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The effects of cumulative stress on immune relevant genes in Atlantic salmon (*Salmo salar* L.)

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Bodø, November 16th, 2020

Brandon Robert H Garcia

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Abbreviations

$\Delta\Delta C_t$	delta-delta cycle threshold
µg	microgram(s)
µl	microliter(s)
APP	acute-phase protein(s)
APR	acute-phase response
bp	base pair
°C	degrees Celsius
cDNA	complementary deoxyribonucleic acid
Cq	quantification cycle
CRDs	carbohydrate-recognition domains
dl	deciliter
DNA	deoxyribonucleic acid
gDNA	genomic deoxyribonucleic acid
h	hour(s)
HPI	hypothalamic-pituitary-interrenal
IL-1	interleukin-1
IL-1β	interleukin-1 beta
kg	kilogram(s)
LABWIs	laboratory-based welfare indicators
m	minute(s)
mmol	millimole
NF- κB	nuclear factor kappa beta
ng	nanogram(s)
NOK	Norwegian kroner

OWIs	operational welfare indicators
PAMP	pathogen-associated molecular patterns
PCR	polymerase chain reaction
pg	picogram(s)
PRR	pattern recognition receptor(s)
qPCR	quantitative polymerase chain reaction
RIN	ribonucleic acid integrity number
RNA	ribonucleic acid
RNAi	ribonucleic acid interference
RQ	relative quantification
rRNA	ribosomal ribonucleic acid
RT	reverse transcription
RT-qPCR	quantitative reverse transcription polymerase chain reaction
s	second(s)
TBE	Tris-borate-EDTA
T_m	primer melting temperature
v	volt(s)
WIs	welfare indicators
α	Greek small letter alpha
β	Greek small letter beta

Abstract

The aquaculture industry in Norway has been increased since the 60s and in 2018, reached 1.28 million metric tons of Atlantic salmon (*Salmo* salar L.) at a landed value of 64.5 billion NOK. Due to increased global demand more research has gone into fish welfare as it is not only beneficial to the fish's well-being, but also economically as increased welfare is usually associated with healthier fish. When investigating fish welfare, determining if a fish is stressed by use of physiological indicators can be done. When an organism is stressed there is an activation of the hypothalamus-pituitary-interrenal (HPI) axis which changes levels of cortisol, lactate, glucose, and magnesium (Mg^{++}) content in blood plasma while also modulating gene expression and immune function. In this study, we observed the effects of cumulative stress on physiological parameters. In addition, ribonucleic acid (RNA) extraction methods were compared while investigating gene expression of both published and novel primers in genes relating to immune function. For our study, 78 Atlantic salmon smolts were used. The duration of the experiment was one week, and sampling of unstressed fish (pre-stress, control) was performed prior to the start of the trial. The fish were subjected to crowding and 90 seconds of air exposure by emptying the tank of water. Samples of blood, gill, muscle, skin and hindgut were taken at 0, 0.5, 1, 2, 3, 4, 6, 12, 24, 48, 168 hours post stressor. Plasma Mg^{++} was measured and total RNA was extracted using two different methods from gill and skin, where they were reverse transcribed and subjected to quantitative reverse transcription polymerase chain reaction (RT-qPCR). The genes of interest were eukaryotic translation elongation factor (*ef1a*), interleukin-1 beta (*il1β*), serum amyloid A-5 (*saa5*), transforming growth factor beta 1(*tgfb1*), tumor necrosis factor alpha 2 (*tnfa*) and with novel primers being made for Galectin-3, 4, 8 (*leg3*, *leg4*, *lgals8*). Mg^{++} was significantly changed and correlated with plasma cortisol (0.875, $p < 0.05$). Column based RNA extractions showed the best results for this study. Not all genes of interest generated products in polymerase chain reaction (PCR) and only *saa5* and *leg3* were used for gene expression analysis. *Saa5* showed a significant increase in expression 24 and 168 h post stressor in gill while a significant decrease in expression 6 h post stressor in skin. Gene expression in *leg3* showed a significant increase at 6 h post stressor in gill and a significant increase 24 and 168 h post stressor in skin. Stress showed to have an influence on Mg^{++} and expression of immune relevant genes. When considering the gene expression, they must be taken with caution as gene expression does not necessarily mean translation into proteins. Additional studies would be needed to support these findings.

1.0 Introduction

1.1 Atlantic salmon and aquaculture in Norway

Atlantic salmon (*Salmo salar* L.) is an anadromous teleost found in the North Atlantic Ocean on both the European and American coasts. Their lifecycle is categorized into the egg (roe), fry, parr, smolt and adult stage. In the wild, eggs are fertilized in streams and rivers, and once hatched will spend its juvenile and fry stage in freshwater for 2 - 4 years. During the parr stage physiological and behavioral changes occur as the salmon prepares to make the transition from freshwater to saltwater during its smolt and adult stage where it will be at sea for at least 1 -5 years until sexually mature. When spawning, adult salmon return to their home rivers with high precision via an oceanic homing mechanism (Hansen & Quinn, 1998; McCormick et al., 2013)

The global population is expected to grow to 9.8 billion people by 2050 (United Nations, 2019), which is expected to have high environment impact with current food cultivating practices. Aquaculture-based food production is the fastest growing animal production globally and may be able to help meet the worlds protein demand while keeping impact on the environment to a minimum compared to terrestrial meat production. Atlantic Salmon culture was started in the 19th century in the United Kingdom in freshwater by stocking rivers with parrs and was really successful by the introduction of sea cages in Norway during the 1960's (FAO, 2004). Since the introduction of sea cages, salmon aquaculture achieved commercial status in the 1980's and as of 2018, 142 different companies registered their slaughtering of salmon in Norway (Fiskeridirektoratet, 2019; Phyne, 2010). Behind oil, and oil products the fourth largest export from Norway is fish accounting for 11.5 % of all exports in 2019 and in 2018 Atlantic salmon accounted for nearly 94.6 % of all aquaculture exports from Norway at 1.28 million metric tons with a landed value of 64.5 billion NOK (Figure 1) (Statistisk sentralbyrå, 2019a). As production increases, fish health needs to be monitored because if it is not, there can be outbreaks of disease and welfare issues which can lead to serious economic loss for the industry (Hjeltnes et al., 2016).

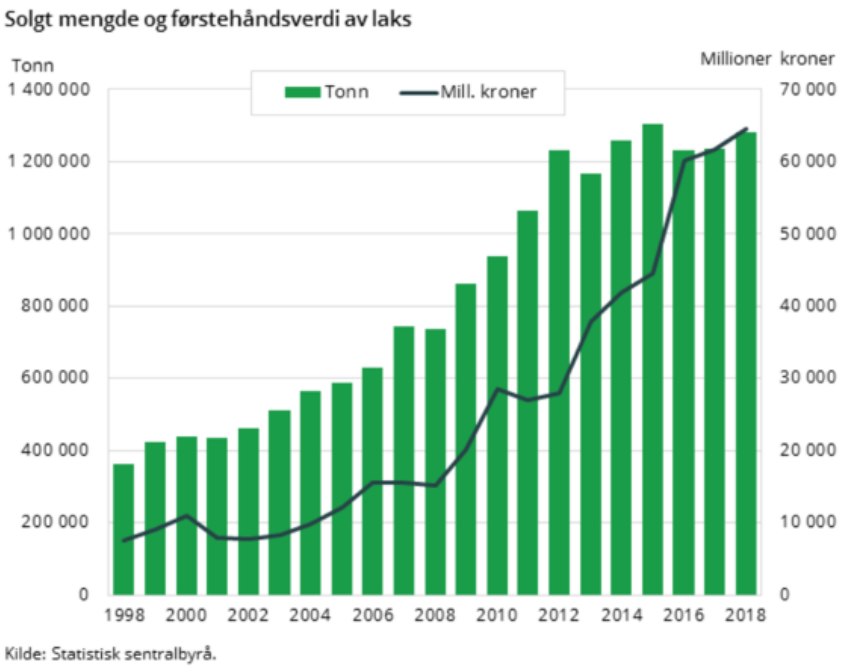

Figure 1. *Metric tons and landed value in NOK of salmon from 1998 to 2018*

Retrieved from a Statistisk sentralbyrå (2019b).

1.2 Fish welfare and welfare indicators

In the last decades interest in fish welfare has grown internationally and efforts to increase the base knowledge by use of fish behavior, fish physiology and fish health as ways to evaluate fish welfare based on the animals behavioral needs (Damsgård, 2006). Assessing fish welfare can be difficult due to the major differences in environment, physiology and behavior of teleost compared with the more studied terrestrial mammals. Branson (2008), considers an animal to be in a good state of welfare if one of the three following conditions apply:1) The animal can adapt to its environment and is in good health; 2) The animal can live a natural life, as it would in the wild, with all needs met; 3) The animal is free of negative experiences and has access to positive ones. Volpato et al. (2007) suggest that "Fish welfare is not only freedom of suffering but also access to comfort states" and proposes that welfare be based on "the internal state of a fish when it remains under conditions that were freely chosen". Another approach to assessing welfare is by use of five domains outlined by Mellor & Stafford (2001) where the animal is considered to be in a good state of welfare if they are free from 1) hunger and thirst; 2) environmental challenges; 3) disease or injury; 4) Behavioral or interactive restriction; and 5) mental/physical suffering.

These conditions are broad, difficult to assess/measure and when assessing fish welfare are further complexed by the debate whether fishes experience pain and suffering, or even experience consciousness (Stevens et al., 2017). One theme that is common among all definitions of welfare is that good health and optimal status of the of the immune system is associated with good welfare though it must be noted this is not always the case, as fish can be lacking other aspects of good welfare such as appropriate social environment (Huntingford & Kadri, 2014). A combination of these definitions would be good welfare characterized as an organism experiencing optimal conditions they would experience in nature with adequate food, shelter, and social interactions in combination with a minimal amount of negative experiences. By using a combination of these definitions, assessing welfare can be investigated scientifically by comparing suggested welfare factors between captive and wild species or by monitoring changes in behavior, immunity, growth, stress hormones etc. when conditions are altered (Carl B Schreck et al., 2016).

In an aquaculture setting, scoring systems and guides such as Salmon Welfare Index Model (SWIM 1.0) proposed by Stien et al. (2013) or the "FISHWELL Morfologiske operative velferdsindikatorer for atlantisk laks" (Noble et al., 2018) have been used as a guideline for people working in the industry as a way to assess Atlantic salmon welfare. This system is a way to attempt to standardize how welfare is assessed in salmon farming by assessing key welfare indicators (WIs). WIs can be direct-animal based or indirect resourced-based and are separated into Operational Welfare Indicators (OWIs) and Laboratory-based Welfare Indicators (LABWIs) (Noble et al., 2018). OWIs are indicators that are for on-farm use (physical damage, presence of parasites, behavior, mortality rate etc.) while LABWIs must be sampled on site and sent off to a laboratory for further analysis (blood cortisol, osmolality, hematocrit, ion concentration etc.).

1.4 Stress response in teleost

In aquaculture it is important to consider the relation between stress and fish welfare. Stress can compromise the health and survival for farmed fish and by improving fish welfare (e.g., by reducing stress) the survival and economic success of farmed fish increases (Ashley, 2007; Martos-Sitcha et al., 2020). Factors such as stocking density, diet, feeding technique, and management procedures have strong effects on stress levels, subsequent stress tolerance, health, and the presence of aggressive behavior which feedback into one another influencing welfare (Ashley, 2007). As with welfare, stress can be hard to define. Wendelaar Bonga (1997) defines stress as "a condition in which the dynamic equilibrium of animal organisms called homeostasis

is threatened or disturbed as a result of the actions of intrinsic or extrinsic stimuli, commonly defined as stressors" while Schreck (2010) suggest a broader definition of stress as a "… physiological cascade of events that occurs when the organism is attempting to resist death or reestablish homeostatic norms in the face of insult".

Stress is a response to a stressor that an organism encounters and causes disruption of homeostasis (Selye, 1975). Stressors can be physical, chemical, or even any type of perceived threat (Barton, 2002; Wendelaar Bonga, 1997) (Figure 2). There are three types of response to stress: Primary, secondary, and tertiary responses (Barton, 2002). The primary response is involved with neuroendocrine responses that include the release of catecholamines from chromaffin tissue and the stimulation of the hypothalamic-pituitary-interrenal (HPI) axis which releases corticosteroid hormones into the blood circulation (Barton, 2002; Reid et al., 1998; Wendelaar Bonga, 1997). Secondary responses to stress relate to physiological changes in processes such as metabolism, respiration, acid-base status, hydromineral balance, immune function and cellular responses which are influenced by changes in plasma, tissues, ions, metabolites, hematological features and proteins related to stress (Barton, 2002; Lockridge, 1981; Wendelaar Bonga, 1997). The tertiary response can be described as where the overall animal performance is changed, such as changes in growth, condition, disease resistance, activity, behavior and in general its survival (Barton, 2002; Wendelaar Bonga, 1997). These groupings are oversimplified by these general statements and are dependent on the magnitude and duration of stress which can have effects on the fish on all levels of organization (molecular/biochemical all the way to the community level) (Barton, 2002).

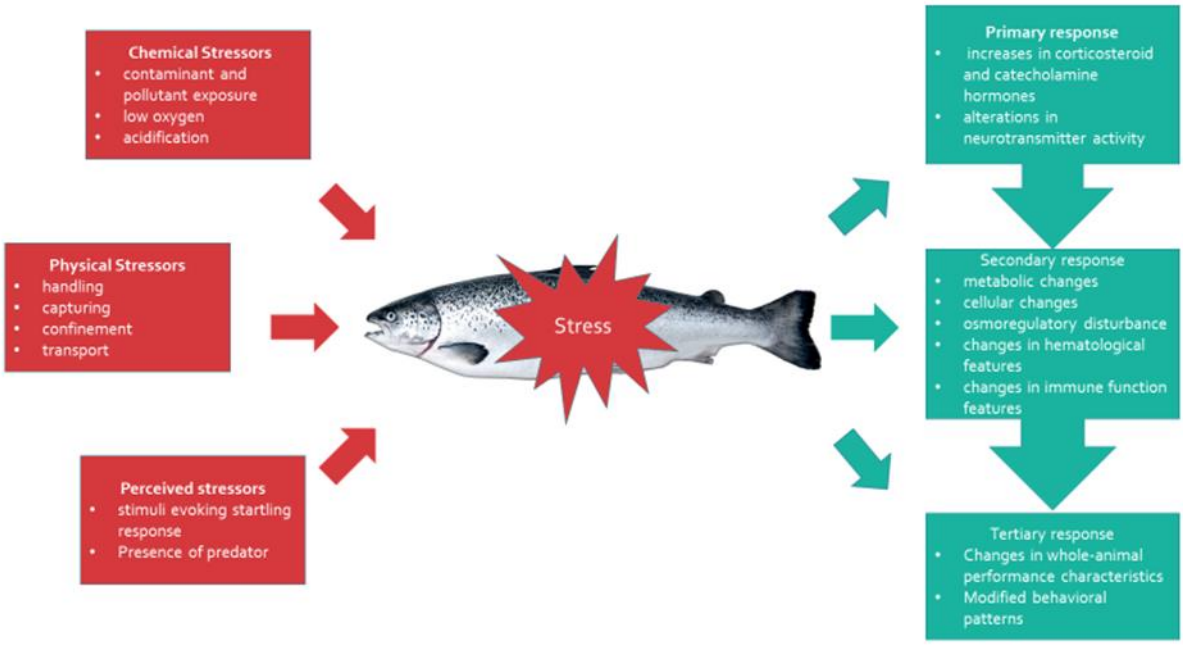

Figure 2. *The stress response in fish*

Adapted from Barton 2002.

The corticosteroid, cortisol, that is released in the activation of the HPI axis in the primary response, is the most used stress indicator in fish (Barton, 2002; Ellis et al., 2012; Noble et al., 2018; Wendelaar Bonga, 1997). Changes in cortisol, glucose, osmoregulation and other physiological features are used to quantify the degree of stress and are dependent on how long they remained changed and how quickly they return to levels before the stressor was introduced (Iversen & Eliassen, 2009, 2014; Mommsen et al., 1999). In the presence of an acute stressor, plasma cortisol levels rise rapidly a few minutes and return to normal levels after one or more hours while during chronic stress cortisol levels may remain elevated consistently but well below peak levels (Wendelaar Bonga, 1997).

Living organisms acquire, utilize, and store energy reserves that can be threatened by both predictable and unpredictable factors (McEwen & Wingfield, 2003). Stress and allostasis are some of these factors and are both energetically costly in their attempts to maintain homeostasis (Carl B. Schreck, 2010). McEwen and Wingfield (2003) describe the concept of allostasis as "maintaining stability through change" and allostatic load referring to the cumulative cost to the body from this change to maintain stability. Allostatic overload occurs when there is an imbalance of resources in allostatic load. Resource consumption of the organism can result in

allostatic overload which is described as the balance between energy expenditure and energy input with two types, Type 1 and Type 2 (McEwen & Wingfield, 2003). When energy demand exceed the energy supply Type 1 allostatic overload occurs resulting in the activation of emergency-life history stage. If there is sufficient or excess energy available in an organism chronic deleterious challenges lead to a sustained allostatic state which is categorized as Type 2 allostatic load. The allostatic load associated with stress is a short term solution to increase fitness, while long term effects in fact reduce fitness (Carl B. Schreck, 2010).

1.5 The innate immune system

In general, fish have similar immune system mechanisms compared to other higher vertebrates but with key differences due to their environment. The immune system of fish can be divided into two systems, the innate immune system, and the adaptive immune system. The innate immune system evolutionary appeared before the adaptive immune system and is common in all animals while the adaptive immune system is evolutionarily relatively recent and only found in jawed vertebrates. From the early embryonic stage, fish are already free-living in an aquatic environment and consequently rely heavily on their innate immune system (Rombout et al., 2005). The innate immune response is non-discriminate to pathogen type, and focuses on few highly conserved structures present in a large group of microorganisms which are referred to as pathogen-associated molecular patterns (PAMP). The detection of a PAMPs or physical damage will cause an inflammation response that is caused by the innate immune system. Common PAMPS are molecules such as polysaccharides, lipopolysaccharide (LPS), peptidoglycan, bacterial DNA, and viral RNA which are detected by receptor proteins that play a key role in immune response to maintain homeostasis and can be recognized by both the innate and adaptive immune system (Medzhitov & Janeway, 2000; Schluter et al., 1999; Uribe et al., 2011). The immune system primarily relies on pattern recognition receptors (PRRs) to detect PAMPs and fall into two major classes: toll-like receptors (TLRs) and the retinoic acid inducible gene I (RIG-I) -like receptors (RLRs) (Taylor & Mossman, 2013). PRRs activate several signaling pathways such as nuclear factor kappa B (NF-κB) pathway. Once activated, NF- κB proteins translocate to the nucleus and regulate the expression of many immune, growth and inflammation genes (Bianchi, 2007; Serasanambati & Chilakapati, 2016). Dendritic cells, macrophages and mast cells specialize in detecting pathogens by use of PRRs and once their NF- κB pathway is activated, they induce the secretion of inflammatory cytokines IL-1, IL-6, IL-12, TNF-α and chemokines (Iwasaki & Medzhitov, 2015; Liu et al., 2017). The release of cytokines during inflammation causes the acute-phase response (APR) which is a complex

reaction that involves both local and systematic effects. APR most notable effect is by changes in the concentration acute-phase proteins (APPs) in blood plasma which have varying effects on the immune system such as direct interaction with microbes (destruction/inhibit growth) and negative feedback on the inflammatory response (Jain et al., 2011). In addition to APR, cytokines and chemokines facilitate the recruitment of natural killer (NK) cells, monocytes, neutrophils, eosinophils and basophils to the site of activation where once present release more proinflammatory cytokines and attempt to contain the pathogen until highly specific adaptive immune cells are recruited to clear the infection (Liu et al., 2017).

1.5.1 Mucosal surfaces

In an aquatic environment, fish rely heavily on their mucosal epithelia as a main organ of defense as they are in constant contact with their immediate environment which has a range of biological, physical, and chemical hazards. The gill, gut, and skin are mucosal-associated lymphoid tissues (MALTs) that are characterized by the presence of a mucus layer that is secreted mainly by goblet and club cells and acts as an effective capturing mechanism of foreign particles, bacteria, and viruses before they are able to make contact with epidermal tissue (Esteban & Cerezuela, 2015). In addition to immobilization, the mucus layer acts a as a chemical barrier which contains a multitude of immune-related factors such as lectins, mucins, antimicrobial peptides, toxins and immunoglobulins (Lazado & Caipang, 2014; Salinas & Magadán, 2017). The immune related factors will detect PAMPs and activate the innate immune response if any injury or pathogen is detected.

1.5.2 Galectins

Galectins are lectins that that are characterized as having a high binding specificity to β-galactoside sugars by use of its evolutionarily conserved carbohydrate-recognition domains (CRDs), though this binding is not exclusive to β- galactoside sugars as galectins have been shown to interact with other non-galactose-containing binding partners (Hirabayashi et al., 2002; Johannes et al., 2018; Wang et al., 2019) . The CRDs of galectins are ∼130 amino acids long and separated into three major groups (Figure 3) , prototypical galectins (Galectin-1, -2, -5, -7, -10, -11, -13, -14, and -15) chimera-type galectins (Galectin-3), and tandem-repeat galectins (Galectin-4, -6, -8, -9, and -12) which are based on the number of CRDs present and CRD organization (Cummings et al., 2017; Li et al., 2013; Wang et al., 2019). Galectins are found in the nucleus, cytosol, outer plasma membrane, and extracellular matrix (Cummings et al., 2017). They lack the signal peptide for classical secretion and are synthesized on free

polyribosomes in the cytoplasm where they accumulate in the cytosol or nucleus until they are secreted by non-classical means (Cummings et al., 2017; Johannes et al., 2018). Galectins are involved in many biological reactions including mRNA splicing, and cell apoptosis, regulation, activation, adhesion, migration and differentiation (Cummings et al., 2017). In relation to immune function, there is evidence that when galectins bind to glycosylated receptors it modulates how cytokines engage with their receptors and therefore their signaling pathways (Gordon-Alonso et al., 2018). In relation to teleost, previous studies in our lab showed Galectin-1 is present in among others mucosal tissues including skin and gill in Atlantic cod (Rajan et al., 2013) and that a truncated Galectin-3 consisting of the CRD C-terminal domain of the galectin is present in skin mucus of Atlantic salmon (Patel et al., 2020). In Atlantic salmon a Galectin-1 like, Galectin– 9 and Galectin-3 binding protein showed modulated gene expression (by microarray) after infection with infectious salmon anemia virus (ISAV) (Jørgensen et al., 2008).

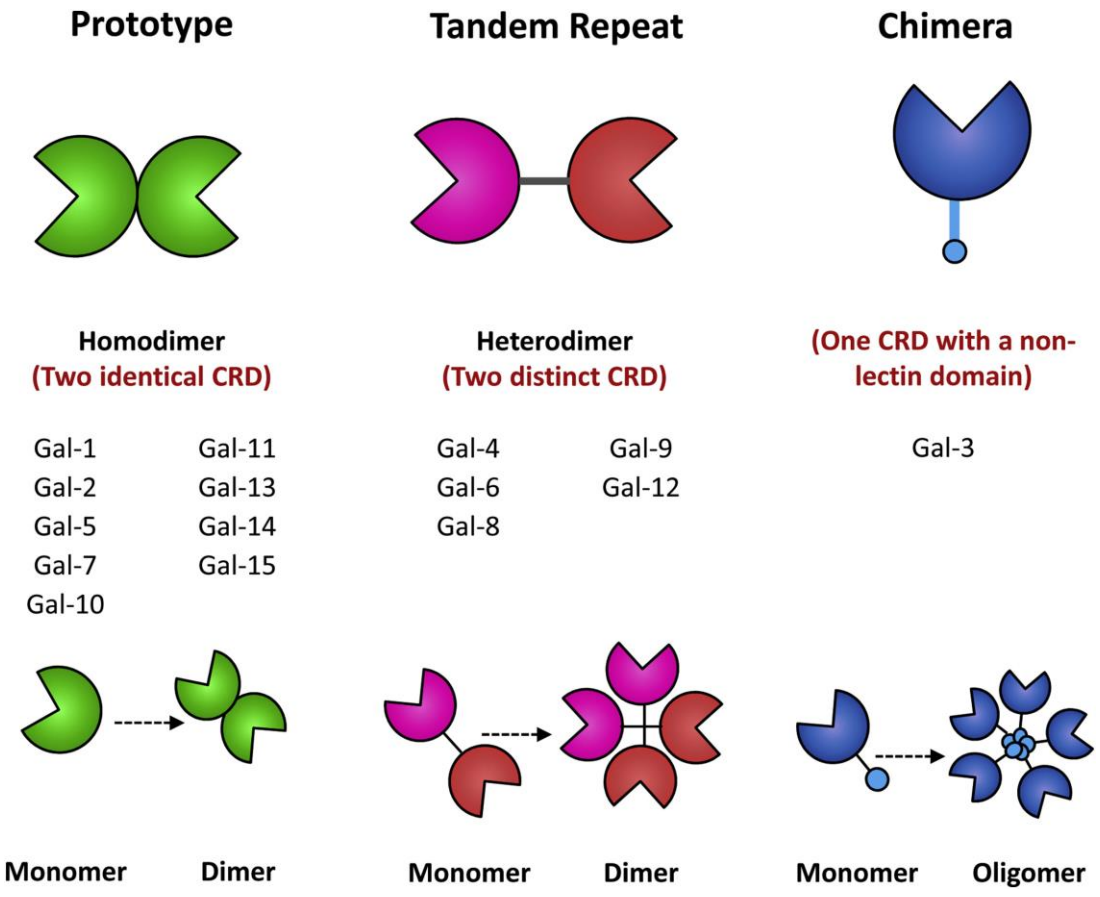

Figure 3. *The three major group of galectins and their structures*

The three major groups of galectins and their structure. From Wang et al. (2019).

1.5.3 Stress and the immune system

The stress response is a complex network involving both the endocrine and immune system with many of the hormones from the endocrine system having influence on immune mechanisms and responses (Tort, 2011). Stress can both induce and reduce the immune function of organisms with evidence in Atlantic salmon of acute stress making the immune system more responsive to challenges whilst chronic stress suppresses the immune response (Uren Webster et al., 2018). Short term crowding stress has been shown to stimulate the immune system (Caipang et al., 2009), whilst long term/chronic stress lowers or delays immune response for example with repeated hypoxia exposure (Kvamme et al., 2013). Cortisol has been shown to modulate cytokine responses, apoptosis and proliferation of immune cells that are responsible for the activation and deactivation of the teleostean immune response though the fine details of regulation, especially its bi-directional nature, is still unclear (Ellis et al., 2007; Verburg-Van Kemenade et al., 2009).

1.6 Methodology

1.6.1 RNA extraction methods

Stress can modulate how genes are expressed which can lead to changes in immune function. Messenger RNA (mRNA) that encode for proteins related to the immune function can be extracted and isolated for use in downstream applications such as gene expression studies. Eukaryotic mRNA for gene expression studies should be intact, and this can be difficult as some decay in under 5 minutes (Sachs, 1993), which is why RNA extraction, including its first step of nuclease inactivation, should be as quick as possible without sacrificing sample integrity. Generally total RNA is extracted by use of either organic (phenol) or filter-based methods. Organic extraction offers rapid denaturation of nucleases and stabilization of RNA, while drawbacks include it being laborious and dangerous due to the use of phenols that can cause severe burns and chloroform which is suspected of causing cancer. Filter based extractions is more convenient to use than the organic extraction method because it is faster, easier, and safer but disadvantages include clogging of the filter due to particular waste, retention of gDNA and a fixed binding capacity of the filter (Thermo Fisher Scientific, 2020b). Tests on different extraction methods have showed that highest concentration and best purity is achieved with organic extraction while filter based methods offered lower RNA yields, especially for smaller RNA's, and lower purity at the cost of ease of use and speed (Xiang et al., 2001).

1.6. 2 Quantitative reverse transcription polymerase chain reaction (RT-qPCR)

In the early eighties "the golden standard" of nucleic acid analysis, polymerase chain reaction (PCR), was invented as a simple, rapid technique used to amplify DNA. This is achieved by subjecting oligonucleotide primers, dNTPs and a heat stable Taq polymerase to a series of heating and cooling steps that are optimal for amplification to occur (Pfaffl, 2010). The PCR protocol was further modified by the addition of a reverse transcription (RT) step to make DNA from RNA before PCR to produce the RT-PCR method, a technique to indirectly amplify any type of RNA which further expanded the applications of how PCR could be used to answer biological questions. Quantitative reverse transcription polymerase chain reaction (RT-qPCR) is a more sensitive and reproducible method compared to RT-PCR as it allows quantification of the template using fluoresce signals during the exponential phase of amplification whereas in RT-PCR, fluorescent data is collected at the end of the PCR reaction (Agilent Technologies, 2012). RT-qPCR can be used for many applications such as: gene expression analysis, RNA interference (RNAi) validation, microarray validation, pathogen detection, genetic testing, and disease research (Thermo Fisher Scientific, 2020a).

Depending on the biological question, RT-qPCR has different options such as one-step vs two-step amplification and detection methods (Figure 4 A) which each have their advantages and disadvantages (Table 1) when setting up the analysis. In addition, there are a list of adjustments that can be made to optimize the assay but there are minimum, key elements that must be considered for the results to be valid. Each reaction must efficiently amplify a single product, amplification efficiency must be independent of template concentration and the amplification of other templates and there must be appropriate controls depending on the use of the assay. This can be shown by use of a standard curve to calculate efficiency, a melting curve or gel analysis to ensure one product is amplified, and the use of positive/negative control or passive reference dye (Agilent Technologies, 2012; Bio-Rad, 2020; Bustin et al., 2009).

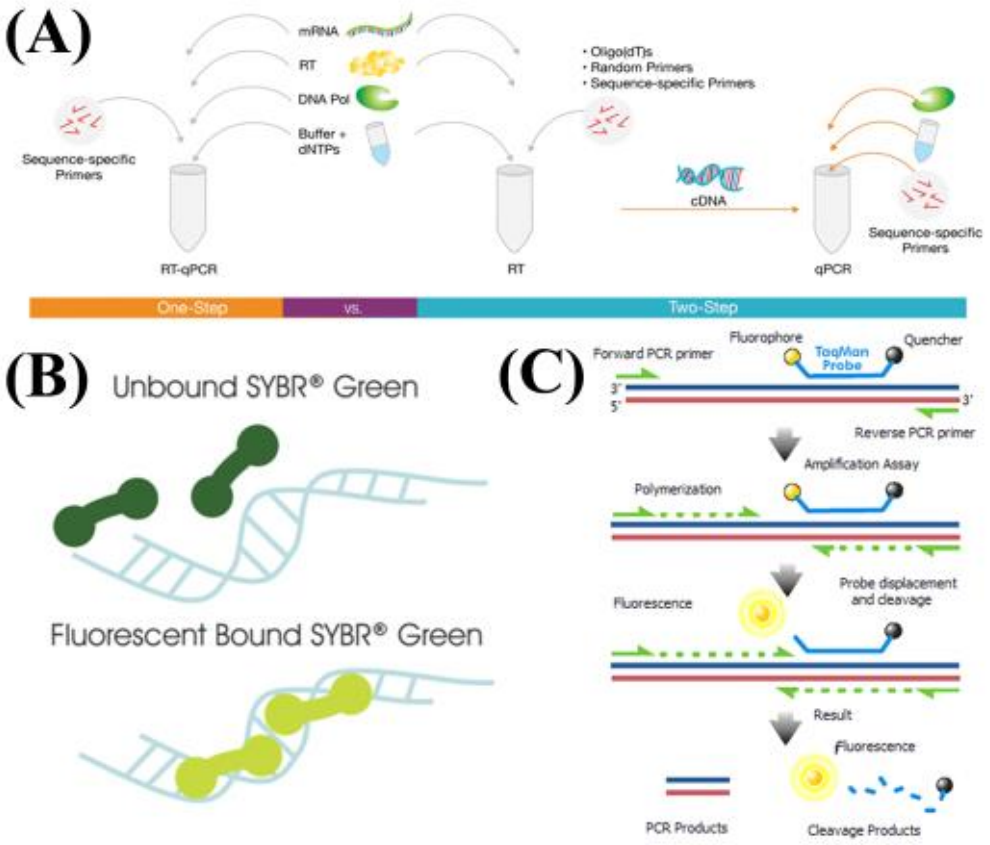

Figure 4. *qPCR reaction types and chemistry options*

(A) comparison of one-step and two-step RT-qPCR. In one-step RT-qPCR all reagents are put together and reverse transcription is performed in tandem with the qPCR reaction. In two-step RT-qPCR reverse transcription is performed first and cDNA is generated. This cDNA is then mixed with the reagents necessary for qPCR. (B) SYBR® Green detection mechanism. When bound to double stranded DNA or cDNA SYBR® Green is 1000-fold more fluorescent than in the unbound state. As the PCR reaction occurs, the signal increases proportionately with the amplification. (C)hydrolysis probe-based detection method. The dual labeled probe is cleaved by the nuclease activity of Taq DNA polymerase during the hybridization of the complementary sequence. Retrieved from (Agilent Technologies, 2012; Thermo Fisher Scientific, 2020a).

Table 1. *Advantages and disadvantages of one-step and two-step RT-qPCR retrieved from (Thermo Fisher Scientific, 2020a)*

	Advantages	Disadvantages
One-step	• Less experimental variation since both reactions take place in the same tube • Fewer pipetting steps reduces risk of contamination • Suitable for high throughput amplification/screening • Fast and highly reproducible	• Impossible to optimize the two reactions separately • Less sensitive than two-step because the reaction conditions are a compromise between the two combined reactions • Detection of fewer targets per sample
Two-step	• A stable cDNA pool is generated that can be stored for long periods of time and used for multiple reactions • The target and reference genes can be amplified from the same cDNA pool without multiplexing • Optimized reaction buffers and reaction conditions can be used for each individual reaction • Flexible priming options	• The use of several tubes and pipetting steps exposes the reaction to a greater risk of DNA contamination • Requires more optimization than one-step • Time consuming

Amplification occurs in two phases, the exponential phase and the nonexponential plateau phase (Figure 5). Depending on the efficiency of the polymerase, the amount of PCR product roughly doubles in each cycle during the exponential phase until reaction components become limited (usually primers) as they are consumed causing a slowing of the reaction during the nonexponential plateau phase (Freeman et al., 1999). Quantification cycle (C_q) (Figure 5) is the cycle number at which the PCR products accumulate enough to produce a detectable fluorescent signal greater than the background levels. C_q values are dependent on initial template concentrations. Higher amounts of initial templates will take fewer cycles to reach the detection threshold (lower C_q) while lower amounts of initial template will take more cycles to reach the detection threshold (higher C_q) forming the basis for the quantitative aspect of qPCR (Agilent Technologies, 2012; Bio-Rad, n.d.).

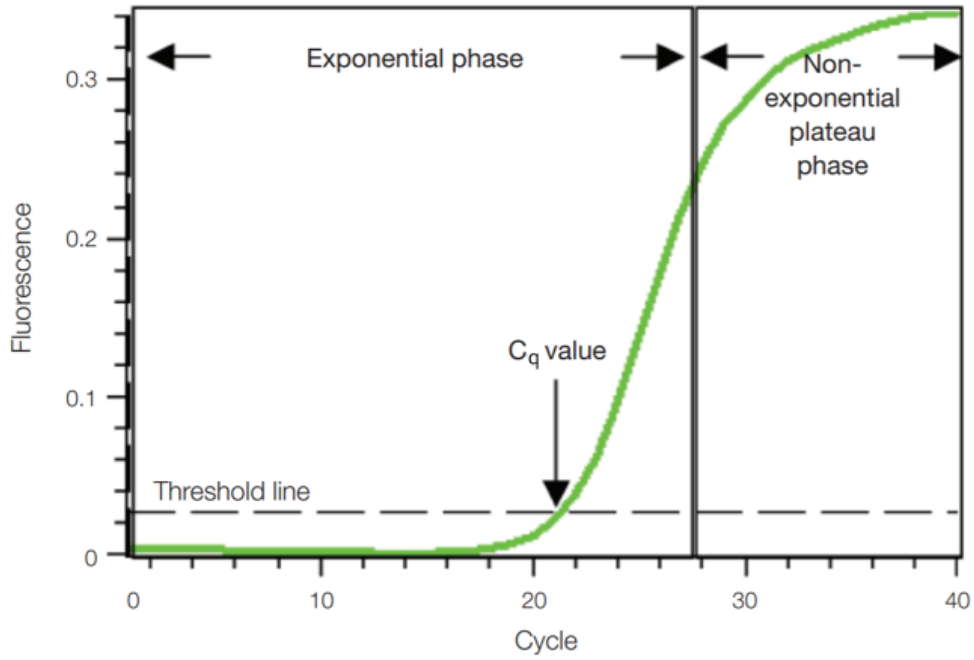

***Figure 5**. Amplification plot in qPCR*

Amplification plot showing qPCR output data with labels indicating the exponential phase, Non-exponential plateau phase and example C_q value. Retrieved from (Bio-Rad, n.d.).

1.6.3 Target genes of interest

Cortisol modulates the gene expression of target tissues through binding to glucocorticoid receptors (reviewed in Mommsen et al. (1999)) (Figure 6) which can have effects on the adaptive immune system response. While many genes are potentially modulated, in this study we will be analyzing gene expression in proinflammatory cytokines transforming growth factor beta 1 (*tgfb1*), tumor necrosis factor alpha 2 (*tnfa*), interleukin-1 beta (*il1β*); the acute phase protein serum amyloid A-5 (*saa5*); and Galectin-3,4,8 (*leg3, leg4, lgals8*) which are multifunctional proteins that bind to β-galactoside sugars (Johannes et al., 2018; Niklasson et al., 2011; Sack, 2018). Baseline reference genes are needed for gene expression studies (Bustin et al., 2009) and beta-actin (*β-actin*), eukaryotic translation elongation factor 1AB (*ef1a*) have been shown to be established reference genes in Atlantic salmon (Olsvik et al., 2005), and were used in this thesis.

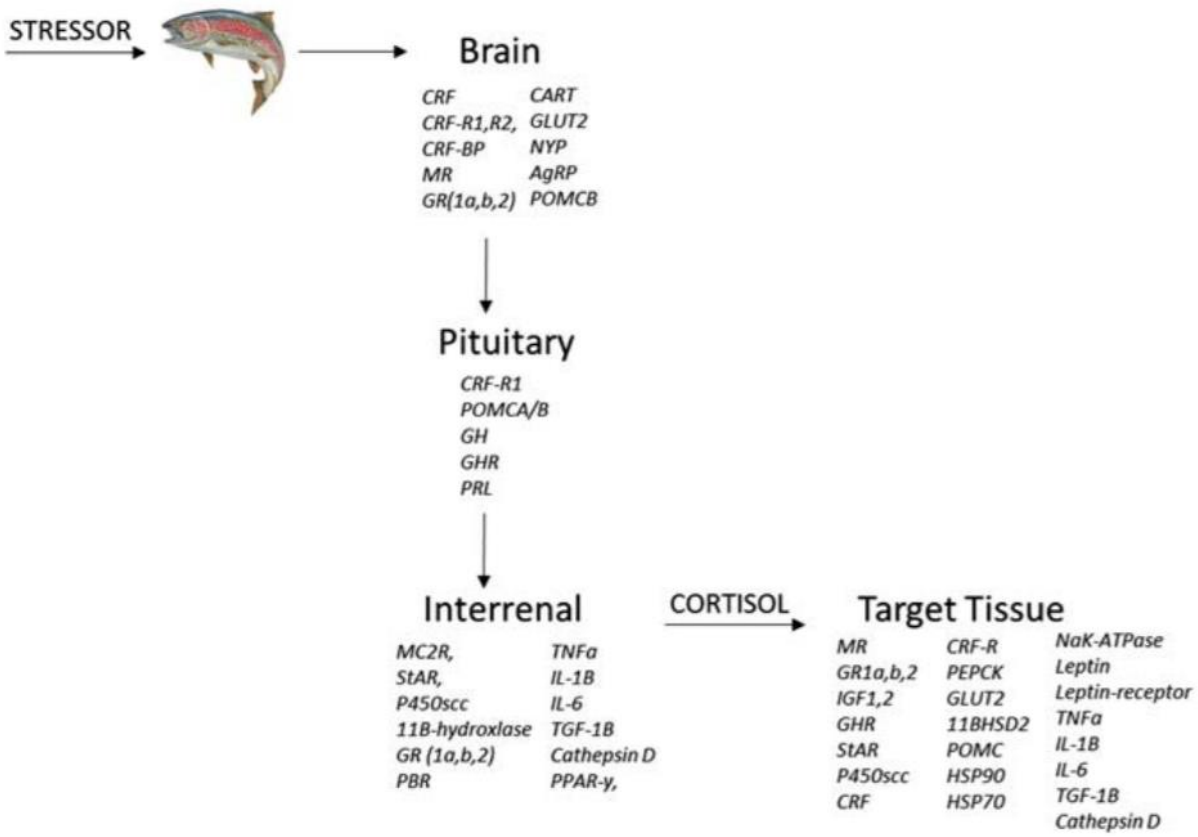

Figure 6. *Overview of stress response from stressor to target tissues*

Stress related genes activated by HPI axis activation with extra attention to TNF-α, IL-1β and TGF-1β in target tissues. After the HPI axis is activated cortisol modulates the expression of genes in the target tissue (Carl B Schreck et al., 2016).

1.7 Objective

The objective of this study is to analyze the effects of cumulative stress on immune relevant gene expression in the skin and gill of Atlantic salmon. This objective would be reached by:

1) Conducting a controlled fish stress experiment that replicates practices in the aquaculture industry and analyzing physiological indicators of stress.
2) Determining the best extraction method of RNA for gill and skin in Atlantic salmon.
3) Evaluate the suitability of primer pairs of immune genes found in published papers.
4) Establishment and verification of new immune relevant primer pairs in the galectin family.

2.0 Materials and method

2.1 Approval for use of animals in research

The experiment was approved by Mattilsynet by letter on 24.05.19 and is registered with approval FOTS ID 19447.

2.2 Challenges before experiment

One month prior to the beginning of the experiment, construction of a new school began, and dynamite explosions took place daily, multiple times throughout the day. The pre-stress fish were kept as far away as possible from the explosions, but it must be noted that conditions were not optimal pre experiment for the pre-stress group. Demolition was halted for the duration of the experiment once it began (0 h – 168 h).

2.3 Experimental setup

250 Atlantic salmon strain QTL-IPN from Salmobreed were hatched 02.03.2018 at Salten Smolt avdeling Breivik and received at Mørkvedbukta research station on 08.05.2019 by boat. The fish were placed in a 1.5 m^3 tank and climatized until the first sampling time on 18.11.2019. Water oxygen was on average at 85 % ± 6.1 % saturation with an average temperature of 7.8℃. 78 fish with an average weigh of 544.30 ± 258.03 g and an average length of 33.47 ± 4.8 cm were used for the experiment. To create a cumulative stress challenge, 66 fish were subjected to stress by air exposure for 90 s directly followed by transport via tractor in a transport tank (transport time 15 min) to a new experimental hall. After transfer, 6 fish were immediately sampled, and the rest were divided into 10 groups of 6 fish per tank (Figure 7 B).

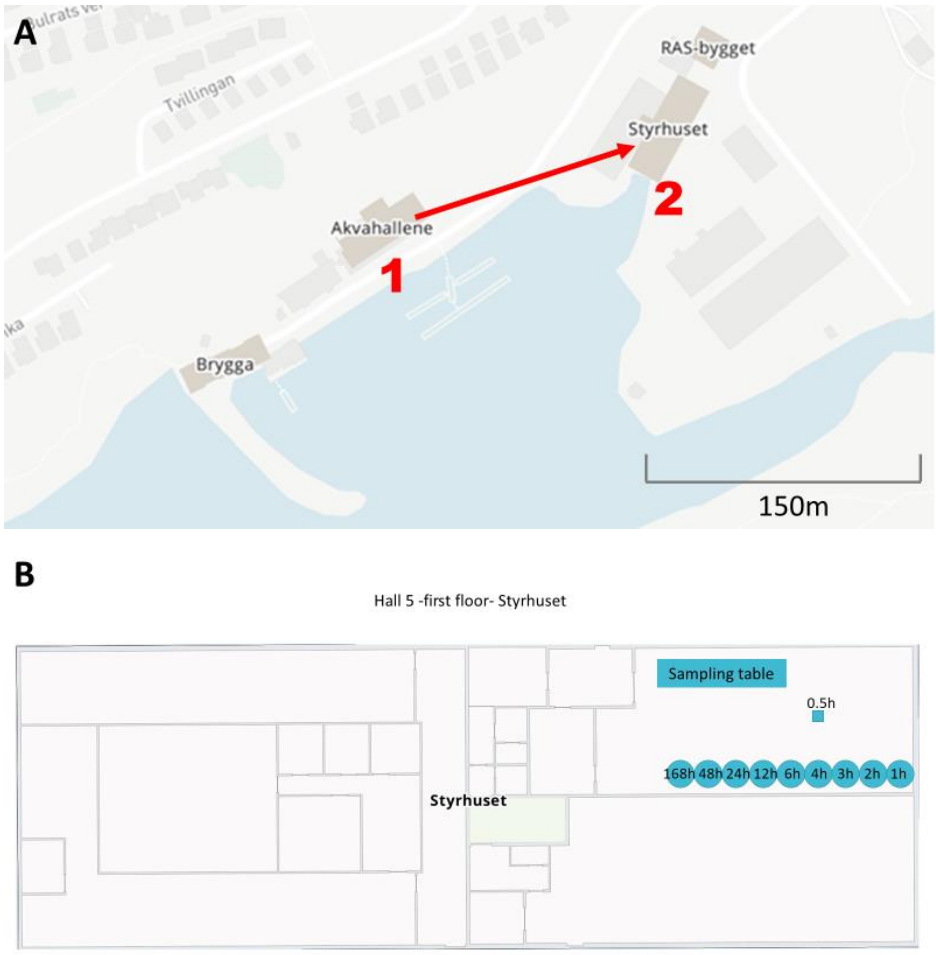

Figure 7. *Map overview of Mørkvedbukta research station in Bodø, Norway*

A) Atlantic salmon were stressed at Akvahallen (1) and immediately transported by tractor in a tank to Styrhuset (2). B) An overview of hall 5 in the basement of Styrhuset depicting how the sampling area was setup. All groups besides 0.5h, which was placed in a small bucket for short term storage, were placed into separate tanks until sampling. (MazeMap.com, n.d.).

2.4 Sampling method

Before the cumulative stress challenge, 12 pre- stress salmon were anesthetized in a 5 mg/l metomidate bath until stage 4 anesthesia (failure to respond to external stimuli) (Iversen et al., 2003) was achieved, euthanized by using a blow to the head and were used as the control for this experiment. All six fish from each group were anesthetized and euthanized in the same manner as the pre-stress group at the time points of 0, 0.5, 1, 2, 3, 4, 6, 12, 24, 48, and 168 h after stressor. The fish were sampled for blood, gill, skin, muscle, and hindgut (Figure 8 A and

B) and were immediately put into 1.2 ml cryotubes (VWR, USA) where they were frozen immediately using liquid nitrogen. All samples were stored at -80°C until further analysis.

2.5 Tissue sampling

The skin sample was taken by removing the scales using a scalpel blade and making a 1 cm^2 incision through the skin and muscle on the dorsal anterior side, above the pectoral fin (Figure 8 A). Forceps were used to separate the skin from the muscle tissue and a scalpel was used to divide the skin into ½ pea sized subsamples for cryopreservation.

For muscle samples a 1 cm^3 piece of scales, skin, and muscle was excised from the dorsal posterior side, above and between the pelvic and anal fin (Figure 8 A). This location was chosen as its located in what is referred to as the "Norwegian Quality Cut" and is where flesh quality samples are taken in the industry. A subsample of muscle was removed from the excised piece of tissue and was divided into ½ pea sized subsamples for cryopreservation.

Gills samples were taken from the middle of the second gill arch on the left lateral side.

Hind gut samples were taken by cutting the open the fish from the anus to the ventral anterior end until the body cavity was opened. From there intestine was were manually stripped by squeezing out the feces. A 0.5 x 1 cm section of hind gut was excised roughly 1 cm after the beginning of the hindgut (Figure 8 B). As with all other samples the hindgut sample was divided into ½ pea sized subsamples in preparation for cryopreservation. Due to time restrain the muscle and hind gut samples, as well as some timepoint of skin and gull samples were not analyzed in the thesis.

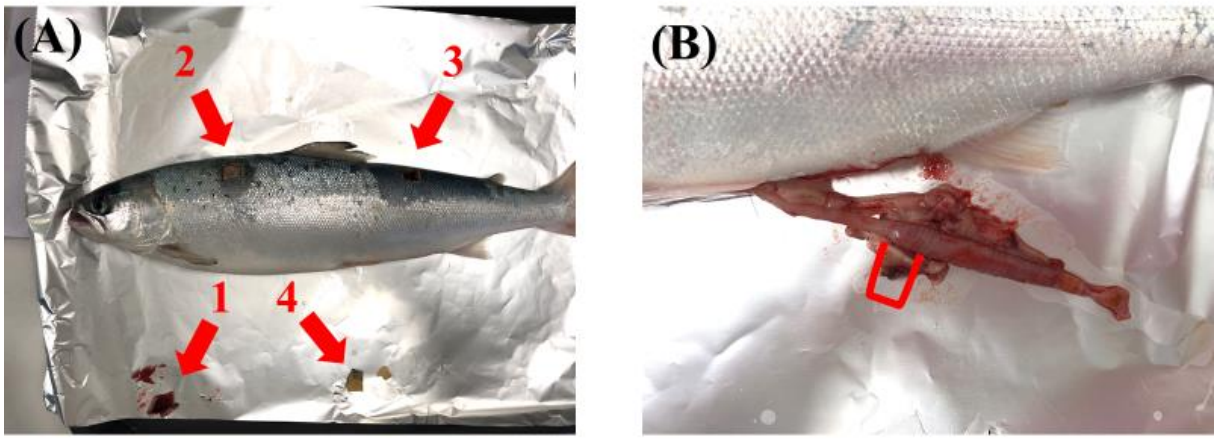

Figure 8. *Overview of tissue sampling*

Four tissues, gill, skin, muscle, and hindgut were sampled in Atlantic salmon for the experiment. (A) The photo shows where gill, skin and muscle tissues were taken with red arrows indicating tissues/locations. 1) the second gill arch was removed and sampled. 2) Skin samples were taken by removing the scales, cutting a 1 cm² square, and removing the skin with forceps. 3) location of where muscle samples were excised. 4) the excised muscle with skin still attached. (B) Example of hindgut sampling with the red bracket indicating the sampling location.

2.6 Mg^{++} analysis

Blood samples were taken from the caudal artery with a 0.6 x 25 mm heparinized syringe (1 ml) centrifuged and transferred to a 1.5 ml Eppendorf LoBind tube (VWR, Norway). Blood was centrifuged at 2,400 g (Heraeus Fresco 21, Thermo, Germany) for 5 min to isolate plasma and stored at -40°C. Mg^{++} content was measured in the isolated plasma using the Respons®910 bench top analyzer (DiaSys diagnostic systems, Germany).

2.7 Gene and primer selection

Primers were selected from published papers for beta-actin (β-actin), eukaryotic translation elongation factor $1A_B$ (*ef1a*), interleukin-1 beta (*il1β*), serum amyloid A-5 (*saa5*), transforming growth factor beta 1(*tgfb1*) and tumor necrosis factor alpha 2 (*tnfa*) (Table 2). Multiple primers for Galectin-3, 4, 8 (*leg3, leg4, lgals8*) (Table 3) were designed by the Primer-BLAST tool (National Center for Biotechnology Information, n.d.). The primers were designed specifically

for Atlantic salmon (taxid:8030), spanned exon-exon junctions and primer melting temperatures (T_m) were set to be within the range of 55℃ - 60℃ with a max T_m difference of 4℃ and the optimal T_m of 58℃. Primers were verified by comparing amplicon size to the predicted amplicon size after RT-PCR and visualized in a 2 % agarose gel in TBE buffer. Primers that generated a PCR product were then further checked in RT-qPCR by looking at amplification efficiency and melting peaks to check for off target products.

***Table 2**. Immune relevant primers used in this study, from the literature*

Gene name Accession nr.	Primer Direction	Sequence (5'→3')	Amplicon size	Reference for primers
β-actin BG933897	Forward	CCAAAGCCAACAGGGAGAA	91 bp	(Olsvik et al., 2013)
	Reverse	AGGGACAACACTGCCTGGA		
ef1a NM_001123629.1	Forward	CCCCTCCAGGACGTTTACAAA	57 bp	(Olsvik et al., 2013)
	Reverse	CACACGGCCCACAGGTACA		
il1β NM_001123582.1	Forward	GCTGGAGAGTGTGTGGAAGA	72 bp	(Haugland et al., 2005)
	Reverse	TGCTTCCCTCCTGCTCGTAG		
il1β NM_001123582.1	Forward	CCGTCCCCATTGAGACTAAAG	156 bp	(Lee et al., 2017)
	Reverse	TGTCGCTCTGCTGGCTGA		
il1β NM_001123582.1	Forward	TGAAGTCCATCAGCCAGCAG	195 bp	(Chalmers et al., 2018)
	Reverse	GGATGGTGAAGGTGGTGAGG		
tgfb1	Forward	ATCGGAGAGTTGCTGTGTGC	108	(Fast et al., 2006)

XM_014129261.1	Reverse	GGGCCGATGCAGTAGTTAGC		
tgfb1	Forward	AGTTGCCTTGTGATTGTGGGA	194	(Lilleeng et al., 2009)
XM_014129261.1	Reverse	CTCTTCAGTAGTGGTTTGTCG		
tnfa	Forward	TGCTGGCAATGCAAAAGTAG	178	(Jensen et al., 2019)
AY848945.1	Reverse	AGCCTGGCTGTAAACGAAGA		
tnfa	Forward	ATGGAAGACTGGCAACGATG	130	(Lee et al., 2017)
NM_001123590.1	Reverse	TCACCCTCTAAATGGATGGC		
tnfa	Forward	TGCTGGCAATGCAAAAGTAG	178	(Vasanth et al., 2015)
AY848945.1	Reverse	AGCCTTGGCTGTAAACGAAGA		
saa5	Forward	GGTGAAGCTGCTCGAGGTGC	172	(Lee et al., 2017)
NM_001146565.1	Reverse	CCATCTCCCGGCCATTACTGAT		
saa5	Forward	GCAGCAGCAGTCATCAGTA	151	(Vasanth et al., 2015)
NM_001146565.1	Reverse	AGTTCCTTGGGAGTCCATTT		

saa5	Forward	ACAAGTACTTCCACGCTCGG	125	(Chalmers et al., 2018)
NM_001146565.1	Reverse	TCCTCATGTCCTCGACCACT		

Table 3. *Primers designed in this study for Galectin-3, 4, 8*

Gene abbreviation	Primer direction	Sequence (5'→3')	Amplicon size	NCBI Reference Sequence used in Primer-BLAST
leg3	Forward	ACGGAGCTACTAACAGATACA	826	NM_001140833.1
	Reverse	CAATCTCCACAGACGTGAGG		NM_001140833.1
leg3	Forward	CCTGTCTACTGTATCTCTGCC	720	NM_001140833.1
	Reverse	CATGATCTTCATCTCGAAGGGC		NM_001140833.1
leg3	Forward	TGAACGGAGCTACTAACAGATACA	935	NM_001140833.1
	Reverse	GATGGAAGGTCACTGGAACC		NM_001140833.1
leg3	Forward	GAACGGAGCTACTAACAGATACA	91	NM_001140833.1
	Reverse	CCACTACTCTGCTGGTTGTT		NM_001140833.1
leg3	Forward	AACGGAGCTACTAACAGATACAA	91	NM_001140833.1
	Reverse	CCCACTACTCTGCTGGTTGTT		NM_001140833.1
leg4	Forward	TAGTATCCCCTATGTGGGGC	98	NM_001146582.1

	Reverse	TGTTGAAACTGGTGATCTCGT		NM_001146582.1
leg4	Forward	CCTCACGAGATCACCAGTTTC	103	NM_001146582.1
	Reverse	CCTTGTCCCAGTTGTCGAAG		NM_001146582.1
leg4	Forward	CTACCAGCCCGTCTACAATC	120	NM_001146582.1
	Reverse	ATGTTGAAACTGGTGATCTCGT		NM_001146582.1
*lgals*8	Forward	TCTGAATCCGACCATTCCCT	118	NM_001140306.1
	Reverse	GGTGAAGTCCACCTGAAACC		NM_001140306.1
lgals8	Forward	TCTGAATCCGACCATTCCCT	110	NM_001140306.1
	Reverse	CCACCTGAAACCTCTCACAA		NM_001140306.1
lgals8	Forward	CTGAATCCGACCATTCCCTT	111	NM_001140306.1
	Reverse	GTCCACCTGAAACCTCTCAC		NM_001140306.1

2.8 Organic based tissue RNA extraction – QIAzol/ chloroform method

Tissues were added to 1000 µl of QIAzol (Qiagen, Germany) lysis reagent in 2 ml reinforced homogenizer tubes (VWR, USA) with 1.4 mm ceramic beads (Qiagen, Germany). The samples were then homogenized at 9,600 g for 40 s. The homogenate was incubated on ice for 5 min. The supernatant was transferred to 1.5 DNA LoBind tubes (Eppendorf®, Germany) where 200 µl of ice-cold chloroform (Sigma Aldrich, USA) was added, shaken vigorously for 15 s and left to incubate for 2 min on ice. After incubation, the samples were centrifuged at 12,000 g for 15 min at 4°C. The water phase containing RNA was transferred to a new 1.5 ml DNA LoBind tube. 500 µl of ice cold 2-Propanol (Sigma Aldrich, USA) was added to precipitate the RNA and was placed in a -20°C freezer for 20 min. After incubation, the sample was centrifuged at 21,100 g f1or 15 min. The supernatant was removed, and the pellet was rinsed with 1 ml of ice-cold 75 % ethanol. Once again, the sample was centrifuged for 5 min at 21,100 g. The ethanol was removed, and the sample left on the benchtop for a maximum of 10 min to allow any excess ethanol to evaporate. The pellet was resuspended in 20 µl of molecular biology grade water (Corning, USA).

2.9 Filter-based tissue RNA extraction – E.Z.N.A.® Total RNA Kit I

All samples were re-extracted with an E.Z.N.A.® Total RNA Kit I (Omega bio-tek, USA) following the manufacturers protocol. Tissues were added to 700 µl of TRK Lysis Buffer in 2 ml reinforced homogenizer tubes (VWR, USA) with 1.4 mm ceramic beads (Qiagen, Germany). The samples were then homogenized at 9,600 g for 40 s and then centrifuged at 21,100 g for 5 min. The supernatant was transferred to a 1.5 ml DNA LoBind tube where one volume of 70 % ethanol was added. The total sample was then transferred to a HiBind® RNA Mini Colum and centrifuged at 10,000 g for 1 min where the remaining filtrate was discarded. 500 µl RNA Wash Buffer I was rinsed through the HiBind® RNA Mini Colum by centrifuging at 10,000 g for 30 s followed by 500 µl of RNA Wash Buffer II centrifuged at 10,000 g for 1 min. The rinse for RNA Wash Buffer II was repeated for a second time. The HiBind® RNA Mini Colum was dried to remove trace ethanol by centrifuging the sample at maximum speed for 2 min. RNA was eluted by adding 40 µl of DEPC water to the HiBind® RNA Mini Colum into a new 1.5 ml DNA LoBind tube. All samples were stored at -80°C.

2.10 RNA quantification and integrity

For RNA quantification and standardization, 1 µl subsamples were taken for each sample for use in the Qubit™ RNA BR Assay Kit (Thermo Fisher, USA). Following the protocol from the manufacturer, RNA concentrations were determined using the Qubit™ 3.0 fluorometer (Invitrogen, USA) and recorded. RNA integrity was checked by taking a 3 µl subsample of extracted RNA, 7 µl of molecular grade water, 1 µl of BlueJuice™ (Thermo Fisher, USA), the denaturing it at 65°C for 3 min and running gel electrophoresis on a 2 % agarose gel made with 1x Tris-borate-EDTA (TBE) buffer with an electric current of 50 V for 70 min. The extracted RNA was stored in a -80°C freezer until further use. Any samples that had low concentration or low RNA integrity were re-extracted. In addition to gel electrophoresis, 28 of the 36 samples that were extracted using QIAzol/chloroform extraction were randomly selected to further check for RNA integrity using the Agilent High Sensitivity RNA ScreenTape® (Agilent Technologies, USA) on the 2200 TapeStation system (Agilent Technologies, USA). All samples were not able to be analyzed due to lack of consumables (High Sensitivity RNA ScreenTape®). Samples were diluted to 5,000 pg/µl and the manufacturers protocol was followed.

2.11 Reverse transcription

Using RNA concentrations from the Qubit™, RNA concentrations were standardized to 1 µg and cDNA was generated from the extracted RNA using the QuantiTect® reverse transcription

kit (Qiagen, Germany) with some modifications. After standardizing samples to1 µg, 2 µl of gDNA Wipeout Buffer 7x was added to each sample, incubated for 8 min at 42℃ and immediately placed on ice. 6 µl of Reverse-transcription master mix was added to each sample and incubated for 30 min at 42℃ followed by a 3 min incubation at 95℃ to inactivate the Quantiscript Reverse Transcriptase. The generated cDNA was stored at -20℃ until qPCR was performed.

2.12 RT- qPCR

cDNA products were diluted 10, 25, and 50 times. In addition, 2, 5, and 10-fold serial dilutions were used to determine the drop off of the standard curve. It was determined that a 25 times dilution with a 10-fold serial dilution would be used for the experiment. cDNA products were combined with FastStart Universal SYBR Green Master (Rox) (Sigma Aldrich, USA) and primer pairs on a LightCycler® 480 Multiwell Plate 96 (Roche, Germany). Standards were prepared for each 96 well plate by using cDNA generated from pooled RNA for each tissue type. Five ten-fold serial dilutions were made for each plate in triplicate to generate the standard curve for the polymerase efficiency. Negative control and a non-reverse transcription control in duplicate were used for every plate. Samples were run in duplicate with total of eight 96 well plates where each plate was had primers for one gene. The run editor in the light cycler 96 SW 1.1 software Application Software Version: 1.1.0.1320 (Roche, Germany) was used to create the cycling conditions for real- time PCR for use on a LightCycler® 96 Instrument Software Version: 1.01.01.0050 (Roche, Germany). The cycling condition were as follows: Preincubation at 95℃ for 600 s, 40 cycles of 2-step amplification at 95℃ for 10 s then 60℃ for 30 s, followed by a melting cycle at 95℃ for 10 s, 65℃ for 60 s, 97℃ for 1 s and cooling at 37℃ for 30 s.

2.13 Statistical analysis

For analysis of RT-qPCR data, the Delta-Delta C_t method ($\Delta\Delta$ C_t) was used. The data was prepared for analysis by taking the arithmetic mean Cq for all samples was taken followed by taking the geometric mean of the arithmetic Cq mean for the reference genes. Relative quantification (RQ) fold changes were calculated ($2^{\wedge}\Delta\Delta$ C_t) with the pre-stress group being the calibrator (RQ = 1) where all samples were compared to the calibrator group i.e. RQ = 10 is a tenfold increase while RQ = 0.1 is a tenfold decrease. Fold decreases were calculated by using the equation [fold change = - (1/RQ) if RQ < 1] for better graphical representation. BestKeeper (https://www.gene-quantification.de/bestkeeper.html) was used to determine the stability of β-actin and *eef1a* to verify their use as reference genes. RStudio for Windows (ver. 1.1.456) was

used for statistical analysis and GraphPad Prism 9 (GraphPad Software, USA) was used to make graphs. Shapiro wilks test for normality and Levene's test for homogeneity was performed on all the data. By use of one-way ANOVA test change of each of the parameters in time in comparison to the pre-stress group was tested. Dunnett's test was used for post hoc analysis. If assumptions for the one-way ANOVA test were not met, the non-parametric Kruskal–Wallis ANOVA was used with Dunn's test as the post hoc analysis instead.

For Mg^{++} data, the program SPSS for Windows (ver. 18.00) was used to complete statistical analyses. Kolmogorov–Smirnov test for normality and Levene's test for homogeneity was performed on all the data. Afterward, change in each of the parameters in time in comparison to the pre-stress situation was tested with one-way ANOVA test (Sokal & Rohlf, 1987). Depending on the result another test was performed: if the F-values were significant, Bonferroni post hoc test was done to verify if there were differences between groups and between times of experiment. The Kruskal–Wallis ANOVA (nonparametric) and Mann–Whitney U test with a Bonferroni-adjusted significance level were used when requirements for parametric statistics were not met. Significant differences were established at level 0.05. Results are presented as the mean ± standard deviation (SD). Sign * at the figures indicates significant difference at a given sampling compared to pre-stress levels within the same experimental group.

To study the relationship between plasma cortisol and secondary stress responses such as magnesium (Mg^{++}), a non-linear regression analysis was performed (Pearson r). Significant difference was determined at the 0.05 level. All results are expressed on average with standard deviation (ñ ± SD). Significant differences in the figures within a group at different sampling times compared to pre-stress were indicated by * symbol.

3.0 Results

3.1 Physiological characteristics of stress

The stress experiment in this study was done together with two other Master students, Benedikte Hokland Ottestad and Vilde Charlotte Alsos. Cortisol, glucose, lactate, and hemoglobin data included here are from their theses (Table 4). This data is included to complement Mg^{++} data in the stress response.

Table 4. *Physiological parameters relating to stress*

*Glucose, lactate, and hemoglobin measurements for all time points in the experiment to show the stress response. Significant differences from pre-stress concentrations is indicated by a * symbol.*

Group	Glucose (mM)	Lactate (mM)	Hemoglobin (mM)
Pre-stress	2.87 ± 0.47	6.68 ± 1.90	10.30 ± 0.94
0 h post stress	3.03 ± 0.39	9.28 ± 1.14*	10.87 ± 0.90
0.5 h post stress	3.28 ± 0.54	9.88 ± 1.23*	10.72 ± 0.98
1 h post stress	3.63 ± 0.29	15.12 ± 2.24*	11.52 ± 0.59*
2 h post stress	3.78 ± 0.78*	13.17 ± 2.45*	10.08 ± 1.16*
3 h post stress	3.93 ± 0.55*	12.08 ± 3.91*	9.85 ± 0.65
4 h post stress	4.53 ± 0.76*	17.48 ± 3.13*	10.00 ± 1.08
6 h post stress	5.33 ± 0.38*	12.38 ± 4.73*	10.03 ± 0.78
12 h post stress	5.28 ± 0.55*	4.10 ± 0.90	9.85 ± 0.62
24 h post stress	4.25 ± 0.51*	3.53 ± 0.72	10.50 ± 0.58
48 h post stress	2.83 ± 0.23	3.98 ± 1.20	10.75 ± 1.04
168 h post stress	3.18 ± 0.43	4.33 ± 0.72	9.87 ± 0.43

To assess possible ion imbalance after stress exposure, Mg^{++} in plasma was measured (Figure 9). Mg^{++} levels were increased after stress and were significantly higher than pre-stress levels from 1 h post stress, reached a peak at 2 h and remained significantly higher than pre-stress levels until the end of the experiment (Figure 9).

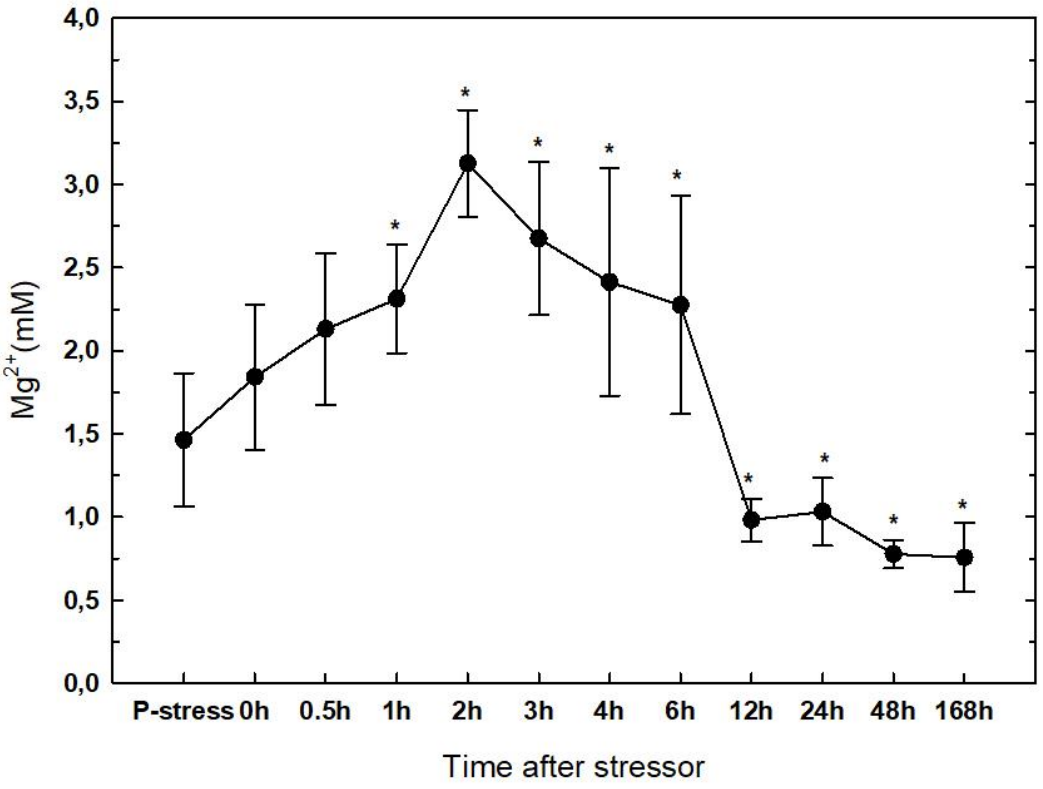

Figure 9. *Mg^{++} concentrations in plasma before and after air exposure and transport*

*Sign * at the figures indicates significant difference at a given sampling compared to pre-stress levels within the same experimental group.*

At 0 h, cortisol levels in plasma had already increased from pre-stress levels, (Figure 10), peaked at 1 h and remained significantly higher than the pre-stress level until 24 h post stressor, with an exception for 12 h post stressor (Figure 10). There was a high correlation of 0.0875 (Pearson *r*) between Mg^{++} and cortisol levels.

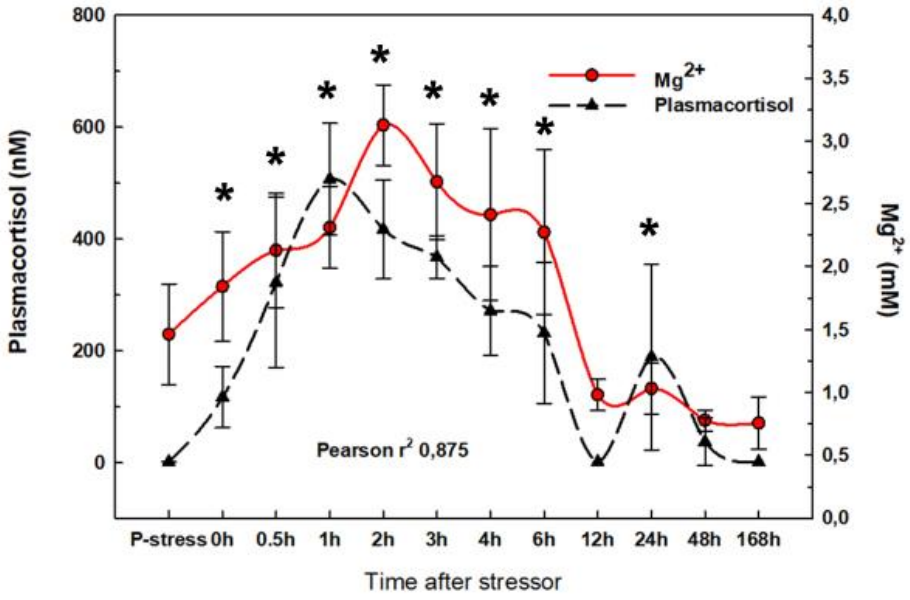

Figure 10. *Mg^{++} and cortisol concentrations in plasma before and after air exposure and transport*

*Significant difference was determined at the 0.05 level. All results are expressed on average with standard deviation (ñ ± SD). Cortisol levels significantly different from the pre-stress group are indicated by * symbol.*

3.2 Comparison of RNA extraction methods

Two different RNA extraction methods were used to obtain RNA for use in downstream applications. Two main factors when comparing the methods are RNA quality and potential impact of polymerase inhibitors in RT-qPCR that were introduced during the extraction process. Both QIAzol/ chloroform extraction (Figure 11 A) and E.Z.N.A.® Total RNA Kit I (Figure 11 B) extractions resulted in intact 18s and 28s ribosomal RNA (rRNA) seen as two clear band in agarose gel (Figure 11 A and B). The column-based extractions have more biological debris than the QIAzol/ chloroform extracted samples and is visible as weak bands above the 28s rRNA band (Figure 11 B). Lack of consumables allowed for only 28 samples to be analyzed by the TapeStation 2200 system, therefore only one type of extraction method (QIAzol/ chloroform) samples were analyzed on the TapeStation 2200 system. These samples were selected at random and showed acceptable RNA integrity number (RIN) >7.8. (Figure 11 C).

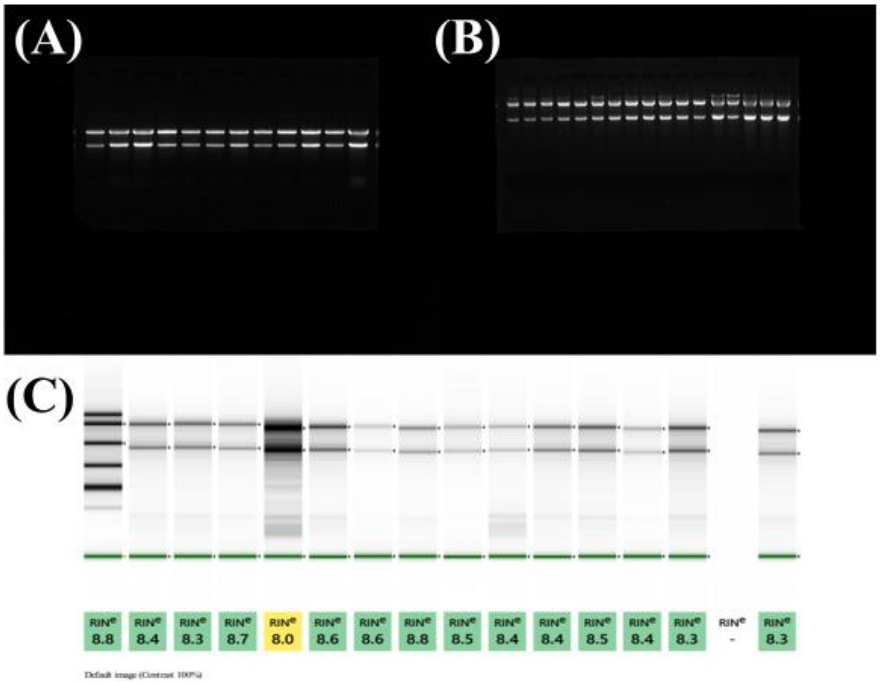

Figure 11. *RNA quality check*

(A) Total RNA extracted from gill using the QIAzol/chloroform extraction method. (B) Total RNA extracted from gill using the E.Z.N.A.® total RNA extraction kit I. (C) Total RNA extracted from skin using QIAzol/chloroform method run on the TapeStation 2200 system which assigns a RIN value to each sample.

The polymerase efficiency was compared between the two type of extraction methods for the target gene *saa5* (Figure 12 A and B). Samples extracted with QIAzol/ chloroform had a linear regression standard curve slope of -2.3983 (Figure 12 A) and samples extracted with E.Z.N.A.® total RNA extraction kit I (Figure 12 B) -3.3350 which calculate to a theoretical TAQ polymerase efficiency of 161.19 % and 99.46 % respectively. The calculations for gene expression require and efficiency of 100 ± 10 % which corresponds to a slope between -3.58 and -3.10 (Arya et al., 2005; Thermo Fisher Scientific, 2014) therefore it was determined that all samples should be extracted using the E.Z.N.A.® total RNA extraction kit I for reliable TAQ polymerase efficiency.

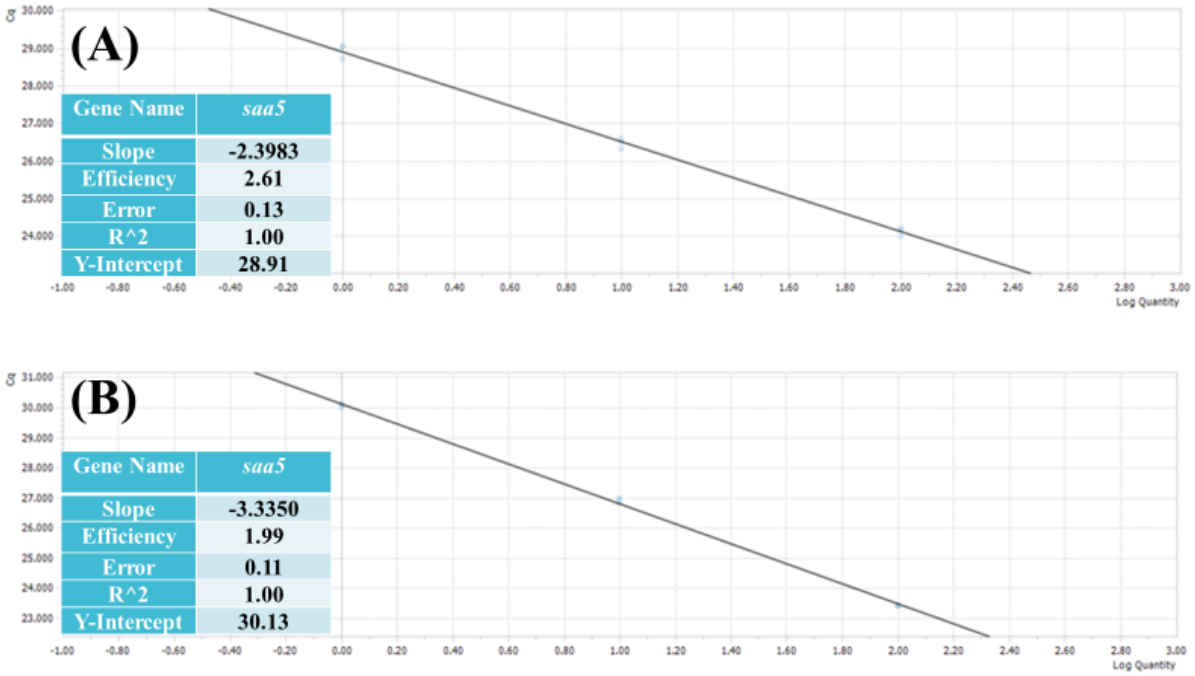

Figure 12. *Efficiency of the TAQ polymerase in the RT-qPCR reaction*

(A) Standard curve of samples extracted with QIAzol/chloroform where the back calculated slope indicates unrealistic polymerase activity. (B) Standard curve of samples extracted with the E.Z.N.A.® Total RNA extraction kit I where the back calculated slope indicates realistic polymerase activity.

All mean C_q values for target genes and reference genes were assessed by BestKeeper (https://www.gene-quantification.de/bestkeeper.html) to validate the stability of *β-actin* and *eef1a* for use as reference genes (Table 5).

Table 5. *BestKeeper analysis of reference genes*

Gene		*β-actin*	*eef1a*
coeff. of corr. [r]		1.00	0.99
coeff. of det. [r^2]		0.99	0.98
intercept [CP]		-4.46	4.74
slope [CP]		1.18	0.81
SE [CP]		±0.171	±0.189
p-value		0.001	0.001

3.3 Amplification of established primers in RT-PCR and RT-qPCR

β- actin, ef1a, il1β, saa5, tgfb1, tnfa primers were selected from published papers and tested for validity using RT-PCR and RT-qPCR. Nine of 13 primer pairs generated a RT-PCR product (Figure 13) from E.Z.N.A.® extracted tissues for all target genes except for *tgfb1*where one of two primer pairs gave a product and *tnfa* where no primer pairs gave a product. The PCR products had the expected size (Table 2) for *β-actin* (91 bp), *ef1a* (57 bp), *il1β* (72 bp, 156 bp, and 195 bp), *tgfb1* (108 bp), *saa5* (172 bp, 151 bp, and 125 bp). In RT- qPCR primers were tested on multiple plates for amplification (Figure 14 A and B). Of the nine, only three (Figure 14 A and B) of primer pairs amplified products at the desired range of C_q< 32 with the current protocol. Of the three, two of the primer pairs were for refence genes (*β- actin* & *ef1a*) (Figure 14 A and B) and the third was for the target gene *saa5*. (Figure 14 B).

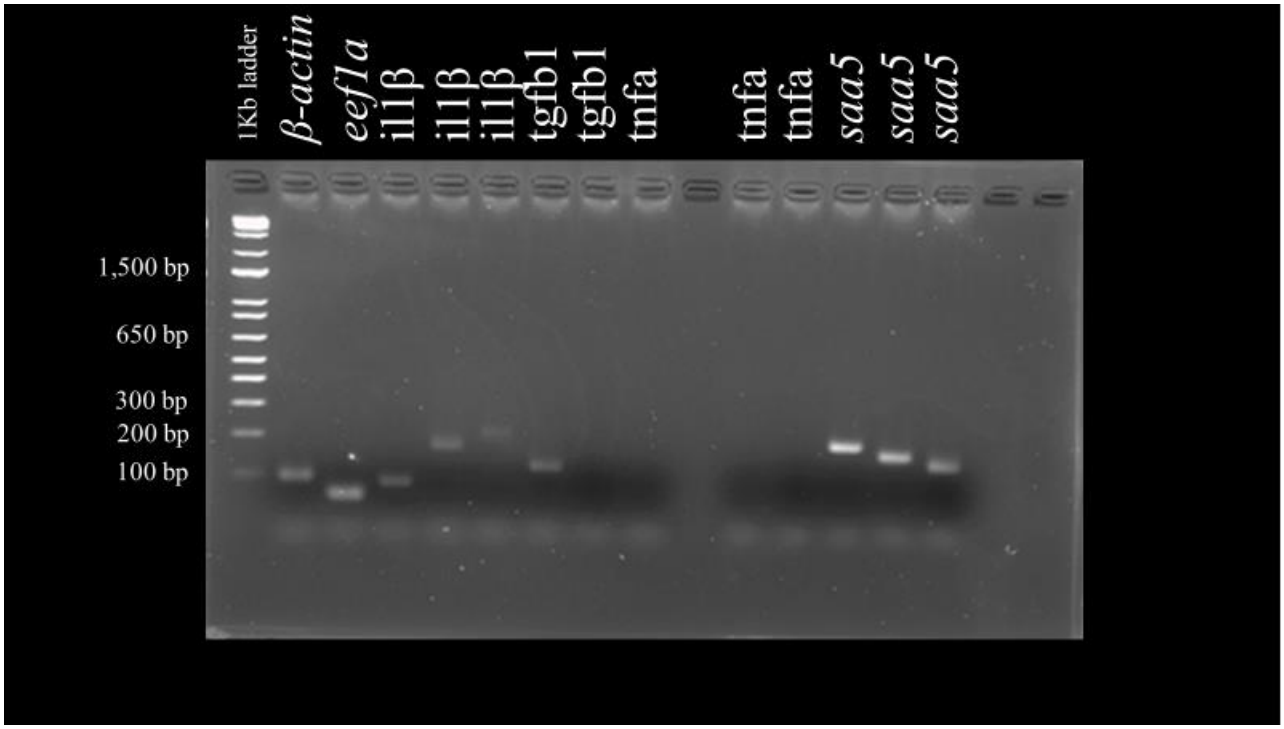

Figure 13. *RT-PCR primer testing for published genes*

2% agarose gel with TBE as the butter showing amplification of target genes using RT-PCR. RNA was isolated from skin and gill of experimental Atlantic salmon (pre-stress and stress), cDNA generated, and PCR was run with primers against selected genes. In the first lane is a 1Kb Plus DNA Ladder for reference sizes (Invitrogen, USA). cDNA was stained with Invitrogen SYBR Safe DNA Gel Stain (Invitrogen, USA).

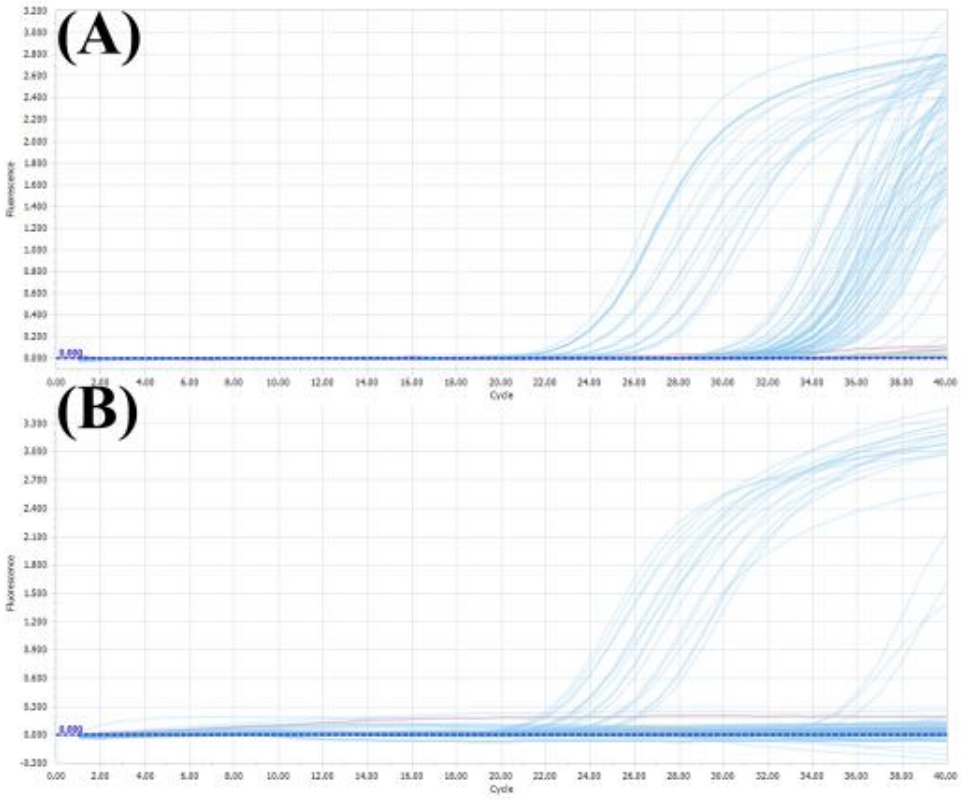

Figure 14. *Amplification plot of primers*

Primers were tested for amplification using RT-qPCR. Pooled cDNA that was generated from skin samples was used. (A) ef1a (arrow a), three il1β, one tgfb and one saa5 primer pairs tested for amplification. While many primer pairs amplified, they did not meet the desired target range of having Cq <32. (B) β-actin, ef1a, one il1β, and one tgfb primer pairs tested for amplification where only β-actin, ef1a amplified well as shown by their C_q values and amplification curves.

For analysis of RT-qPCR data, the Delta-Delta C_t method ($\Delta\Delta$ C_t) was used and mean RQ values were calculated and transformed using the equation [fold change = - (1/RQ) if RQ < 1] for better graphical representation in comparison with the calibrator group (pre-stress group, RQ=1). While not significant, the *saa5* expression trend in gill was decreased at 1 and 6 h post stressor followed by a significant increase in expression at 24 and 168 h post stressor (Figure 15).

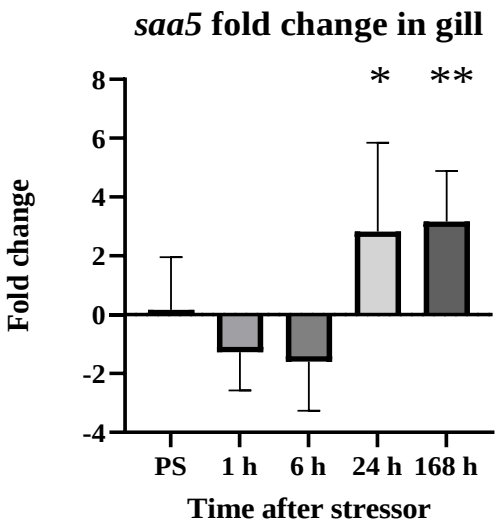

Figure 15. *Gene expression of saa5 in gill of Atlantic salmon*

*Pre-stress (PS), 1, 6, 24 and 168 h groups after stress. Quantification by use of RT-qPCR. Transcript levels are normalized to the reference genes: β-actin and eef1α with RQ fold change values indicated as means ± SD (n=6) by use of the calculation - (1/RQ) if RQ < 1 and * indicating a significant difference (P<0.05) in delta-delta C_t values compared to the PS group.*

In skin, *saa5* showed a significant decrease in expression 6 h post stressor, a non-significant increased trend in expression 24 and 168 h post stressor (Figure 16).

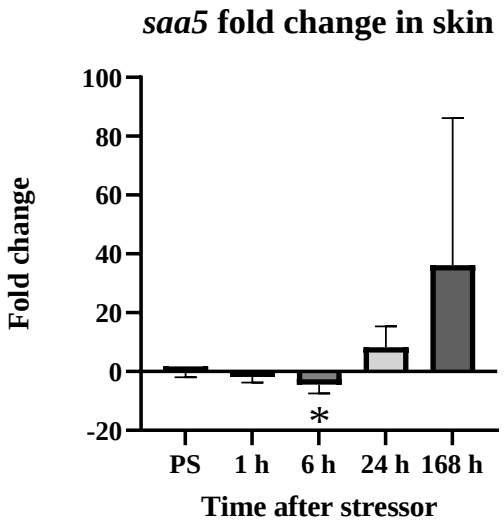

Figure 16. *Gene expression of saa5 in skin*

*Pre-stress (PS), 1, 6, 24 and 168 h groups after stress. Quantification by use of RT-qPCR. Transcript levels are normalized to the reference genes: β-actin and eef1α with RQ fold change values indicated as means ± SD (n=6) by use of the calculation - (1/RQ) if RQ < 1 and * indicating a significant difference (P<0.05) in delta-delta C_t values compared to the PS group.*

3.4 Amplification of novel galectin primers in RT-PCR and RT-qPCR

GenBank was used to find transcript (mRNA) data for Atlantic salmon galectins. Galectins-3, 4 and 8 were selected because their mRNA transcripts had the most complete REFSEQ status compared to other galectins in Atlantic salmon. Primers were designed using Primer-BLAST and had the criteria of spanning exon-exon junctions. Of the 11 primer pairs made, nine generated a RT- PCR product (Figure 17). The primers are shown in the same order as they are listed in Table 3, and they show the expected product lengths. For *leg 3* RT-PCR products of 826 bp (lane 2), 935 bp (lane 4) and 91 bp (lanes 5 and 6) were amplified. Amplification that would generate a 720 bp product (lane 3) did not occur and generated no product. The primer pairs for *leg4* gave products sized 98 bp (lane 7) and 103 bp (lane 8). No amplification was seen in lane 9, with the *leg4* primer pair that would have generated a 120 bp product. All *lgals8* primer pairs gave amplification with amplicons of 118 bp (lane 10), 110 bp (lane 11) and 111 bp (lane 12).

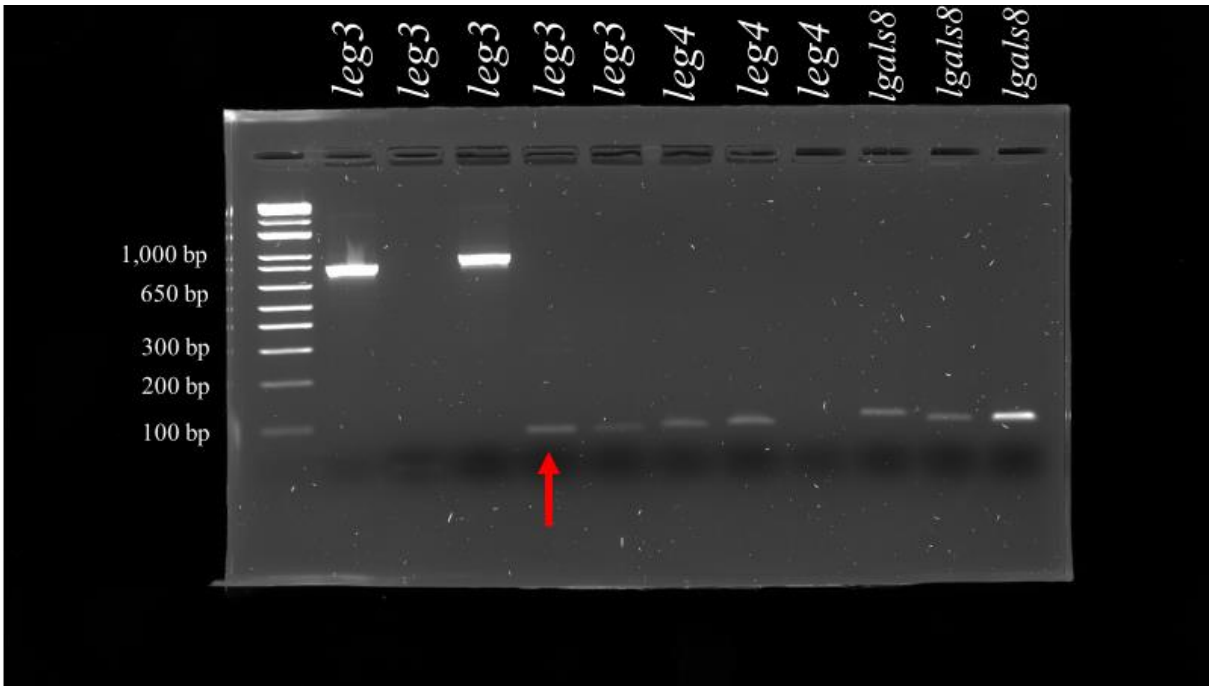

Figure 17. *Galectin primers*

2% agarose gel with TBE as the butter showing amplification of novel galectin primers using RT-PCR. The red arrow indicates the primer pair used for RT-qPCR. RNA was isolated from skin and gill of experimental Atlantic salmon (pre-tress and stress), cDNA generated, and PCR was run with primers against selected galectin genes. In the first lane is a 1Kb Plus DNA Ladder for reference sizes (Invitrogen, USA). cDNA was stained with Invitrogen SYBR Safe DNA Gel Stain (Invitrogen, USA).

Primers against *leg3* were previously used by our lab to study the gene's tissue distribution in Atlantic salmon (Patel et al., 2020) where their study identified a C-terminal truncated Galectin-3 (Galectin-3C) in skin mucus. Primers in their study were designed to target the corresponding, downstream section of the gene and did not span an exon-exon junction (Figure 18). In the current work new primers were designed for RT-qPCR use (Table 3 and Figure 17). Galectin-3 has six exons (Figure 18); Exon 1 (nucleotide 1-55), exon 2 (nucleotide 56-66), exon 3 (nucleotide 67-462), exon 4 (nucleotide 463- 551), exon 5 (nucleotide 552-720), and exon 6 (nucleotide 721-1230). Of the several primer sets designed for *leg3* Figure 17 shows the one used for further work in this study. Primers were designed using PrimerBLAST where the forward primer spanned the junction between exon one and two (nucleotide 39-61) and the reverse primer was in exon three (nucleotide 129- 109, Figure 18).

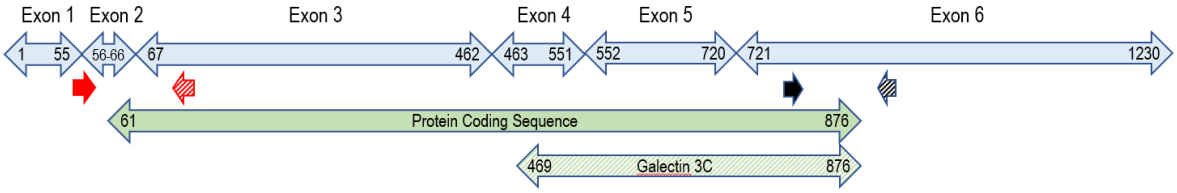

Figure 18. *Localization of published and new primer sets on leg3 mRNA*

leg3 (gi|213514684|ref|NP_001134305|) has six exons indicated by light blue arrows. The protein coding sequence is indicated by the dark green double-sided arrow, and the truncated Galectin-3 is represented by the light green double-sided arrow. Primers from Patel et al. ,2020 are indicated by dark blue arrows and, the primers designed in this study indicated by red arrows. The forward primers are indicated by a solid arrow and the reverse primer with striped arrow.

RQ values were calculated in the manner as they were for *saa5*. The fold change of *leg3* in gill showed a trend of increasing in 1, 6, and 24 h post stressor with only 6 h post stressor being significant compared to the pre-stress group (Figure 19). While not significant, 168 h post stressor showed a negative fold change compared to the pre-stress group (Figure 19).

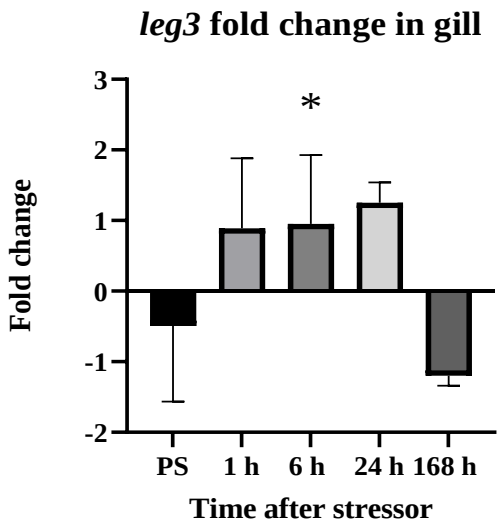

Figure 19. *Gene expression of leg3 in gill*

*Pre-stress (PS), 1, 6, 24 and 168 h groups after stress. Quantification by use of RT-qPCR. Transcript levels are normalized to the reference genes: β-actin and eef1α with RQ fold change values indicated as means ± SD (n=6) by use of the calculation - (1/RQ) if RQ < 1 and * indicating a significant difference (P<0.05) in delta-delta C_t values compared to the PS group.*

The fold change in *leg3* in skin showed a trend towards positive fold change in all groups compared to the pre-stress group with 24 h and 168 h being statistically significant (Figure 20).

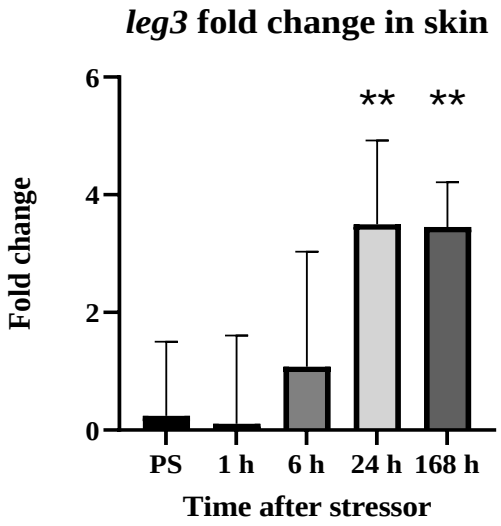

Figure 20. *Gene expression of leg3 in skin*

*Pre-stress (PS), 1, 6, 24 and 168 h groups after stress. Quantification by use of RT-qPCR. Transcript levels are normalized to the reference genes: β-actin and eef1α with RQ fold change values indicated as means ± SD (n=6) by use of the calculation - (1/RQ) if RQ < 1 and * indicating a significant difference (P<0.05) in delta-delta C_t values compared to the PS group.*

4.0 Discussion

In recent years fish welfare has become a major focus of the aquaculture industry as good welfare is generally associated with good health and good health is associated with a better-quality product. One major factor in welfare is stress, where different physiological WIs are used as a tool to assess welfare. In current aquaculture practices, fish experience stress through different stressors such as handling, disease, crowding, perceived threats etc. which has detrimental effects on growth, disease resistance, or even death. The effects of stress are far reaching with primary, secondary, and tertiary responses. During the secondary response, immune function is modulated.

In the innate immune system, MALTs are in direct contact with the external environment and act as a physical and chemical barrier. These tissues are coated in mucus which is rich in immune factors (reviewed in Brinchmann (2016)) that can immobilize and neutralize pathogens. When the innate immune system is triggered, the inflammation response starts, and immune relevant cells are recruited via cytokines and chemokines. Cytokines activate multiple systems such as the APR which causes the release of APPs.

In this study, a physiological indicator of stress, RNA extraction methods, and gene expression of both establish and novel primers were studied. Many clinical signs of stress will return to normal within a day after acute stress, but other physiological systems such as the immune system can take weeks to return to pre stress levels (Carl B Schreck et al., 2016) To view genetic and physiological changes due to stress, timepoints from pre-stress, 1, 6, 24, and 168 hours post stress were used.

4.1 Physiological measures of stress

This stress experiment was conducted along with for Master students, Benedikte Hokland Ottestad and Vilde Charlotte Alsos, where cortisol, glucose, lactate, and hemoglobin were used from their theses to compare with Mg^{++} in this study. At 0 h, cortisol levels in plasma had already increased from pre-stress levels, (Figure 10), peaked at 1 h and remained significantly higher than the pre-stress level until 24 h post stressor, with an exception for 12 h post stressor (Figure 10). There was a high correlation of 0.0875 (Pearson *r*) between Mg^{++} and cortisol levels. Mg^{++} levels were increased after stress and were significantly higher than pre-stress levels from 1 h post stress, reached a peak at 2 h and remained significantly higher than pre-stress levels until the end of the experiment (Figure 9).

Total blood plasma Mg^{++} concentration stays relatively low (>1mM) and generally does not exceed 2 mM, as it plays a determinant role in cell function due to its control over catalytic reactions (Bijvelds et al., 1998). Fishes actively regulate the ion levels and osmolality in their bodily fluids and stress hormones such as cortisol can influence their water, ion and hydromineral balance when in seawater (Iversen & Eliassen, 2009; Carl B Schreck et al., 2016). In sea bream, *Sparus aurata*, after air exposure, Catecholamines that stimulate the HPI axis have been show to increase gill permeability by stimulating oxygen uptake, consequentially increasing Mg^{++} plasma levels (Arends et al., 1999). This increase is caused during severe stress by the seawater being hyperionic compared to the blood plasma, and as permeability of the gills increases there is an influx of ions and an efflux of water in the blood plasma (Arends et al., 1999). This rapid spike in Mg^{++} plasma after air exposure is consistent with our study's findings particularly in relation to cortisol. After cortisol significantly increased post stressor (0.5 h) Mg^{++} plasma levels were significantly higher at the next timepoint (1 h) where both Mg^{++} plasma levels and cortisol were correlated at significantly raised levels until 6 h post stressor (Figure 10). In a transport experiment by Iversen & Eliassen (2009), Atlantic salmon sedated with AQUI-S® showed a recovery of Mg^{++} plasma levels to pre-stress levels 12 h after transport while the unsedated group showed no recovery and had significantly higher plasma Mg^{++} at 12, 48, 96, and 168 h post transport. Our data was inconsistent with either group in their experiment and showed a significant decrease in Mg^{++} plasma levels 12, 24, 48 and 168 h post stressor suggesting recovery may be dependent on stressor type.

4.2 RNA extraction methods and downstream applications

A comparison of organic versus column-based RNA extraction was done in this study. While not tested in the study, organic extractions generally offer the highest concentration and best purity compared to filter based methods (Xiang et al., 2001). Both methods generated quantifiable amounts of good quality RNA for RT-qPCR, and amplification of target genes. Problems arose with the organic based extractions when calculating the polymerase efficiency for relative gene quantitation. For this study, the calculated efficiencies were 161.19 % for the QIAzol/chloroform extraction method with the E.Z.N.A.® Total RNA Kit I had an efficiency of 99.46 %. Inhibition of the polymerase is often the cause of unrealistic PCR efficiency calculations (>100 %) which can be caused by primers/sequence and or structure, contamination from upstream processing, reagent concentration (limiting factors), and competing reactions (Svec et al., 2015). The $\Delta\Delta C_t$ method relies on the reference genes and target genes to have approximately equal calculated efficiencies at or 100 ± 10 % for reliable

results (Arya et al., 2005; Thermo Fisher Scientific, 2014), therefore the samples extracted using the E.Z.N.A.® Total RNA Kit I were used for relative gene quantitation. The assay was re-worked from every aspect and the efficiency problem persisted in QIAzol/chloroform extracted samples. It was concluded that there was most likely phenol contamination in the samples as all upstream applications are unaffected by phenol, but in RT-qPCR if phenol from RNA extraction is not properly removed they can denature enzymes critical in the PCR reaction (Wilson, 1997). This is further backed up by the fact that in this study the manufactures protocol was followed with very minor modifications while the consensus of multiple in house protocols had drastic changes that added additional washing steps to remove contaminants. In retrospect a spectrophotometer such as NanoDrop^{TM} (Thermo Fisher Scientific, USA) should have been used to quantify and assess the purity of RNA, as it would have detected phenol contamination in the sample.

4.3 Published primers

Of the 13 published primer pairs tested in this study, two reference genes (*β*-actin, *eef1a*) and one target gene (*saa5)* were used for gene expression in gill and skin. In RT-PCR, the target genes *Il1β* and *tgfb1* showed products while no products were observed for *tnfa*. In RT-qPCR both *Il1β* and *tgfb1* showed amplification, but the C_q value were < 32. C_q cutoff values are necessary but run the risk of false positives/negatives (Type I and Type II errors) (Burns & Valdivia, 2008). When researching qPCR limits of detection, Burns & Valdivia (2008) suggested C_q cut-off value of 36 cycles was too stringent while a high C_q value of 40 or more cycles was less stringent but more susceptible to Type I error. With this said, in this study the C_q cycle of < 32 is too stringent but was chosen when considering making tenfold serial dilutions for the standard curve. It was considered that if samples were showing high C_q values at the current dilution, their standard curve tenfold serial dilutions would not only be unreliable (high C_q values) but might be out of the detection limit. This problem could have been fixed by modifying initial dilutions after cDNA synthesis and it is possible that the primers for *Il1β* and *tgfb1* could have generated confident RT-qPCR results.

4.4 Fold changes in gene expression

4.4.1 saa5 gene expression

Serum amyloid A-5 protein was the only target gene that generated results in gill tissue and was chosen as target gene because SAA proteins are APPs that are released during the APR as consequence of inflammation, infection, trauma and other events (Sack, 2018). SAA-5 have been identified in the mouse and human genome with Atlantic salmon having a single copy of

the SAA gene where its expression has been consistently shown to be increased in response to bacterial pathogens or bacterial related PAMPs (Lee et al., 2017). In this experiment *saa5* in gill showed a decreasing trend in fold change of expression 1 and 6 h post stress while increasing significantly 24 and 168 h post stressor in comparison the pre-stress group. In skin, *saa5* showed minimal fold change of expression 1 h post stressor, until 6 h post stressor where a significant decrease in fold change of expression was observed, followed by a trend of increasing fold change of expression in 24 and 168 h post stressor compared to the pre-stress group.

In mammals that SAA proteins remain at quite low levels until APR where their levels can rise up to 1000- fold 48 hours after onset and fall rapidly after the APR pattern ends (Sack, 2018). In Holstein calves, SAA proteins, cortisol and proinflammatory cytokines increased significantly 2-3 days post stress (Kim et al., 2011). This is consistent with our observations of *saa5* expression in gill, as levels remain relatively low 1 h and 6h post stressor with 24 h and 168 h showing a significant increase post stressor. To further validate our results, it would have been better to take more samples beyond 24 h and 168 h to see the full APR start and end. As for saa5 expression in skin, the trend towards positive fold gene expression was not significant due to the 168 h post stress group having a lot of variation within the group with two of six replicates being outliers in fold change (80.34 and 116.84) compared to the average of the other four replicates being a 4.91 fold increase. The statistical test used for this group (Kruskal-Wallis ANOVA) is very stringent and in addition removing two of six replicates would not represent the group properly. These outliers may have been caused by possible errors when conducting the analysis as there are many steps involved in RT-qPCR. Another explanation is that these values do represent the individual variance in the population. Another factor is the demolition occurring close to the research facility, which could cause the "pre-stress" base expression to not be correct and in reality, showing the expression of chronically stressed fish. If this is the scenario, fold changes may represent chronically stressed fish reacting to the absence of stress as demolition was halted from 0 h to 168 h during the experiment.

4.4.2 leg3 gene expression

Galectins contain a CRD with a high binding to β-galactoside sugars and are involved in multiple processes of the cell such as mRNA splicing, and cell apoptosis, regulation, activation, adhesion, migration, differentiation and even evidence of cytokine modulation (Cummings et al., 2017; Gordon-Alonso et al., 2018). Previous studies in our lab found that Galectin-1 is present in many tissues also mucosal tissues as skin and gill in Atlantic cod

(Rajan et al., 2013) whilst our work in Atlantic salmon identified a truncated Galectin-3 consisting of the CRD C-terminal domain as the galectin in skin mucus (Patel et al., 2020). In this study, novel primer pairs for Galectin-3, 4, and 8 were created using PrimerBLAST to quantitate any gene expression changes after stress. Nine of 11 primer pairs generated PCR products in RT-PCR with only one *leg3* primer pair was tested in gill and skin in RT-qPCR due to time restrains. Verification for this primer pair and all galectin RT-PCR products is pending, as they have yet to be verified by use of sequencing in combination with RT-qPCR. The *leg3* primer used in this study generated a RT-PCR product that was the predicted size (Table 3) and generated one specific product in RT-qPCR. All other galectin primer pairs that generated a product in RT-PCR had only one band at the predicted (Table 3) size. This indicates that the primers pairs were successful in amplifying the targeted galectins but need further validation by use of sequencing and RT-qPCR.

Fold change expression of *leg3* in gill showed a trend of increasing in 1, 6, and 24 h post stressor with only 6 h post stressor being significantly different compared to the pre-stress group followed by a trend of a negative fold change of expression at 168 h post stressor (Figure 19). In skin, a trend towards positive fold change of expression in all groups compared to the pre-stress group with the 24 h and 168 h upregulation being statistically significant (Figure 20). There are limited studies on Galectin-3 in relation to stress. In one study of on human glioblastoma cells in a cell culture, cells were subjected to heat shock and showed an initial reduction of Galectin-3 proteins levels but after 24 h protein levels returned to pre-stress levels (Dumic et al., 2000). UV-C irradiation increased Galectin- 3 protein levels for the duration of the experiment (24 h) while alkylating damage showed increased Galectin-3 protein levels at 4 h post exposure with protein levels returning to pre-stress levels at 24 h (Dumic et al., 2000). In another study by Al-Salam and Hashmi (2018) they were looking at the role of Galectin-3 during myocardial ischemia reperfusion injury. The hypoxia in myocardial ischemia can lead to cell death through apoptosis which can be prevented if blood flow is reestablished. In Galectin-3 knock-out mice (Al-Salam & Hashmi, 2018) more apoptosis is observed than in control mice and knock-out mice also have lower level of antioxidant enzymes which suggest Galectin-3 affects redox pathways and plays a protective role on the myocardium (Al-Salam & Hashmi, 2018). In our study for this thesis, *leg3* was modulated which in turn could influence proteins levels, however gene expression does not necessarily mean protein synthesis will occur. With this said, this study cannot be directly

compared with other studies as it is possibly the first study to show modulated galectin-3 gene expression after stress.

4.5 General comments

When designing a gene expression study, using RT-qPCR experimental design and assay optimization is key in generating valid results. For this study, there are quite a few changes that would be made in hindsight. To begin, shortcuts were unintentionally taken when conducting RT-qPCR which led to many mistakes which cost time and money. In hindsight, optimizing the RT-qPCR from the beginning would have avoided this problem and more results could have been obtained in the given time. Additionally, more tissues should have been used as skin and gill data could have been backed up by the addition of head kidney and liver as they are also key tissues in the stress response and the APR. Finally, the demolition that occurred right before the sampling was not optimal for a stress experiment and it must be noted that for all results this must be considered.

On March 12th, 2020, "Regjeringen" announced a national lockdown of schools, kindergartens, fitness centers etc. in Norway due to the COVID-19 pandemic. This lockdown meant that the University laboratories were closed for an extended period throughout the summer and valuable time was lost. In addition, consumables for RT-qPCR were scarce (especially from the USA) as they were in high demand for COVID-19 testing. With this said, much of the work presented in this thesis is not complete and can be expanded on greatly.

5. 0 Conclusion

The results of this study showed that cumulative stress had effects on the primary and secondary responses to stress. It was showed that Mg^{++} correlated significantly with cortisol in the stress response and that it may be used as an indicator of stress. The QIAzol/chloroform RNA extraction method generates good quality RNA but is prone to contamination during the extraction process that inhibits polymerase activity in RT-qPCR. Column based RNA extraction proved to be the best option for RT-qPCR as there is a lower chance of contamination at the cost of sample purity. Primers published in the literature worked in our assay while some fine details need to be worked out before they can be used for gene expression in our lab. For galectin primers, new primer pairs can be used in further work once verified by sequencing of gene products. This sets the foundation for further studies of galectins in our lab and as far as we know this is the first in vivo study of effects of stress on the modulation of Galectin-3. As for gene expression, there is an intricate relationship between cortisol, cytokines, galectins, and the APR but there is evidence of some modulation of *saa5* and *leg3* gene expression in both skin and gill after cumulative stress.

6.0 Further work

From this study, all timepoints for muscle and hindgut have yet to be analyzed in addition to the remaining timepoints for gill and skin. The additional timepoints and tissue may give a more detailed view of the effects of stress on genes in the innate immune system.

All galectins that produced a RT-PCR product need verification by use of sequencing and RT-qPCR. For primer pairs that generated a RT-PCR product and or had a high C_q value, the assay needs to be modified by changing the concentration of cDNA in an attempt to reduce C_q or by changing the factor of dilution in the serial dilutions for the standard curve. If these steps are successful, so gene expression can be assessed, it should give an overview of how the innate immune system modulates when encountering cumulative stress. With infinite resources and time, it would be interesting to use additional techniques such as proteomics or RNA- sequencing to see the effects of cumulative stress on the global proteome/transcriptome level.

It is hard to determine how much influence the demolition prior to the experiment had on the final results of the experiment, the best option would be to repeat the experiment again and take additional tissues such as liver and head kidney as they are important sites to the HPI axis and APR.

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