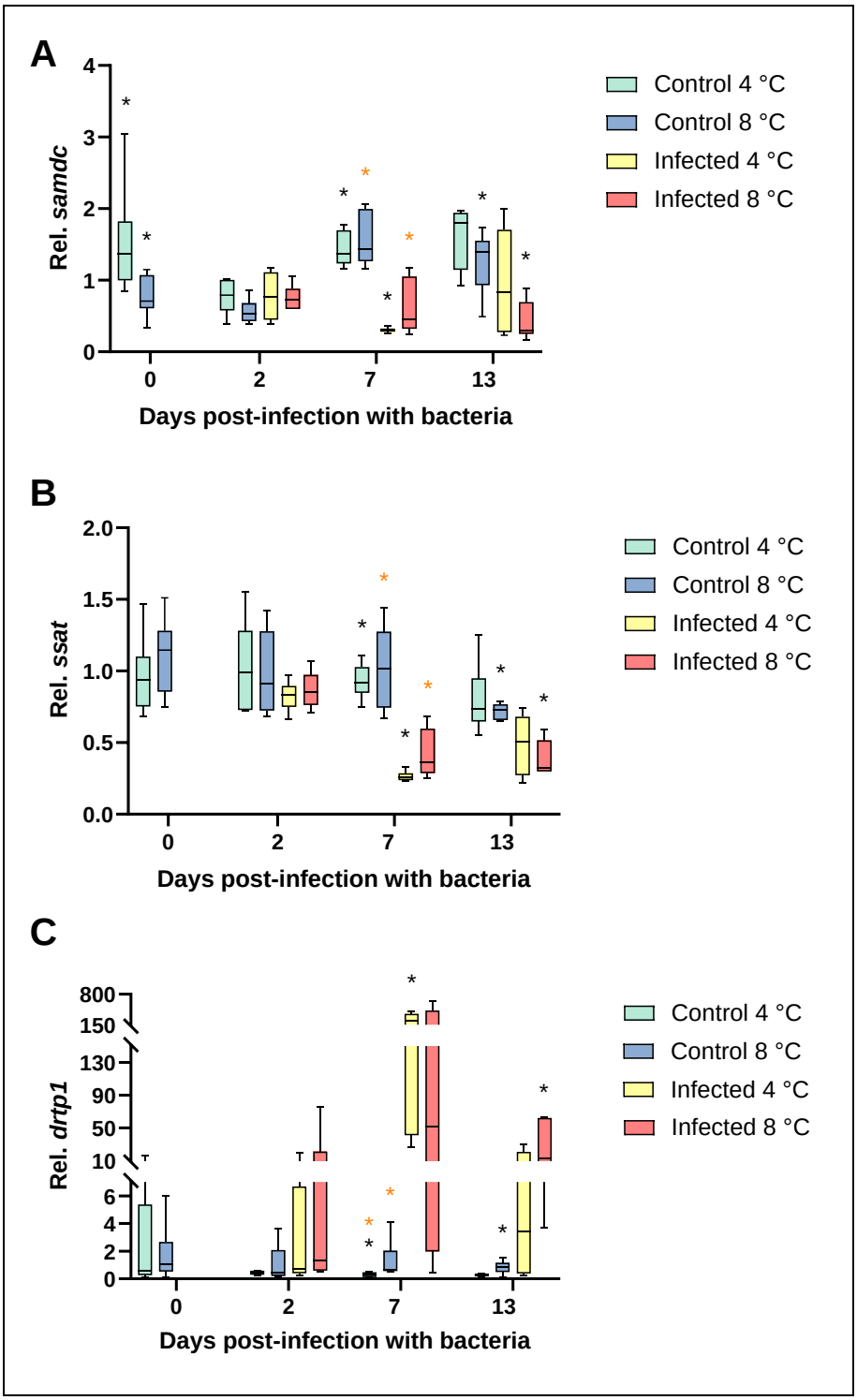

Figure 12: Immune-related genes (1/2). Relative (rel.) transcript (mRNA) levels (relative to the control genes. A) *samdc* (n = 96). B) *ssat* (n = 96). C) *drtp1* (n = 90). The asterisk mark represents significant difference between groups and the different colors (black and orange) is to separate which comparison was significant within one timepoint. Significant difference between timepoints is not shown.

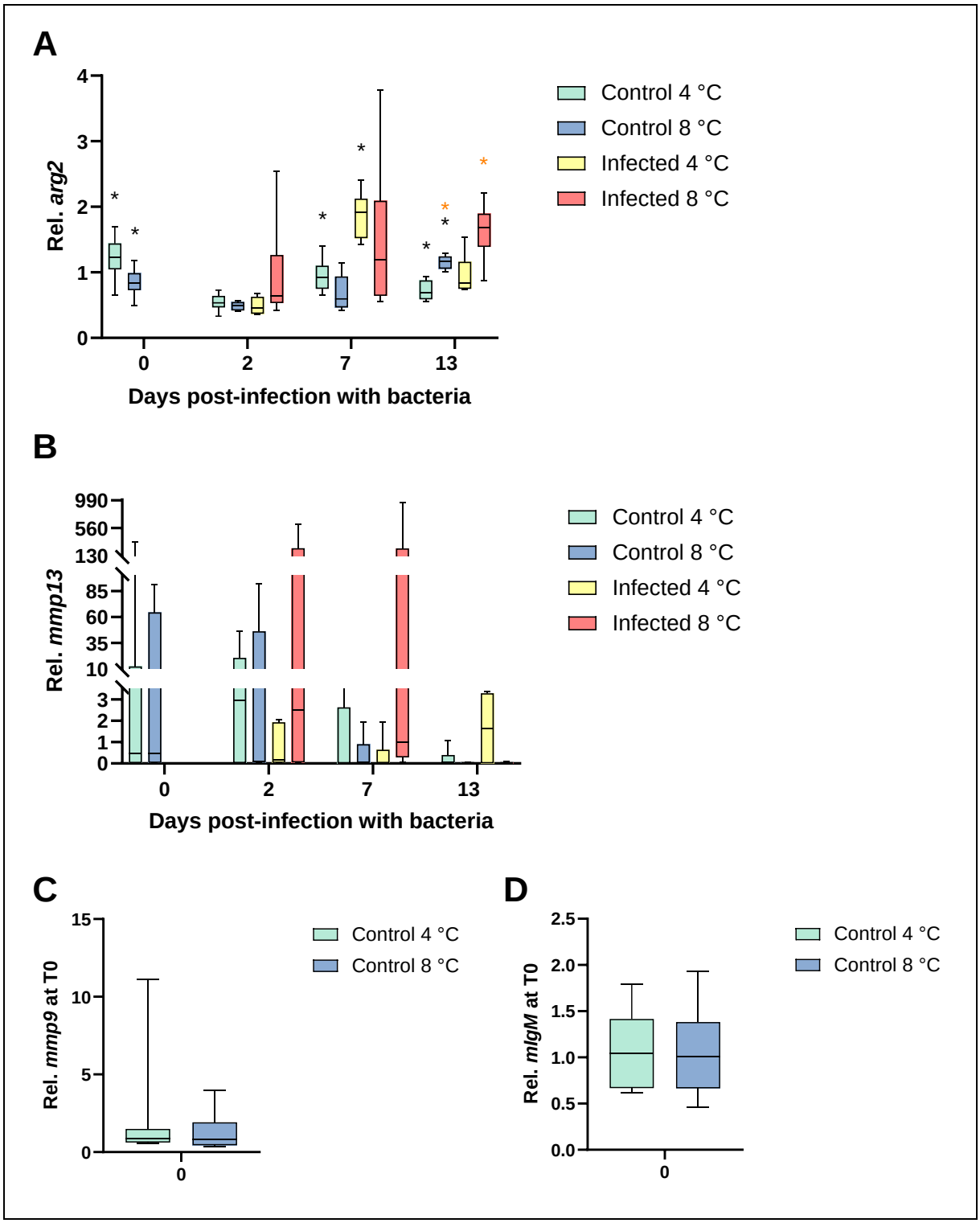

Figure 13: Immune-related genes (2/2). Relative (rel.) transcript (mRNA) levels (relative to the control genes. A) *arg2* (n = 96). B) *mmp13* (n = 90). C) *mmp9* (n = 24). D) *mIgM* (n = 24). *Arg2* and *mmp13* have samples from all timepoints, while *mmp9* and *mIgM* have only from 0 dpi. The asterisk mark represents significant difference between groups and the different colors (black and orange) is to separate which comparison was significant within one timepoint. Significant difference between timepoints is not shown.

4 Discussion

The heart is often considered as one of the vital organs for performance and robustness in many aspects of fishes' life and is capable of remodeling to suit an organism's thermal environment. Temperature-dependent plasticity can involve changes in cardiac performance, heart morphology, and immune response. There have been reporting's of high mortality of harvest-ready salmon with deviating hearts on multiple levels which have been accredited to high ambient water temperatures during early freshwater incubation (97). In the present study, we examined morphological cardiac remodeling and immune response using histological and gene analysis, with a particular focus on how they may contribute to maladaptive changes that can impede performance. When such changes are observed in sensitive stages of early life development like the fish used in this study, chances of developing or not recovering from deviating heart morphology and immune system, becomes very likely to happen in later life stages when the fish must adapt to challenging and intensive farming conditions.

4.1 Observational changes

Clinical symptoms typical of Yersiniosis were observed in both fish behavior and tissue. Together with the positive PCR-results and many altered gene expressions, it is safe enough to assume successful infection of *Y. ruckeri*. Looking at the results from fish body mass and shape, it seemed that CON4 group at the best growth and K (significant) while INF8 had the lowest (not significant). Infection could have had an impact on these results since it can affect immune status, energy expenditure and appetite. Looking at the growth trend (CON4 > CON8 > INF4 > INF8), it appears that the 4 ℃ groups were superior in both growth and robustness against infection. Interestingly, CON8 had a seemingly growing K in contrast to the other groups, a sign that weight was growing more than length, but it was not significant.

4.2 Histology

Histology-wise, pathological signs were within expectations, but not particularly severe compared to other recorded cases of rainbow trout (e.g., stronger cellular inflammatory reaction and large degenerative changes) (61). Furthermore, IHC did not detect many strong positive signals, which may imply low amounts of *Y. ruckeri* in cardiac tissue. However, the staining was too strong which probably hid the signal. The relatively mild symptoms may be ascribed to the experimental units. For example, Atlantic salmon may have been affected differently than rainbow trout, heart may be a less exposed organ, or the fish may have died before any severe symptoms occurred due to their young age and small size. It was generally difficult observing

histopathological changes in the ventricle and especially the BA tissue, since cells are not clearly defined and can contain multiple nuclei. As a result of lacking evidence, the degree of cardiac infection and impact on fish health remains uncertain and other organs may have been affected more severely.

The variable of interest when comparing Ventricular height : width ratio and rel. compact thickness were first and foremost temperature dependent, as previous research has shown such changes induced by early life history (65, 98). Physiological changes have also been observed in infected hearts of larger fish, but recordings of juvenile fish are few (97, 99), making this an interesting study as well. Muir et al. (98) showed that wild juvenile Atlantic salmon reared at elevated temperature (0 vs. 4 ℃) developed increased rel. compact thickness, but no other changes in ventricle parameters (e.g., shape, size) were observed. In addition, Klaiman et al. (100) proved that acclimation (cold and warm) of rainbow trout had effects on cardiac morphology and tissue composition. In contrast, no clear indication of temperature or pathological effects were observed when comparing the ratio and compact thickness in this study, and the results appeared quite random. For instance, CON4 had a decreasing ventricular height : width ratio over time, which was not expected to change. On the other hand, CON4 showed highest growth at this timepoint, which could have affected heart morphology. There were also big variations within each group. These results are not surprising since observed heart shapes varied a lot, as well as having a very small sample size due to many low-quality histological sections. It is also possible that the analysis did not pick up on anatomically shape differences due to the small fish size and their developing hearts. Hearts in bigger fish would for example have more time at (mis)developing and be less prone to postmortem changes (shrinking, contraction, etc.), making it easier to spot changes. These could be reasons why aforementioned studies had much more clear observations in their experiments. It is quite likely that a different analysis, one that measures heart roundness with a different method for example, would be better suited to this experiment since it was more often anecdotally observed than elongated/shortened hearts. The big differences in heart shapes are an indication nonetheless, that the fish had varying heart health and future risk for maladaptive cardiac morphological changes. To confirm or deny details and gain more knowledge of temperature-dependent plasticity in farmed salmon heart, a more in-depth analysis with more temperature groups (0 ℃ vs. 4 ℃ vs. 8 ℃) and bigger sample size are recommended.

4.3 Gene expression

Proteins and genes that regulate muscle metabolism have been shown to be affected in cardiac tissue by water temperature during different life stages (37, 65, 101). However, an infection is also likely to have an impact since it is affecting the whole energy metabolism and how developmental plasticity in heart can handle this must be considered. Little research on the regulation of *mafbxb* in cardiac tissue has been done, but it is believed that it can be used as a marker of atrophy (63). An explanation to why highest expression was observed in the 8 ℃ groups, especially at 0 dpi, could therefore be that the transfer, stress etc., affected the 8 ℃ groups more, which upregulated this atrophic gene (102). That would also explain the low expression at 13 dpi in all groups, as the fish may have had time to acclimatize to its new environment and calm down. However, 10-11 days of acclimation should have been enough, while the trial period (0-13 dpi) should have been stressful and increased the expression. Maybe the CON8 fish was getting used to handling, netting etc. after many days of sampling. Interestingly, infection did not seem to increase upregulation, rather the opposite. In contrast, a viral infection in Atlantic salmon has shown upregulation of the gene (103). This could be a sign that the hearts were not severely affected, which is supported in findings of induced atrogin-1 in failing mammalian hearts (104). On the other hand, viral and bacterial infections differ in nature, and while viruses need host cells and their intact machinery, bacteria need nutrients to proliferate. Maybe limiting free nutrients by inhibiting *mafbx* works as a countermove to a bacterial infection.

Both *anp* and *vegf* had higher expressions in the 4 ℃ groups at 0 dpi. This indicates that the 4 ℃ groups had higher hypertrophic growth and more formation of blood vessels in the heart, a possible sign of maladaptive cardiac remodeling (37, 65, 69). However, several points contradict this conclusion. Firstly, *anp* did not show large significant differences ($P \approx 0.05$). Besides, the other twin-marker of cardiac pathology, *bnp*, showed little difference between the temperature groups, and tended to be higher in the 8 ℃ groups. Secondly, these two natriuretic peptides play a role in circulatory and fluid homeostasis, and the parr being closer to smoltification than the adult fish used in the other studies, may have affected the results. It has for example been shown that landlocked salmon differ critically in heart physiology (105). Thirdly, general growth was highest in the 4 ℃ groups (by a small margin), which could mean that the hypertrophic muscle growth and blood vessel formation were within normal range of growing juvenile fish. Lastly, *pcna* expression showed highest levels in the 4 ℃ groups, which indicate higher cell proliferation, and therefore higher hyperplastic muscle growth in this group

(65). In other words, considering that *pcna* expression was much higher in the 4 ℃ groups (P = 0.02) than *anp* was lower in the 8 ℃ groups, there exist two possible reasons (or combined), either the 4 ℃ groups had highest cardiac growth and no maladaptive remodeling or growth took place in any of the groups, or the 8 ℃ groups had a tendency towards maladaptive hypertrophic cardiac growth compared to the 4 ℃ groups. The last conclusion would match previous observations in the study by Burgerhout et al. (37)

When it comes to immunological genes, the biggest change in expression happened at 7 dpi, which is in line with the time-period Ohtani et al. (58) observed the most severe signs of cardiac infection. At this timepoint *samdc* was less expressed in the infected groups. In contrast, Clark et al. (106) observed a slight upregulation of *samdc* in muscle of bacteria infected juvenile rainbow trout after 2 dpi, although not significant. This does not, however, differ much from what was observed at 2 dpi in our fish. While the reasons for downregulation in infected fish is unknown, fact remains that the 4 ℃ groups had generally more expression of s*amdc* compared to the 8 ℃ groups, which was also closer to the control groups. This could imply that the 4 ℃ groups were less affected by the infection. Furthermore, high levels of *samdc* (70, 107) are favorable when facing a bacterial infection and may imply that the 8 ℃ groups was deficient in some part of the polyamine metabolism (70, 107). The co-fluctuation of regulation in the control groups (slight downregulation at 2 dpi) is probably a result of stress from handling, new environment etc.

ssat was also less expressed in the infected groups, especially when the infection was the most severe at 7 and 13 dpi. An explanation could be that fish deficient in amino acids and polyamines, for example during an infection, would pointlessly lose energy on producing enzymes for PA turnover (70). This is further supported by the hypothesis that healthy fish transcribe and produce *ssat* mRNA and *ssat* enzymes in excess, to be utilized by the immune system later when needed (108). No difference in *ssat* gene expression was found between the temperature groups. A non-significant downregulation in the control groups was also observed at 13 dpi. Since stress has been reported to increase *ssat* expression (70), this could indicate that both groups became more acclimatized with time.

drtp1 had large individual differences in expression. Upregulation is expected during acute phase response, stress, and bacterial infections (78), and not surprisingly did the infected groups show higher expression of *drtp1* (78). CON4 also had lower expression than CON8 at 7 dpi, and INF8 had the individuals with highest expression (but INF4 median was highest at 7 dpi),

which may imply that the 8 ℃ groups were more stressed and affected by the new environment, handling, and infection.

arg2 expression had some significant differences between the control groups at different timepoints, but no clear trend was observed. The infected groups had higher expression than the control groups, which implies that the infection caused cellular damage in some of the fish, since Arg2 is a marker for regenerative processes (76, 77). In addition, INF4 had a weak, not significant, lowering of *arg2* expression towards the end, which may imply that the 4 ℃ groups had less cardiac injuries and maybe less affected by the infection.

Mmp13 levels are expected to increase after injury or disease in the heart, especially during inflammatory response or wounds (109), but no significant differences were observed between any groups, which could be due to the short time from infection to sampling. However, the 8 ℃ groups tended to have higher expression than 4 ℃ groups (not significant), as well as having the individuals with highest expression. Thus, implying a small difference in how the temperature groups handled the infection, with the 4 ℃ groups being less affected. On the other hand, individual differences (e.g., sex, size) could also have an effect. Another point being that some individuals from the 8 ℃ groups, control and infected, showed very high expression of *mmp13* which could indicate some sort of chronic maladaptive occurrence in the hearts, and could originate pre-bacterial challenge (74, 75). Maybe 8 ℃ incubation had a negative effect on heart development in the early life stages or the fish experienced an infection from another pathogen pre-challenge. Nonetheless, there was high variation in most of the groups, which makes any conclusion less certain and should be confirmed in future studies. The forementioned studies also found upregulation of *mmp*9, however, no trends or individuals with high expression were observed in this study. However, sample size was smaller than preferred (n = 12 pr. group) and does not necessarily undermine the results from *mmp13* which had much larger sample size in total and many individuals with high expression. Lastly, *mIgM* did not have any noteworthy differences at 0 dpi. However, the sample size of this gene was also low which may have not detected affected individuals. On the other hand, the humoral immune system should not have been excessively activated since only the control groups were tested and indicates that most of the fish in these groups were not under infection.

This study did not find any histological differences between the temperature groups, while gene expression analyses revealed interesting results. The regulation of many genes indicated less stress, less burdened immune system, and improved, non-maladaptive cardiac growth in the

4 ℃ groups compared to the 8 ℃ groups. Furthermore, general growth seemed higher in the 4 ℃ groups, which may be a sign of growth compensation. These results confirm speculations from multiple studies (see 1.3 Embryogenetic temperature effects) and may indicate that Atlantic salmon are not well adapted to 8 ℃ incubation temperature which may inflict multiple impairments in cardiac tissue. Furthermore, a study by Muir et al. (101) found many differences in heart physiology between fishes reared at 7 and 11 ℃, but they did not find differences in signature markers of cardiac pathology between the two groups. Instead, they found that fish incubated and reared at elevated temperatures (+4 ℃) to have improved thermal performance. Together with our results, this points to both benefits and drawbacks of both "low" and "high" temperatures regimes. Further studies should explore other incubation temperatures such as 1- 5, 6, 7 and 9 ℃ to pinpoint exactly how and where suboptimal plasticity occurs.

There were multiple points during the whole experiment that could have affected the results. The biggest source of error may have been that the bacterial infection happened on different dates for the two temperature groups. Even though biological development was calculated by DD, the large disparity between 4 and 8 ℃ will widen the relationship between actual DD and biological DD, and the 4 ℃ groups develop faster than predicted (34). However, the similar weight and length at 0 dpi for the two temperature groups, makes the comparison well grounded. Another problem when infecting two groups at different timepoints, is the ability to duplicate the method accurately. Changes such as different amounts of bacteria given to the fish could alter the biological response between the temperature groups drastically and make the results obsolete. In this case, CFU was a little lower for the 8 ℃ groups but taken into consideration that the streak was not done correctly, and that the value was an underestimation, the actual number of bacteria in the water should not have been much lower for the 8 ℃ groups.

5 Conclusion

This experiment yields a simple overview of the cardiac performance in fish reared early at 4 ℃ and 8 ℃ incubation temperatures, as well as providing more information regarding bacterial infection in cardiac tissue in response to temperature dependent developmental plasticity in the heart. The study is first of its kind, linking incubation temperature to cardiac physiology and immunocompetence against a particular bacterial disease in juvenile Atlantic salmon. Gene analysis and general observations such as growth and deformities, showed that incubation temperature had a significant impact on cardiac health and its ability to resist a bacterial infection. In most aspects, 4 ℃ was superior to 8 ℃ incubation temperature. Observations indicate more severe cardiac pathology in fish reared at 8 ℃, and a lower temperature regime is recommended to improve fish welfare and performance. However, if fish are going to be reared at high temperatures in later life stages, low incubation temperatures may not be able to improve acute thermal tolerance. Future research should include different incubation temperatures to pinpoint when maladaptive changes start to occur, and more histological analyses are needed to confirm any morphological changes. In today's industry, salmon hearts are under constant stress due to diseases, handling stress and intense farming conditions, and these results highlight the importance of optimizing early life environment and development, such that cardiac health can be improved.

References

1. Sprague J, Doerry E, Douglas S, Westerfield M. The Zebrafish Information Network (ZFIN): a resource for genetic, genomic and developmental research. Nucleic Acids Res. 2001;29(1):87-90.
2. Lønnsomhetsundersøkelse for produksjon av laks og regnbueørret: matfiskproduksjon. In: Fiskeridirektoratet, editor. 2022.
3. Johnsen PFF, Rognsås LL, Erraia J, Grønvik O, Fjose S, Blomgren A, et al. RINGVIRKNINGER

AV SJØMATNÆRINGEN I 2021. Norce, Nofima and Menon Economics; 2022.
4. Summary Report on the statistics on the use of animals for scientific purposes in the Member States of the European Union and Norway in 2018. Brussels: EUROPEAN COMMISSION; 2021.
5. BRUK AV DYR I FORSØK I 2020. In: Mattilsynet, editor. 2022.
6. Sommerset I, Wiik-Nielsen J, Oliveira VHSd, Torfinn, Moldal GB, Haukaas A, et al. Fiskehelserapporten 2022. www.vetinst.no: Veterinærinstituttet; 2023. Contract No.: nr. 5a/2023.
7. Sneddon LU, Wolfenden DCC, Leach MC, Valentim AM, Steenbergen PJ, Bardine N, et al. Ample evidence for fish sentience and pain. Animal Sentience. 2018;3(21).
8. Lambert H, Cornish A, Elwin A, D'Cruze N. A Kettle of Fish: A Review of the Scientific Literature for Evidence of Fish Sentience. Animals. 2022;12(9):1182.
9. Lov om dyrevelferd, (01.01.2010, 2009).
10. Salmon on soy beans - deforestation and land conflict in Brazil. Oslo; 2018.
11. Tobi RCA, Harris F, Rana R, Brown KA, Quaife M, Green R. Sustainable Diet Dimensions. Comparing Consumer Preference for Nutrition, Environmental and Social Responsibility Food Labelling: A Systematic Review. Sustainability. 2019;11(23):6575.
12. Rudd MA, Pelletier N, Tyedmers P. PREFERENCES FOR HEALTH AND ENVIRONMENTAL ATTRIBUTES OF FARMED SALMON AMONGST SOUTHERN ONTARIO SALMON CONSUMERS. Aquaculture Economics & Management. 2011;15(1):18-45.
13. Winkler K, Fuchs R, Rounsevell M, Herold M. Global land use changes are four times greater than previously estimated. Nature Communications. 2021;12(1):2501.
14. Wennevik V, Hansen T. Topic: Salmon – Atlantic2020. Available from: https://www.hi.no/en/hi/temasider/species/salmon--atlantic.
15. Mobley KB, Aykanat T, Czorlich Y, House A, Kurko J, Miettinen A, et al. Maturation in Atlantic salmon (Salmo salar, Salmonidae): a synthesis of ecological, genetic, and molecular processes. Reviews in Fish Biology and Fisheries. 2021;31(3):523-71.
16. Jensen B, Boukens BJD, Wang T, Moorman AFM, Christoffels VM. Evolution of the Sinus Venosus from Fish to Human. Journal of Cardiovascular Development and Disease. 2014;1(1):14-28.
17. Carmona R, Ariza L, Cañete A, Muñoz-Chápuli R. Comparative developmental biology of the cardiac inflow tract. Journal of Molecular and Cellular Cardiology. 2018;116:155-64.
18. Yousaf MN, Amin AB, Koppang EO, Vuolteenaho O, Powell MD. Localization of natriuretic peptides in the cardiac pacemaker of Atlantic salmon (Salmo salar L.). Acta Histochemica. 2012;114(8):819-26.
19. Kryvi H, Poppe T. Fiskeanatomi. Bergen Fagbokforlaget; 2016.

20. Foddai M, Carter CG, Hilder PE, Gurr H, Ruff N. Combined effects of elevated rearing temperature and dietary energy level on heart morphology and growth performance of Tasmanian Atlantic salmon (Salmo salar L.). Journal of Fish Diseases. 2022;45(2):301-13.
21. Yousaf MN, Poppe TT. Cartilage in the bulbus arteriosus of farmed Atlantic salmon (Salmo salar L.). Journal of Fish Diseases. 2017;40(9):1249-52.
22. Garseth A, Svendsen J, Fritsvold C, Mikalsen A. Kardiomyopatisyndrom (CMS) hos laks - sjukdomsutvikling, agens, epidemiologi2017.
23. Serbedzija GN, Chen JN, Fishman MC. Regulation in the heart field of zebrafish. Development. 1998;125(6):1095-101.
24. Yelon D. Cardiac patterning and morphogenesis in zebrafish. Developmental Dynamics. 2001;222(4):552-63.
25. Glickman NS, Yelon D. Cardiac development in zebrafish: coordination of form and function. Seminars in Cell & Developmental Biology. 2002;13(6):507-13.
26. Martin K, Waxman J. Atrial and Sinoatrial Node Development in the Zebrafish Heart. Journal of Cardiovascular Development and Disease. 2021;8:15.
27. Jensen AJ, Johnsen BO, Heggberget TG. Initial feeding time of Atlantic salmon,Salmo salar, alevins compared to river flow and water temperature in Norwegian streams. Environmental Biology of Fishes. 1991;30(4):379-85.
28. Fleming IA. Reproductive strategies of Atlantic salmon: ecology and evolution. Reviews in Fish Biology and Fisheries. 1996;6(4):379-416.
29. Tuor K, Shrimpton J. Differences in water temperatures experienced by embryo and larval Coho Salmon (Oncorhynchus kisutch) across their geographic range in British Columbia. Environmental Biology of Fishes. 2019;102.
30. Saltveit SJ, Brabrand Å. Incubation, hatching and survival of eggs of Atlantic salmon (Salmo salar) in spawning redds influenced by groundwater. Limnologica. 2013;43(5):325-31.
31. Finstad AG, Jonsson B. Effect of incubation temperature on growth performance in Atlantic salmon. Marine Ecology Progress Series. 2012;454:75-82.
32. Fraser TWK, Fleming MS, Poppe TT, Hansen T, Fjelldal PG. The effect of ploidy and incubation temperature on survival and the prevalence of aplasia of the septum transversum in Atlantic salmon, Salmo salar L. Journal of Fish Diseases. 2014;37(3):189-200.
33. Fraser TWK, Hansen T, Fleming MS, Fjelldal PG. The prevalence of vertebral deformities is increased with higher egg incubation temperatures and triploidy in Atlantic salmon Salmo salar L. Journal of Fish Diseases. 2015;38(1):75-89.
34. Atlantic salmon hatchery manual: Egg to first feeding2022 02.12.22; 2. Available from: https://www.bmkgenetics.com/library/.
35. Richter A, Kolmes SA. Maximum Temperature Limits for Chinook, Coho, and Chum Salmon, and Steelhead Trout in the Pacific Northwest. Reviews in Fisheries Science. 2005;13(1):23-49.
36. Jonsson B, Jonsson N. Early environment influences later performance in fishes. Journal of Fish Biology. 2014;85(2):151-88.
37. Burgerhout E, Mommens M, Johnsen H, Aunsmo A, Santi N, Andersen Ø. Genetic background and embryonic temperature affect DNA methylation and expression of myogenin and muscle development in Atlantic salmon (Salmo salar). PLOS ONE. 2017;12(6):e0179918.
38. Teichert N, Benitez J-P, Dierckx A, Tétard S, de Oliveira E, Trancart T, et al. Development of an accurate model to predict the phenology of Atlantic salmon smolt spring migration. Aquatic Conservation: Marine and Freshwater Ecosystems. 2020;30(8):1552-65.

39. Johnston IA, McLay HA. Temperature and family effects on muscle cellularity at hatch and first feeding in Atlantic salmon (Salmo salar L.). Canadian Journal of Zoology. 1997;75(1):64-74.
40. Macqueen DJ, Robb DHF, Olsen T, Melstveit L, Paxton CGM, Johnston IA. Temperature until the 'eyed stage' of embryogenesis programmes the growth trajectory and muscle phenotype of adult Atlantic salmon. Biology Letters. 2008;4(3):294-8.
41. Stickland NC, White RN, Mescall PE, Crook AR, Thorpe JE. The effect of temperature on myogenesis in embryonic development of the Atlantic salmon (Salmo salar L.). Anatomy and Embryology. 1988;178(3):253-7.
42. Nathanailides C, Stickland NC, Lopez-Albors O. Influence of prehatch temperature on the development of muscle cellularity in posthatch Atlantic salmon (Salmo salar). Canadian Journal of Fisheries and Aquatic Sciences. 1995;52(4):675-80.
43. Johnston IA, Manthri S, Alderson R, Smart A, Campbell P, Nickell D, et al. Freshwater environment affects growth rate and muscle fibre recruitment in seawater stages of Atlantic salmon (Salmo salar L.). Journal of Experimental Biology. 2003;206(8):1337-51.
44. Matschak TW, Stickland NC, Crook AR, Hopcroft T. Is physiological hypoxia the driving force behind temperature effects on muscle development in embryonic Atlantic salmon (Salmo salar L.)? Differentiation. 1995;59(2):71-7.
45. Lim DD, Milligan CL, Morbey YE. Elevated incubation temperature improves later-life swimming endurance in juvenile Chinook salmon, Oncorhynchus tshawytscha. Journal of Fish Biology. 2020;97(5):1428-39.
46. Johnston IA, Abercromby M, Andersen Ø. Muscle fibre number varies with haemoglobin phenotype in Atlantic cod as predicted by the optimal fibre number hypothesis. Biology Letters. 2006;2(4):590-2.
47. Nicieza A, Metcalfe N. Growth Compensation in Juvenile Atlantic Salmon: Responses to Depressed Temperature and Food Availability. Ecology. 1997;78:2385-400.
48. Burt JM, Hinch SG, Patterson DA. Developmental temperature stress and parental identity shape offspring burst swimming performance in sockeye salmon (Oncorhynchus nerka). Ecology of Freshwater Fish. 2012;21(2):176-88.
49. Jonsson B, Jonsson N. Egg incubation temperature affects the timing of the Atlantic salmon Salmo salar homing migration. Journal of Fish Biology. 2018;93(5):1016-20.
50. Alcorn SW, Murray AL, Pascho RJ. Effects of rearing temperature on immune functions in sockeye salmon (Oncorhynchus nerka). Fish & Shellfish Immunology. 2002;12(4):303-34.
51. Ross AJ, Rucker RR, Ewing WH. DESCRIPTION OF A BACTERIUM ASSOCIATED WITH REDMOUTH DISEASE OF RAINBOW TROUT (SALMO GAIRDNERI). Canadian Journal of Microbiology. 1966;12(4):763-70.
52. Furones MD, Rodgers CJ, Munn CB. Yersinia ruckeri, the causal agent of enteric redmouth disease (ERM) in fish. Annual Review of Fish Diseases. 1993;3:105-25.
53. Tobback E, Decostere A, Hermans K, Haesebrouck F, Chiers K. Yersinia ruckeri infections in salmonid fish. Journal of Fish Diseases. 2007;30(5):257-68.
54. Ellis AE. Immunity to bacteria in fish. Fish & Shellfish Immunology. 1999;9(4):291-308.
55. Ghosh B, Nguyen TD, Crosbie PBB, Nowak BF, Bridle AR. Oral vaccination of first-feeding Atlantic salmon, Salmo salar L., confers greater protection against yersiniosis than immersion vaccination. Vaccine. 2016;34(5):599-608.
56. Coquet L, Cosette P, Junter G-A, Beucher E, Saiter J-M, Jouenne T. Adhesion of Yersinia ruckeri to fish farm materials: influence of cell and material surface properties. Colloids and Surfaces B: Biointerfaces. 2002;26(4):373-8.

57. Glenn R, Taylor P, Pelton E, Gutenberger S, Ahrens M, Marchant L, et al. Genetic Evidence of Vertical Transmission and Cycling of Yersinia ruckeri in Hatchery-Origin Fall Chinook Salmon Oncorhynchus tshawytscha. Journal of Fish and Wildlife Management. 2014;5:197-.
58. Ohtani M, Villumsen KR, Strøm HK, Raida MK. 3D Visualization of the Initial Yersinia ruckeri Infection Route in Rainbow Trout (Oncorhynchus mykiss) by Optical Projection Tomography. PLOS ONE. 2014;9(2):e89672.
59. Wrobel A, Leo JC, Linke D. Overcoming Fish Defences: The Virulence Factors of Yersinia ruckeri. Genes. 2019;10(9):700.
60. Mendez J, Cascales D, Garcia-Torrico AI, Guijarro JA. Temperature-Dependent Gene Expression in Yersinia ruckeri: Tracking Specific Genes by Bioluminescence During in Vivo Colonization. Frontiers in Microbiology. 2018;9.
61. McArdle J. Heart pathology is a consistent finding in enteric redmouth (ERM) Disease of Rainbow Trout (Oncorhynchus mykiss). Fish Veterinary Journal. 2014:47.
62. Kumar G, Menanteau-Ledouble S, Saleh M, El-Matbouli M. Yersinia ruckeri, the causative agent of enteric redmouth disease in fish. Veterinary Research. 2015;46(1):103.
63. Bower NI, de la Serrana DG, Johnston IA. Characterisation and differential regulation of MAFbx/Atrogin-1 alpha and beta transcripts in skeletal muscle of Atlantic salmon (Salmo salar). Biochem Biophys Res Commun. 2010;396(2):265-71.
64. Inoue K, Sakamoto T, Yuge S, Iwatani H, Yamagami S, Tsutsumi M, et al. Structural and Functional Evolution of Three Cardiac Natriuretic Peptides. Molecular Biology and Evolution. 2005;22(12):2428-34.
65. Frisk M, Høyland M, Zhang L, Vindas MA, Øverli Ø, Johansen IB. Intensive smolt production is associated with deviating cardiac morphology in Atlantic salmon (Salmo salar L.). Aquaculture. 2020;529:735615.
66. Volpe M, Rubattu S, Burnett J, Jr. Natriuretic peptides in cardiovascular diseases: current use and perspectives. European Heart Journal. 2013;35(7):419-25.
67. Johansen IB, Sandblom E, Skov PV, Gräns A, Ekström A, Lunde IG, et al. Bigger is not better: cortisol-induced cardiac growth and dysfunction in salmonids. Journal of Experimental Biology. 2017;220(14):2545-53.
68. Kelman Z. PCNA: structure, functions and interactions. Oncogene. 1997;14(6):629-40.
69. Jørgensen S, Castro V, Krasnov A, Torgersen J, Timmerhaus G, Hevrøy E, et al. Cardiac responses to elevated seawater temperature in Atlantic salmon. BMC physiology. 2014;14:2.
70. Martins GP, Espe M, Zhang Z, Guimarães IG, Holen E. Surplus arginine reduced lipopolysaccharide induced transcription of proinflammatory genes in Atlantic salmon head kidney cells. Fish & Shellfish Immunology. 2019;86:1130-8.
71. Espe M, Andersen SM, Holen E, Rønnestad I, Veiseth-Kent E, Zerrahn J-E, et al. Methionine deficiency does not increase polyamine turnover through depletion of hepatic S-adenosylmethionine in juvenile Atlantic salmon. British Journal of Nutrition. 2014;112(8):1274-85.
72. Giordano E, Flamigni F, Guarnieri C, Muscari C, Pignatti C, Stefanelli C, et al. Polyamines in cardiac physiology and disease. Open Heart Failure Journal. 2010;3(1).
73. Cui N, Hu M, Khalil RA. Chapter One - Biochemical and Biological Attributes of Matrix Metalloproteinases. In: Khalil RA, editor. Progress in Molecular Biology and Translational Science. 147: Academic Press; 2017. p. 1-73.
74. Skugor S, Glover KA, Nilsen F, Krasnov A. Local and systemic gene expression responses of Atlantic salmon (Salmo salar L.) to infection with the salmon louse (Lepeophtheirus salmonis). BMC Genomics. 2008;9(1):498.

75. Wargelius A, Fjelldal PG, Grini A, Gil-Martens L, Kvamme B-O, Hansen T. MMP-13 (Matrix MetalloProteinase 13) expression might be an indicator for increased ECM remodeling and early signs of vertebral compression in farmed Atlantic salmon (Salmo salar L.). Journal of Applied Ichthyology. 2010;26(2):366-71.
76. Wiegertjes GF, Wentzel AS, Spaink HP, Elks PM, Fink IR. Polarization of immune responses in fish: The 'macrophages first' point of view. Molecular Immunology. 2016;69:146-56.
77. Dhamotharan K, Bjørgen H, Malik MS, Nyman IB, Markussen T, Dahle MK, et al. Dissemination of Piscine orthoreovirus-1 (PRV-1) in Atlantic Salmon (Salmo salar) during the Early and Regenerating Phases of Infection. Pathogens. 2020;9(2):143.
78. Sun B, van Dissel D, Mo I, Boysen P, Haslene-Hox H, Lund H. Identification of novel biomarkers of inflammation in Atlantic salmon (Salmo salar L.) by a plasma proteomic approach. Developmental & Comparative Immunology. 2022;127:104268.
79. Talbot AT, Smith TJ, Cairns MT. Characterisation of the differentially regulated trout protein 1 (DRTP1) gene in rainbow trout (Oncorhynchus mykiss). Fish & Shellfish Immunology. 2009;26(4):589-98.
80. Friess MD, Pluhackova K, Böckmann RA. Structural Model of the mIgM B-Cell Receptor Transmembrane Domain From Self-Association Molecular Dynamics Simulations. Front Immunol. 2018;9:2947.
81. Ingerslev H-C, Rønneseth A, Pettersen EF, Wergeland HI. Differential Expression of Immune Genes in Atlantic Salmon (Salmo salar L.) Challenged Intraperitoneally or by Cohabitation with IPNV. Scandinavian Journal of Immunology. 2009;69(2):90-8.
82. van der Wal YA, Jenberie S, Nordli H, Greiner-Tollersrud L, Kool J, Jensen I, et al. The importance of the Atlantic salmon peritoneal cavity B cell response: Local IgM secreting cells are predominant upon Piscirickettsia salmonis infection. Developmental & Comparative Immunology. 2021;123:104125.
83. Jorgensen SM, Kleveland EJ, Grimholt U, Gjoen T. Validation of Reference Genes for Real-Time Polymerase Chain Reaction Studies in Atlantic Salmon. Marine Biotechnology. 2006;8(4):398-408.
84. Kortner TM, Valen EC, Kortner H, Marjara IS, Krogdahl Å, Bakke AM. Candidate reference genes for quantitative real-time PCR (qPCR) assays during development of a diet-related enteropathy in Atlantic salmon (Salmo salar L.) and the potential pitfalls of uncritical use of normalization software tools. Aquaculture. 2011;318(3):355-63.
85. Ingerslev H-C, Pettersen EF, Jakobsen RA, Petersen CB, Wergeland HI. Expression profiling and validation of reference gene candidates in immune relevant tissues and cells from Atlantic salmon (Salmo salar L.). Molecular Immunology. 2006;43(8):1194-201.
86. Rønningen EK. Effekter av temperatur gjennom tidlig utvikling på histologi og genuttrykk i hjertet til startfôringsklar atlantisk laks (Salmo salar L.). Tromsø: UiT The Arctic University of Norway; 2020.
87. Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nature Methods. 2012;9(7):671-5.
88. Perry W, Solberg M, Brodie C, Coral Medina A, Pillay K, Egerton A, et al. Disentangling the effects of sex, life history and genetic background in Atlantic salmon: growth, heart and liver under common garden conditions. Royal Society Open Science. 2020;7:200811.
89. Bankhead P, Loughrey MB, Fernández JA, Dombrowski Y, McArt DG, Dunne PD, et al. QuPath: Open source software for digital pathology image analysis. Scientific Reports. 2017;7(1):16878.

90. Julin K, Johansen LH, Sommer AI. Reference genes evaluated for use in infectious pancreatic necrosis virus real-time RT-qPCR assay applied during different stages of an infection. Journal of Virological Methods. 2009;162(1):30-9.
91. Robinson NA, Krasnov A, Burgerhout E, Johnsen H, Moghadam HK, Hillestad B, et al. Response of the Salmon Heart Transcriptome to Pancreas Disease: Differences Between High- and Low-Ranking Families for Resistance. Scientific Reports. 2020;10(1):868.
92. Andersen SM, Holen E, Aksnes A, Rønnestad I, Zerrahn J-E, Espe M. Dietary arginine affects energy metabolism through polyamine turnover in juvenile Atlantic salmon (Salmo salar). British Journal of Nutrition. 2013;110(11):1968-77.
93. Krasnov A, Afanasyev S, Nylund S, Rebl A. Multigene Expression Assay for Assessment of the Immune Status of Atlantic Salmon. Genes. 2020;11(11):1236.
94. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001;25(4):402-8.
95. Excel. 2303 ed: Microsoft Corporation; 2023.
96. GraphPad Prism. 9.5.1 for Windows ed. San Diego, California USA: GraphPad Software, LLC.
97. Brocklebank J, Raverty S. Sudden mortality caused by cardiac deformities following seining of preharvest farmed Atlantic salmon (Salmo salar) and by cardiomyopathy of postintraperitoneally vaccinated Atlantic salmon parr in British Columbia. Can Vet J. 2002;43(2):129-30.
98. Muir CA, Garner SR, Damjanovski S, Neff BD. Temperature-dependent plasticity mediates heart morphology and thermal performance of cardiac function in juvenile Atlantic salmon (Salmo salar). Journal of Experimental Biology. 2022;225(16).
99. Ferguson H, Poppe T, Speare DJ. Cardiomyopathy in farmed Norwegian salmon. Diseases of Aquatic Organisms. 1990;8(3):225-31.
100. Klaiman JM, Fenna AJ, Shiels HA, Macri J, Gillis TE. Cardiac Remodeling in Fish: Strategies to Maintain Heart Function during Temperature Change. PLOS ONE. 2011;6(9):e24464.
101. Muir CA, Bork BS, Neff BD, Damjanovski S. Proteomic analysis of temperature-dependent developmental plasticity within the ventricle of juvenile Atlantic salmon (Salmo salar). Current Research in Physiology. 2022;5:344-54.
102. Aravena-Canales D, Aedo JE, Molina A, Valdés JA. Regulation of the early expression of MAFbx/atrogin-1 and MuRF1 through membrane-initiated cortisol action in the skeletal muscle of rainbow trout. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 2021;253:110565.
103. Heidari Z, Tinsley J, Bickerdike R, McLoughlin MF, Zou J, Martin SAM. Antiviral and metabolic gene expression responses to viral infection in Atlantic salmon (Salmo salar). Fish & Shellfish Immunology. 2015;42(2):297-305.
104. Hanai J-i, Cao P, Tanksale P, Imamura S, Koshimizu E, Zhao J, et al. The muscle-specific ubiquitin ligase atrogin-1/MAFbx mediates statin-induced muscle toxicity. The Journal of Clinical Investigation. 2007;117(12):3940-51.
105. Usui Y, Kimoto M, Hanashima A, Hashimoto K, Mohri S. Cardiac hemodynamics and ventricular stiffness of sea-run cherry salmon (Oncorhynchus masou masou) differ critically from those of landlocked masu salmon. PLOS ONE. 2022;17(11):e0267264.
106. Clark TC, Tinsley J, Macqueen DJ, Martin SAM. Rainbow trout (Oncorhynchus mykiss) urea cycle and polyamine synthesis gene families show dynamic expression responses to inflammation. Fish & Shellfish Immunology. 2019;89:290-300.
107. Clark TC, Tinsley J, Sigholt T, Macqueen DJ, Martin SAM. Arginine, ornithine and citrulline supplementation in rainbow trout: Free amino acid dynamics and gene expression responses to bacterial infection. Fish & Shellfish Immunology. 2020;98:374-90.

108. Holen E, Chen M, Fjelldal PG, Skjærven K, Sissener NH, Remø S, et al. Tailoring freshwater diets towards boosted immunity and pancreas disease infection robustness in Atlantic salmon post smolts. Fish & Shellfish Immunology. 2022;120:377-91.
109. Johansen L-H, Thim HL, Jørgensen SM, Afanasyev S, Strandskog G, Taksdal T, et al. Comparison of transcriptomic responses to pancreas disease (PD) and heart and skeletal muscle inflammation (HSMI) in heart of Atlantic salmon (Salmo salar L). Fish & Shellfish Immunology. 2015;46(2):612-23.

Appendix

Appendix table 1: Deparaffinization process using the Leica® TP1020 Automatic Benchtop Tissue Processor

Step	Solution	Time
1	PBS	1 h
2	Ethanol 50%	1 h
3	Ethanol 70%	1 h
4	Ethanol 96%	1 h
5	Ethanol 100%	30 min
6	Ethanol 100%	30 min
7	Ethanol 100%	30 min
8	Xylen	30 min
9	Xylen	30 min
10	Xylen	30 min
11	Parafin	1 h
12	Parafin	2 h

Appendix table 2: Hematoxylin & Eosin staining using Leica® ST5010 Autostainer XL

Step	Solution	Time
1	Xylen	5 min
2	Xylen	5 min
3	Absolute Ethanol	3 min
4	Absolute Ethanol	3 min
5	Ethanol 96%	2 min
6	Ethanol 96%	2 min
7	Ethanol 70%	2 min
8	Water	3 min
9	Haematoxylin	4 min
10	Water	2 min
11	Ammoniac	1 min
12	Water	30 seconds
13	Hydrochloric acid	5 seconds
14	Water	30 seconds
15	Erythrosine B	30 seconds
16	Water	35 seconds
17	Ethanol 70%	15 seconds
18	Ethanol 96%	15 seconds
19	Absolute Ethanol	30 seconds
20	Absolute Ethanol	1 min
21	Xylen	1 min
22	Xylen	Indefinitely

Appendix table 3: Immunohistochemistry protocol partially developed by Nofima, Ås.

Step	Instruction
1	Incubate slides for 60C for one hour;
2	Deparaffinize in the slide processor (program 1)
3	Put in PBS 1%
4	While samples are in PBS 1% prepare the primary antibody accordingly to the dilution preferred: 1:2000- I µl of primary antibody (Anti- Y.Ruckeri, Clone 4B12/F8; Lot 0Y7381MS, Ref FM-050AW; Ango, CA, USA) : 2 ml TBS 1% + 50 µl BSA (Albumin, Bovine Serum, 10 % AqueousSolution, Nuclease-free; M.W. 66000, Lot 378657; Millipore Corp Massachusetts, USA) For 16 samples 1.5 µl AB: 3 ml buffer + 75 µl BSA Keep at 4C
5	Make a circle around the tissue with hydrophobic pen after removing excess PBS and place in the immune rack;
6	Unmask with trypsin for 15 minutes. Prepare trypsin 1:1 ratio of concentrated trypsin (Concentrated Liquid Trypsin ab970; Lot GR3327482-1; Abcam UK) to buffer (Trypsin Buffer ab970; Lot GR3327482-1; Abcam UK). 30 drops of concentrated trypsin to 30 drops of buffer.
7	Permeabilize with TBS with triton 0.1% for 5 min shaking intermittently in staining jars.
8	Peroxidase Blocking after removing excess buffer with paper. Th peroxidase blocking solution (Bloxall® Blocking solution; Ref SP-6000, Lot ZJ0817; Vector Laboratories, California, USA) should cover all the sample.
9	With the help of paper, remove excess solution without touching the samples and apply enough primary antibody previously prepared to cover all the sample (~150 µl per sample)
10	Place parafilm over the samples and put cover on. Incubate at RT for 2 hours.
11	Rinse 2 times for five minutes with TBS with tween 0.05%;
12	Remove excess buffer without letting it dry and the apply the secondary antibody (Envision + System- HRP, labelled polymer, anti-mouse; Ref K4001, Lot 11462074; Dako, Denmark) covering the sample.
13	Let it incubate at RT for 30 minutes in the dark;
14	Rinse 3 times for 5 minutes with TBS 1% or PBS 1%
15	Calculate how much of the substrate chromogen you need for the slides (150 µl per sample), 17 parts of magenta to 3 parts of substrate buffer (EnVision FLEX, HRP Magenta Substrate, Chromogen System; Ref GV925, Lot 41424219; Dako, Denmark). Careful because it's light sensitive, cover the Eppendorf with aluminum foil;
16	Cover samples with enough solution and incubate at RT for 5-10 minutes.
17	Rinse in distilled water, change the water frequently;
18	Do the contrast stain with haematoxylin (Mayer's Hematoxylin, Lillie's Modification, Histological Staining Reagent; Ref S3309, lot 11401568; Dako, Denmark). Cover the samples with the solution for 2 minutes;
19	Rinse carefully with distilled water until water is clean;
20	Mount the slides and scan the next day;

Appendix table 4: Controls used in qPCR instead of samples. 13 µl reaction mix was added to both the controls and samples.

Controls	Reagents
NRT	Solution made from RT-qPCR of cDNA, but without reverse transcriptase enzyme.
Positive controls	Mix of cDNA (1:40) from all groups.
NTC	Invitrogen™ Nuclease-Free Water (cat.nr: AM9932)