UiT The Arctic University of Norway

Faculty of bioscience, fisheries and economics

Effects of acute and chronic handling stress on antiviral responses in Atlantic salmon (*Salmo salar*) red blood cells and testing of stress target genes.

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Master's thesis in Aquamedicin (60 stp)

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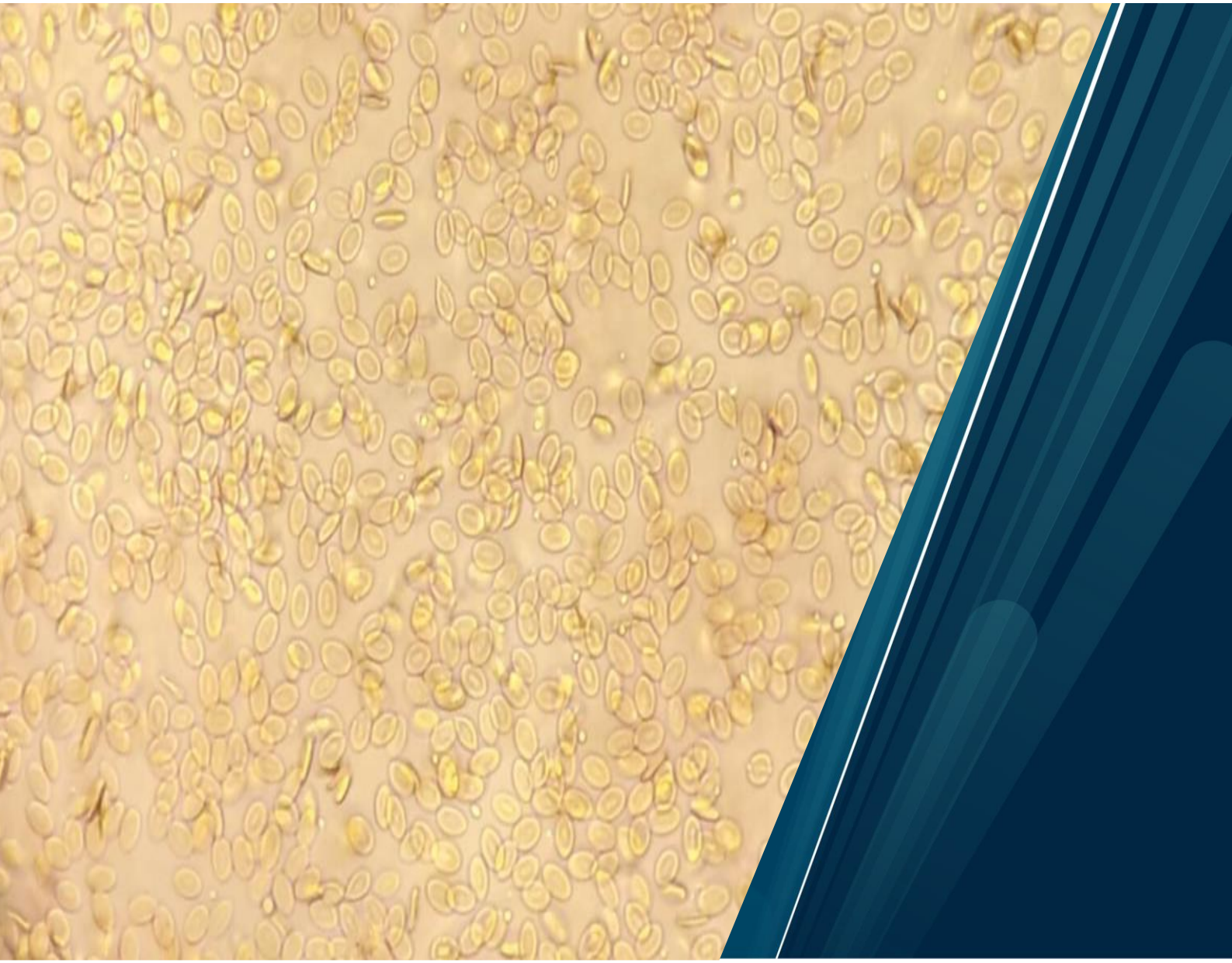

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Even though we live far away from each other, the support from my family has been a blessing. In lonely times, you have only been a phone call away. I love you all!

Tromsø, May 2023

Martine Johansen Aardal

Abstract

The salmonids in today's Norwegian aquaculture are exposed to many stressors, like suboptimal water quality, crowding and handling. This project focuses on red blood cell (RBCs) function in Atlantic salmon and how cellular responses are affected by stress. Since fish RBCs are nucleated and can change their gene expression, one of the main goals of this thesis is to better understand how the RBCs function in the fight against viruses after the fish have been exposed to handling stress. Lastly, one also wants to find a better way to monitor stress levels in Atlantic salmon RBCs by testing and finding stress biomarkers.

A small in vivo stress trial was run, where Atlantic salmon were exposed to acute and chronic handling stress. There were no long-lasting significant changes in stress hormones, haemoglobin levels, or antiviral responses to poly (I:C) in the RBCs when measured after 1 and 4 days. However, the acute stress group had a trend towards higher blood plasma cortisol level and the RBCs had a lower antiviral response to poly (I:C) than samples with lower blood plasma cortisol.

Because of the unconclusive in vivo results, ex vivo trials with cultures of Atlantic salmon RBCs were run to try and find more stable stress biomarkers. Stimulation with the chronic stress hormone hydrocortisone showed an upregulation in gene expression of FkBP prolyl isomerase 1 (FkBP1). For the in vivo stress trial samples, the gene expression of FkBP1 was not upregulated in the RBCs for the stress groups, probably due to the low hormone levels upon sampling.

Cultures of Atlantic salmon RBCs were also stimulated with isoproterenol and epinephrine in hope of finding acute stress biomarkers. There was a significant increase in gene expression of carbonic anhydrase 1 (Cahz) in response to isoproterenol, and heat-shock protein (HSP)90aa in response to epinephrine.

In this ex vivo trial, Atlantic salmon RBCs were also stimulated with poly (I:C) to test the effects of stress hormones on antiviral responses. The response to poly (I:C) on the gene expression of the antiviral proteins ISG15, Mx1 and IRF3 was affected oppositely by acute and chronic stress hormones. Acute stress led to an enhancement, and chronic stress to an

inhibition of the expression of these antiviral proteins. RBCs stimulated with both an acute and a chronic stress hormone, indicated that chronic stress hormone effects are stronger than acute stress hormone effects, as the antiviral responses still were inhibited.

In summary, these results strengthen the foundation for being able to monitor the chronic stress levels of the fish in the field using low invasive blood sampling. The data from ex vivo trials also indicate that chronic stress results in inhibition of antiviral responses in red blood cells of Atlantic salmon, possibly making the fish more susceptible to systemic viral disease.

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1 Introduction

1.1 Atlantic salmon aquaculture

Since the start of Atlantic salmon aquaculture in the 1960s in Norway, there have been many breakthroughs with respect to smolt rearing and development of dry feed, advancing the salmon aquaculture and leading to a large-scale commercial operation taking off in the 1980s. After this period, the number of new cultured locations has expanded, the productivity has been greatly improved, and the husbandry practices enhanced. All this have contributed to a remarkable growth of salmon aquaculture (Y. Liu et al., 2011). From early 1970s to 2022 the Norwegian yearly farmed salmon production has increased from less than 500 tons to 1 500 000 tons with a first-hand export value of over NOK 105 billion (Fisk.no, 2023; Sommerset et al., 2023)

With such a large-scale and intensive production of fish, problems are hard to avoid. According to the Norwegian Veterinary Institute (VI), the mortality of farmed salmon in seawater was 16.1 percent in 2022. The main contributors to this high mortality rate are viral diseases and stress correlated with handling and dealing with the salmon louse parasite (*Lepeophtheirus salmonis*), but also complex gill diseases and bacterial diseases like winter ulcer (Sommerset et al., 2023)

Atlantic salmon is an anadromous fish species. This means that the fish, in the wild, hatch in freshwater rivers and develop to parr before they undergo a process known as smoltification 1-6 years after hatching. Smoltification is a morphological and physiological transformation allowing the fish to migrate downstream of the river and transition to life from freshwater to seawater during the late spring and summer. After 1-4 years of growing in the ocean, the fish reach sexual maturity and return to their natal river (May-October). During the late autumn and winter, spawning takes place and the eggs hatch in late winter. The fry hide between the rocks in the river until the yolk-sac with nutrients has been absorbed/consumed, and later in the spring, the fry leaves the bottom of the river. The fish are now classified as parr (Hansen & Quinn, 1998; Otero et al., 2012).

Atlantic salmon aquaculture tries to mimic this life cycle as much as possible. The roe from broodstock is fertilized and incubated in trays until the yolk sac is absorbed. Then the fish are fed with pellets and transferred to larger tanks to grow. When the fish reach about 50-200

grams smoltification is induced by either the use of photoperiodic changes, which mimics the natural summer-winter-summer cycle, or using a specialized feed. Fully smoltified fish are transferred to sea cages until they reach market size. During this intensive production cycle, the fish are exposed to several types of stress.

1.2 Stress and fish welfare

Animal, or fish, welfare can be defined as the quality of life as perceived by the animal itself. The fish farmers have been trying to ensure the best welfare, as good welfare increases fish growth and health - which in turn provides the best financial returns. Since one cannot measure what the fish experience, one must use indirect methods to measure their welfare. The "FishWell" handbook by Nofima (Noble et al., 2018) uses different Welfare Indicators (WI), and in that way the farmers can get an idea of the welfare situation in their own fish farm. The WI's include Direct WI's, which are observations made on or from the animal, and Indirect WI's, which are observations made on the environment, infrastructure, and processes.

The welfare needs of salmonids can be categorized into available resources, a suitable water environment, good health, and freedom to express different behaviors, as shown in figure 1.

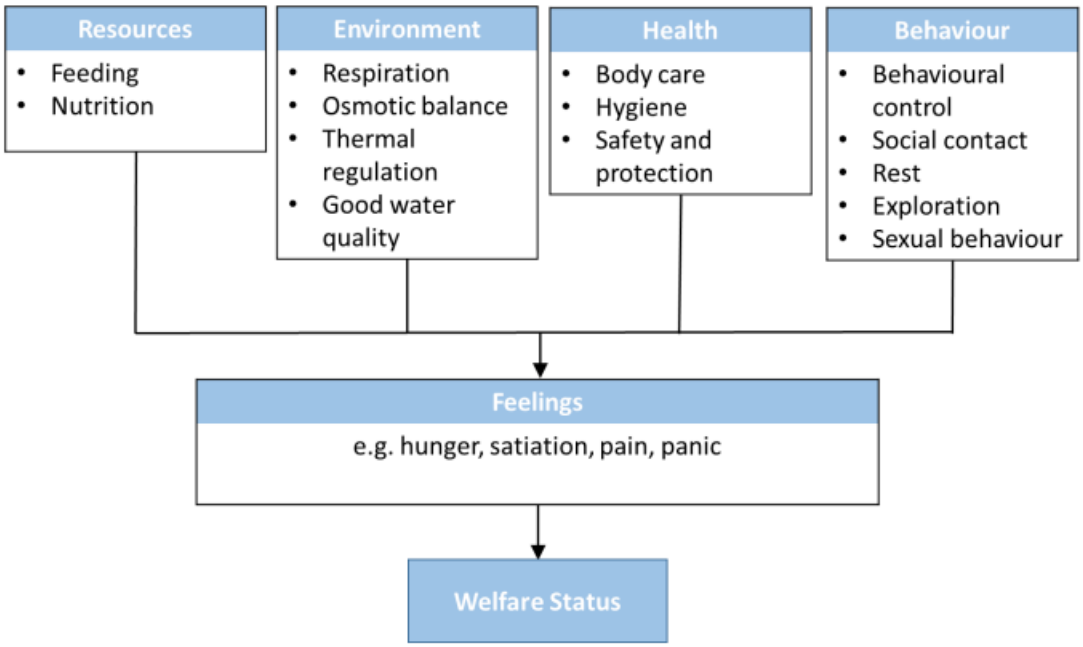


Figure 1: The welfare needs of salmonids as described by Noble et al (2018). The needs are categorized into available resources, a suitable water environment, good health, and freedom to express different behaviours. If some of these needs are not fulfilled, the fish can experience stress.

Some of these needs are essential for welfare and survival for all fish species at all life stages, like for instance respiration. And some of the behavioural needs may be important during, or restricted to, one or more life stages, like for instance sexual behaviour. Other needs may not be crucial for survival, but can still reduce the fish's welfare (Noble et al., 2018). For instance, the fish can have a need of exploration, and the lack of the possibility to explore may result in reduced welfare. If the fish lack any of these needs, it can induce a change in the inner balance of the fish, the homeostasis, and trigger a stress response.

1.2.1 Definition, mechanisms, and effects of stress

One can consider stress as a demanding strain, called stressors, that results in a change in the inner balance of the organism, the homeostasis (Balasch & Tort, 2019). To maintain the homeostatic state, and for the organism to obtain inner balance, adaptive mechanisms are initiated for the organisms to deal with the stressors (Barton, 2002).

Physiological responses to environmental stressors are divided into primary, - secondary, - and tertiary responses (Barton, 2002), as shown in figure 2. When the fish recognises the stressor, neural signals from different sensory cells, such as visual, olfactory and auditory to mention a few, activates cells in the hypothalamus (Madaro et al., 2020). This initiates, via the brain stem and spinal cord, an activation of primary responses, which includes a number of endocrine changes (Mazeaud et al., 1977). For instance, sympathetic fibres from the hypothalamus gets activated and releases catecholamines, like adrenalin/epinephrine and noradrenaline/norepinephrine, from chromaffin cells of the head kidney of the fish, into the bloodstream (Barton, 2002).

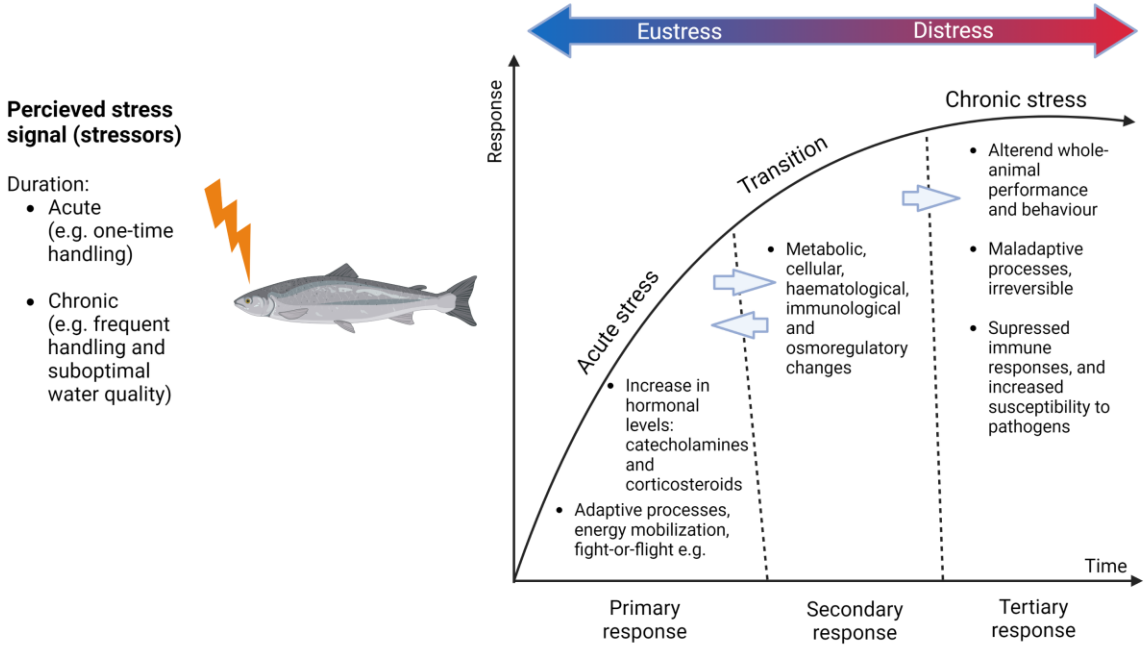
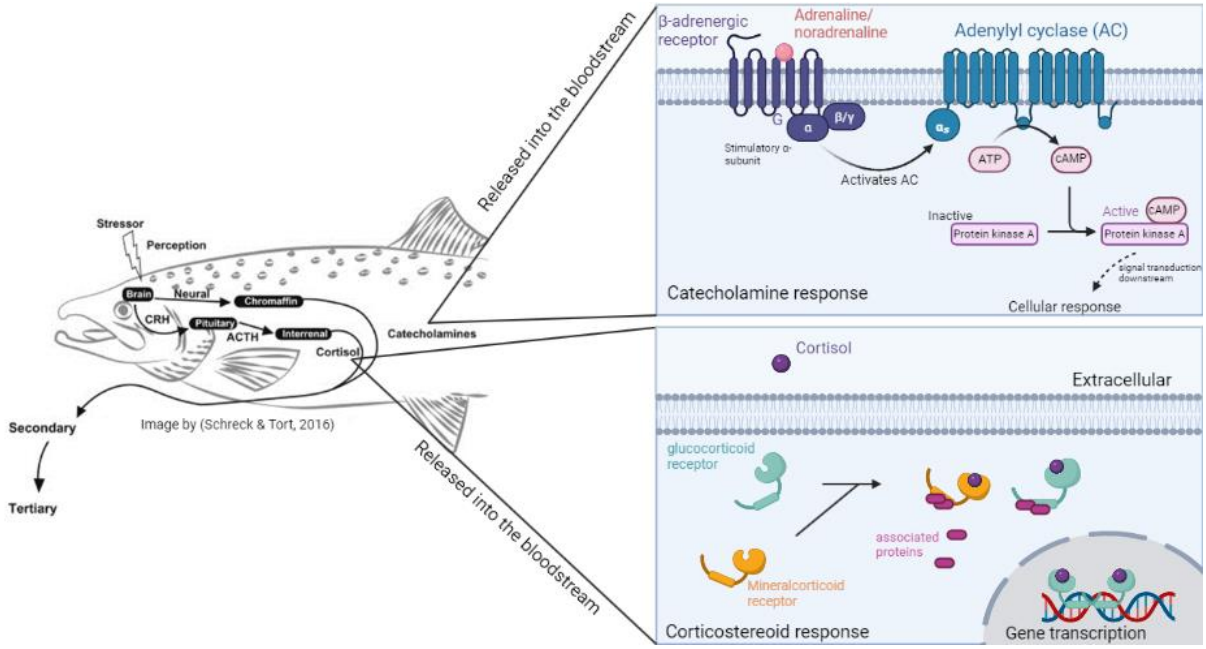


Figure 2: Fish exposed to stressors evoke primary (acute), secondary (transition) and tertiary (chronic) physiological responses. Acute stress increases hormonal levels of catecholamines and corticosteroids, leading to a fight-or-flight response. In between the acute stress and chronic stress, one has the transition phase where there are metabolic, cellular, haematological, immunological and osmoregulatory changes. Chronic stress is when there are a continuous release of corticosteroids, resulting in whole-animal alterations in behaviour and performance. These processes are often irreversible and results in suppressed immune responses and increased susceptibility to pathogens. Figure inspired by Nardocci et al (2014) and created in BioRender.com.

Adrenaline/epinephrine, noradrenaline and other synthetic catecholamines, like isoproterenol, bind to adrenergic receptors on the surface of the cells. These receptors are divided into two groups: α- and β-adrenoceptors (ARs). This receptor is a G-protein-coupled receptor which activates a cAMP dependent signalling pathway though adenylyl cyclase and changes gene transcription and expression to activate an acute, rapid stress response by initiating typical "flight or fight" responses. Such responses include increased ventilation rate and oxygen uptake, perfusion to gills and muscles, and glycogenolysis and lipid degradation for increased glucose and energy supply (Balasch & Tort, 2019).

As a result of the primary responses, the secondary responses occur (Mazeaud et al., 1977). This includes metabolic- and cellular changes, osmoregulatory disturbances and changes in haematological- and immunological functions (Barton, 2002), and occur as a result of catecholamines activating the hypothalamus–pituitary–interrenal gland axis (HPI axis) (Madaro et al., 2020). The hormone cortisol, a corticosteroid, is released from the interrenal cells of the head kidney. Hydrocortisone is the active form of the corticosteroid cortisol, and

binds to nuclear glucocorticoid (GR),- and mineralocorticoid (MR) receptors that initiates changes in gene transcription and protein synthesis, resulting in increased catabolism, immunosuppression and allostasis (Balasch & Tort, 2019) (Figure 3). Dexamethasone is a synthetic corticosteroid working the same way as hydrocortisone.



Figur 3: Illustration of the release of catecholamines and corticosteroids into the bloodstream of teleost fish, binding to its receptors and signalling pathways that gets activated. Catecholamines are released from chromaffin cells of the head kidney and bind to G-protein-coupled β-adrenergic receptors on the cell which activates cAMP dependent signalling pathway though adenylyl cyclase and changes gene transcription. Cortisol is released from interrenal cells of the head kidney and binds to glucocorticoid- and mineralocorticoid receptors inside the cell. With associated proteins, the complex initiate changes in gene transcription. Parts of figure borrowed from Schreck and Tort (2016) Figure created in BioRender.com.

Allostasis is when the organism maintain stability through change and adapts to changing environmental conditions and changes in bodily needs, rather than constancy to optimise survival, growth, and reproduction. Changes include both physiological and behavioural alterations that optimises performance to meet the most likely environmental demands at minimal costs. When the environmental changes are prolonged in time, the organism gets in an allostatic state. This means that the organism's regulatory capacity becomes reduced, and the body cannot return to physiological levels seen prior to the stress exposure (Madaro et al., 2020)

Tertiary response to stressors, is when there are changes in growth, condition, overall resistance against diseases and pathogens, behaviour and survival (Barton, 2002; Tort et al.,

2004). The stressor has reached above the organism's homeostatic capacity, and this has resulted in an allostatic state. The organism is struggling to adapt to the stressors (Schreck, 2000). This state is an "emergency" response and can only be maintained for a short period of time whilst the organism has sufficient energy to support the mechanisms of allostasis (Madaro et al., 2020)

If the organism is either exposed to additional stressors, fails to habituate to repeated challenges or fails to turn off the stress response with a constant release of glucocorticoids, the allostatic load (the cumulative result of an allostatic state) changes to an allostatic overload. This is when the fish experience chronic stress, where there are little resources and energy to maintain all bodily functions (Madaro et al., 2020). This results in a maladaptation where the organism can't compensate the effects of the stressors. Chronic stress is a result of mild, repetitive stressors, as strong stressors often result in death, and mild stressors result in recovery (Balasch & Tort, 2019).

To conclude, adrenalin could be considered as the «acute» stress hormone and initiates an acute stress response (fight and flight) (Balasch & Tort, 2019), while prolonged cortisol secretion is considered a chronic stress response (Madaro et al., 2020). Cortisol is the most stable stress marker (Schaaf et al., 2009), and can be detected in the blood, water, faeces and mucus a period of time after the fish are being exposed to stress (Cau et al., 2017).

1.2.2 Stress in salmonid aquaculture

As farmed fish don't have the ability to escape from different stressors, the fish are often exposed to many stressors at the same time. This includes suboptimal and bad water quality, handling, transport, pumping, vaccination, and crowding (Madaro et al., 2015). Especially in Norwegian aquaculture of Atlantic salmon, the main contributor to stress is the handling of the fish when the fish gets treated against the salmon louse parasite.

To keep the number of parasites per fish as low as possible, both in aquaculture and in wild stocks, several delousing methods have been developed, including both medicinal and non-medicinal methods. Especially the non-medicinal methods can be harsh for the fish, involve a lot of handling, and can cause ulceration, stress, and mortality. Due to the parasites' ability to resist the different treatment methods, the mechanical delousing methods are used more frequently and more intensely (Cerbule & Godfroid, 2019; Torrissen et al., 2013).

In 2022, there were 3145 treatments against salmon lice, where two of the most frequently used non-medicinal delousing methods were thermal delousing (1357 treatments) and mechanical delousing (1074 treatments) (Sommerset et al., 2023). Thermal delousing (Figure 4) involves submerging the fish in water with a temperature of 28-34°C for 20-30 seconds, and mechanical delousing involves either the use of brushes or flushing with water jets. Both of these methods entail crowding, pumping and straining, which may lead to stress, risk of hypoxia and mechanical injuries (Østevik et al., 2022).

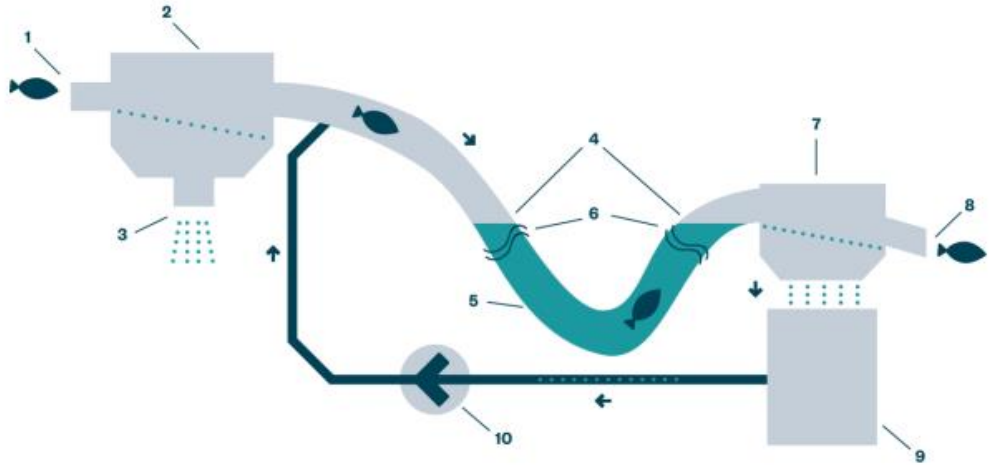


Figure 4: The principle of thermal delousing (Thermolicer). 1. The fish are pumped in from the sea-cage. 2. The fish pass a grate that diverts the sea water away. 3. The sea water is filtered and is transported away from the system. 4. The fish are submerged in warm water (28-34 ℃) for 20-30 seconds. 5. The fish swim in a closed system with warm water. 6. Water surface in the treatment unit. 7. Water separator transports the treatment water. 8. The fish exit the system. 9. The treatment water is transported to a heater for cleaning, aeration, and re-heating. 10. The water is transported back to the treatment unit. Figure borrowed from Grøntvedt et al. (2015).

In a study done by Moltumyr et al (2022), it was shown that Atlantic salmon exposed to warm water (34℃) for 30s had severe panic and pain responses. This also resulted in a higher prevalence of loss of shells, injuries on fins, snout, and eyes, gill damages, as well as reduces specific growth rate. The same injuries can be found on fish treated with mechanical delousing methods like brushing and the use of water jets (Gismervik et al., 2017).

In addition to this, fish health professionals also report an increase in outbreaks of viral diseases such as cardiomyopathy syndrome (CMS) (B. B. Jensen et al., 2019) caused by the piscine myocarditis virus (PMCV), hearth and skeletal muscle inflammation (HSMI) caused by the piscine orthoreovirus (PRV) and pancreas disease (PD) caused by the salmonid alfa-virus (SAV) within two weeks after a such stressful event as delousing (Sommerset et al., 2022). This indicates that highly stressful events can result in a decrease of antiviral responses, making the fish more suspectable to diseases.

1.2.3 Different ways to run stress trials: in vivo, in vitro and ex vivo.

There are different ways one can run stress trials: in vivo, in vitro and ex vivo. In vivo means "within the living" in latin, and refers to experiments performed in whole, living organism. For this thesis, whole, living Atlantic salmon were used to test the stress response in fish red blood cells after the fish were exposed to normal handling stress by crowding, chasing, and transferring fish between tanks with a hand net. That way one can describe the response as closely as possibly as it would have been in the field of salmonid aquaculture.

There are other ways to induce stress in fish in in vivo trials as well. For instance, one can expose the fish to acute hypoxic stress and periodic hypoxic stress, inducing an acute and chronic stress response respectively, as done by Lund et al. (2017). One can also expose the fish to higher temperatures, mimicking a thermal delousing treatment. As recirculating aquaculture systems (RAS) are getting more common, one can also test stress responses regarding suboptimal water quality (other than hypoxia), like exposure to different toxic compounds such as nitrite. Both acute exposure and long term (chronic) exposure to an unstable and fluctuating environment can result in an allostatic overload compromising the fish growth and robustness (Mortensen et al., 2022).

In vitro means "within the glass" and refers to experiments that use components of an organism that have been isolated from their biological surroundings to permit a more detailed or more convenient analysis than what can be done with whole organisms.

Ex vivo is latin for "out of the living" and refers to experiments that takes place outside the organism. In this thesis, one isolates living red blood cells from Atlantic salmon and experiment on them in an artificial environment outside the organism. This allows an experiment under more controlled conditions than what is possible in in vivo experiments (Kamøy et al., 2022).

One can also stimulate cells ex vivo under conditions resembling in vivo trials. In this thesis isolated red blood cells from Atlantic salmon were stimulated with stress hormones ex vivo, to resemble stress exposure similar to in vivo experiments.

1.3 Fish immunology

The immune system is a complex network of lymphoid organs, cells, humoral factors, and cytokines. The system has an essential function in host defense against pathogens, tumors (tumor immunity), and harmless environmental molecules (allergy) (Parkin & Cohen, 2001).

In general, the immune system is divided into two parts: the innate immune system and the adaptive immune system. Innate immune responses can provide immediate protection against microbial invasion, whereas the adaptive immune responses develop more slowly and form a more specialized long-term defense (Abbas et al., 2020).

1.3.1 Innate antiviral immunity

Innate immune responses are the first line of defense against infectious agents. Fish are constantly exposed to a microbial-rich environment which interacts with every epithelial barrier of their body. These barriers include the skin, the gastrointestinal tract, the gills, and the nasopharynx. The barriers are covered in mucus and associated with immune cells and molecules, and are therefore called mucosa-associated lymphoid tissues, MALT for short (Salinas, 2015). In the mucus, one has found different antibacterial molecules, commensal bacteria, IgM and IgT antibodies, and in the underlaying epithelial layers one has found immune cells such as T-cells, B-cells, macrophages, plasma cells and granulocytes (Salinas et al., 2011).

If the infectious agent manages to break through the physical barriers and enter the body, a cascade of events is bound to happen. The innate immune system recognizes structures that are shared by various classes of microbes, but not present on a normal host cell. These microbial molecules are called pathogen-associated molecular patterns (PAMPs) and are recognized by receptors expressed by innate immune cells, called pattern recognition receptors (PRRs) (Abbas et al., 2020).

A type of PRR in teleost fish are toll-like receptors (TLRs). Teleost fish possess a larger and different repertoire of TLRs compared to mammals. In mammals only 13 TLRs have been identified, while in teleost fish, at least 28 functional TLR genes have been identified (Liao & Su, 2021). TLRs on the host cell surface, recognize, in general, molecules associated with the bacterial cell wall, whereas endosomal TLRs recognize nucleic acids of ingested microbes (Abbas et al., 2020). For instance, the endosomal TLR3 and TLR22 (Matsuo et al., 2008)

recognize double stranded RNA (dsRNA) and the endosomal TLR7 and TLR8 recognize single stranded RNA (ssRNA), all being important in antiviral immune responses as RNA viruses are detected and antiviral responses are initiated (Figure 5).

In virus infected mammalian host cells, activation of TLR3 induces transcription and secretion of the interferon (IFN) type 1 IFN-β, whereas TLR7/8 induces transcription and secretion of IFN-α, by first activating interferon regulatory factors, IRFs, that regulates the gene expression (Abbas et al., 2020).

Teleost fish possesses IFN genes encoding six different IFN type 1 subtypes: IFNa, IFNb, IFNc, Find, IFNe and IFNf, where IFNa, IFNb and IFNc are most studied (Robertsen, 2018). All IFNs show antiviral activity in vitro. It is shown that IFNa genes are induced by similar pathways as mammalian IFN-β, and IFNb genes are induced through similar pathways as mammalian IFN-α, even though the genes are not orthologs of the mammalian IFNs (B. Sun et al., 2009). IFNa is induced in most cells by virus RNA and exerts antiviral activity mainly at the site of production, while IFNb and IFNc are induced in selected cell types by virus RNA and show systemic antiviral activity (Robertsen, 2018).

Through TLR3/22, teleost fish can signal the adapter protein TIR domain-containing adaptor-inducing interferon (TRIF) and induce production of IFNa. While through TLR7/8, teleost fish can signal myeloid differentiation primary response protein 88 (MyD88) and induce production of IFNb and IFNc. Both these pathways work via the phosphorylation of IRFs (Liao & Su, 2021; Robertsen, 2018; Skjarveland et al., 2009).

In mammals, IFN type 1 binds to IFN type 1 receptors on other virus-infected or uninfected cells, and JAK-STAT signalling pathways are activated (Langevin et al., 2013). Upon binding to the receptor, Jak1 and Tyk2 are activated and phosphorylated, providing docking sites for the phosphorylation of STAT1 and STAT2. Phosphorylated STAT1 and STAT2 form a dimer, and with IRF9 form the transcription factor ISGF3. ISGF3 moves into the nucleus and activates IFN-stimulated genes (ISGs). STAT1, STAT2, Jak1, Tyk2 and IRF9 have been identified in teleost fish, and that is the reason it is thought that fish have a similar IFN type 1 mediated signalling (Robertsen, 2018).

There are many types of ISGs, including ISG15 (Røknes et al., 2007) and Mx1 (Robertsen et al., 1997) that are both expressed in Atlantic salmon (Robertsen, 2008). ISG15, is a small ubiquitin-like protein that covalently binds to a target protein in a process called ISGylation. (Poynter et al., 2016; Røknes et al., 2007). By covalently binding with host and viral target proteins, ISG15 inhibits the release of viral particles and hinder viral replication (Zhang et al., 2021). Mx proteins also have antiviral mechanisms as it inhibits viral replication by preventing intracellular trafficking of viral nucleocapsids, for instance against orthomyxoviruses (Robertsen, 2006) like the infectious salmon anaemia virus (ISA) causing the listed disease infectious salmon anaemia in Norwegian Atlantic salmon aquaculture (Jansen & Silva de Oliveria, 2022).

TLR7/8 activation by ssRNA also activates NF-κB, which increase the transcription of inflammatory cytokines (Liao & Su, 2021).

An illustration of the signalling pathways just described are shown in figure 5.

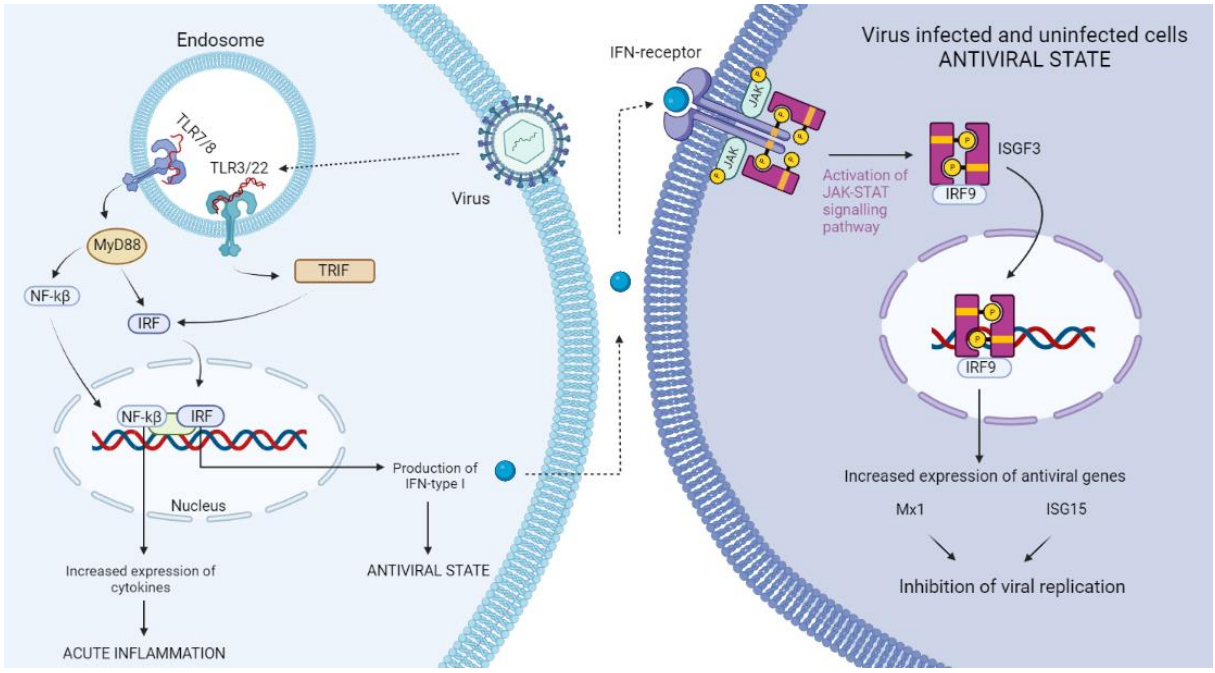


Figure 5: Illustration of the signalling pathways as virus dsRNA and ssRNA are detected by TLR3/22 and TLR7/8 respectively in teleost fish. TLRs activate pathways that result in increased production of IFN-type 1 and cytokines. IFN-type 1 exits the cell and binds to IFN-receptors on virus infected and uninfected cells. JAK-STAT signalling pathway is activated, leading to increased expression of antiviral genes encoding proteins like Mx1 and ISG15. This again results in inhibition of viral replication. Figure created BioRender.com, and inspired by Liao and Su (2021)

1.3.1.1 TLR-agonists imiquimod and poly (I:C)

An agonist is a substance that binds to a receptor and initiate/enhances a physiological response (Page & Maddison, 2008).

Imiquimod is a synthetic substance structurally similar to single-stranded RNA (ssRNA) and acts as a molecular pattern associated with viral infections as it activates the endosomal TLR-7 and initiates an immune response (Angelopoulou et al., 2020). The agonist's antiviral activity was first shown in guinea pigs infected with herpes simplex virus (Miller et al., 1999), and is today used as a topically treatment against genital warts caused by a papillomavirus in humans (Bottrel et al., 1999)

Polyinosinic:polycytidylic acid (poly I:C) is a synthetic substance structurally similar to double-stranded RNA (dsRNA) and acts as a molecular pattern associated with viral infections as it activates TLR3 and TLR22 and initiates an antiviral response (Alexopoulou et al., 2001; Invivogen, n.d.).

1.3.2 Enhancement and suppression of immune responses linked to different types of stress

By a cell-cell contact communication system, including mediators such as cytokines, fish can direct the immune response and communicate the recognition of stressors and regulate the magnitude of the stress response. The immune system acts as a sensor that informs the central nervous system (CNS) to trigger a neuroendocrine response, resulting in communication between the CNS, the autonomic nervous system and the hypothalamus-pituitary-interrenal axis. In that way, the neuroendocrine system signals affect normal functions of the immune system (Nardocci et al., 2014).

In teleost fish, studies have shown that acute stress can have beneficial effects on the immune system by upregulating proinflammatory cytokines IL-1β (Hoseini et al., 2019) and IL-8 (Metz et al., 2006), and increase lysozyme activity (Demers & Bayne, 1997; Lui et al., 2011) and production of complementary C3 proteins as bacteriolytic mechanisms (Jiang et al., 2008; Madaro et al., 2020).

But there are also studies showing opposite results, presenting immunosuppressive effects of acute stress on innate defense. Different fish species and stressors can be the possible explanation of these findings, but it demonstrates that more focused research is needed to

further elucidate the mechanisms of acute stress and the effects on fish immunity in greater detail. Either way, the acute stress response affects the fish immune system in a less suppressive way than chronic stress responses (Guo & Dixon, 2021).

Whereas chronic stress inhibits immune responses (Fast et al., 2008; Nardocci et al., 2014) due to an allostatic load and deficit of the resources needed to support the operative mechanisms of the immune system. Studies have reported that chronic stress supresses phagocytic and lysozyme activity, antibody production and lymphocyte activation and mobilisation, and it appears that cortisol may be the main link between stress and the inhibition of the teleost immune system (Madaro et al., 2020).

1.4 Fish red blood cells

Red blood cells (RBCs) represent the most abundant cell type in the body of vertebrates, circulating in the bloodstream. Respiratory gas exchange is their main function, however other functions such as interactions with the immune system have been reported (Chico et al., 2019; Wessel et al., 2019). The differences between mammalian and other lower vertebrateRBCs are shown in figure 6 (Anderson et al., 2018).

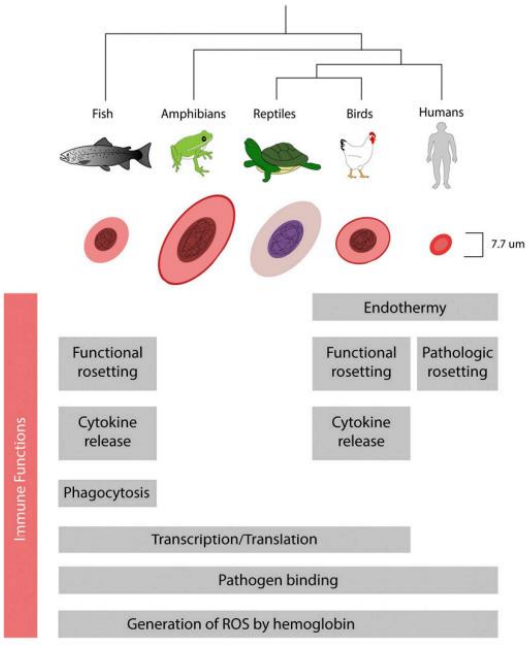


Figure 6: Differences between RBCs in humans, and lower vertebrates like birds, reptiles, amphibians, and fish considering their immune functions'. RBCs of lower vertebrates are nucleated and can undergo transcription and translation, produce and release cytokines, form rosettes and phagocytose pathogens. The haemoglobin in all RBCs makes it possible for host defense by generating antimicrobial reactive oxygen species (ROS). Figure borrowed from Anderson et al. (2018).

RBCs under development in both mammals and lower vertebrates, go through a complex maturation process called erythropoiesis, where the cells change shape, size, structure, and biochemistry. Mammalian RBC has a unique feature in which the nucleus, and other organelles, are extruded by budding off from the immature RBC. This enucleation allows an elevation of haemoglobin levels in the blood and gives the RBC its flexible, biconcave shape (Ji et al., 2011; Moras et al., 2017).

On the other hand, non-mammalian RBCs, like those in fish, are nucleated and have organelles, such as endoplasmic reticulum, ribosomes, Golgi apparatus and mitochondria, in their cytoplasm (Zhu & Su, 2020). Nucleated RBCs can modify their transcriptome and proteome, and they can actively response to environmental cues (Anderson et al., 2018).

Studies done on Nile tilapia (*Oreochromis niloticus*) (Shen et al., 2018) and rainbow trout (*Oncorhynchus mykiss*) (Morera et al., 2011) showed an increase in expression of TLR3 in RBCs in response to poly (I:C). The TLR3 gene also is expressed in Atlantic salmon RBCs (mean 1100 reads). TLR8, TLR7 and TLR 22 mRNA has also been found in RNA sequence data of Atlantic salmon RBCs, but in rather low amounts (mean 20, 6 and 4 transcripts detected) (unpublished research by RED-FLAG). The presence of these PRRs suggest that teleost RBC can detect and specifically respond to PAMPs.

Atlantic salmon (*Salmo salar*) RBCs can upregulate antiviral immune genes like INF type 1 in response to infectious salmon anaemia virus (Workenhe et al., 2008), and IFN type 1, RIG-I (a type of PRR that recognizes viral RNA), Mx and ISG15 transcripts in response to piscine orthoreovirus infection (Haatveit et al., 2017; Wessel et al., 2015). All this likely to contributing to host resistance.

Fish RBCs can also produce antimicrobial peptides (AMPs) such as β-defensin and NK-lysin that are involved in fighting microbes (Zhu & Su, 2020). There are also indications of NK-lysin being involved in the resistance against viral diseases, such as viral haemorrhagic septicaemia virus (VHSV) in turbot during autophagy (Pereiro et al., 2017).

Fish RBCs can also form cellular aggregations, called rosettes. Passantino et al. (2002) showed that particles from the opportunist fungus *Candida albicans* (CA) bind to rainbow

trout RBCs, which in turn engulf/phagocytose the fungus particles. The RBCs form rosettes with macrophages, meaning three or more RBCs surround one or more macrophages, in which the macrophages help kill the fungus. Grass carp (*Ctenopharyngodon Idella*) RBCs has also been observed to engage in phagocytosis of bacteria (Qin et al., 2019).

Passantino et al. (2004) also found out that RBCs from rainbow trout activated with the fungus CA produce a Macrophage migration Inhibition Factor (MIF)-like substance. In human and experimental systems, MIF works as a cytokine important in triggering an acute inflammatory immune response.

Nucleated RBCs may also be capable of inducing an adaptive immune response (Nombela & Ortega-Villaizan, 2018). RBCs from rainbow trout and Atlantic salmon express MHC class I, and it has been reported that a piscine orthoreovirus infection in salmon induces genes involved in antigen presentation via MHC class I (Dahle et al., 2015). It has also been shown that poly I:C upregulates gene ontology categories related to antigen processing, antigen presentation, and MHC class I receptor activity in rainbow trout RBCs (Morera & MacKenzie, 2011). Fish RBCs may also be involved in antigen presentation to T cells and NK-like cells via MHC class II molecules (Puente-Marin et al., 2018).

When it comes to receptors for different stress hormones, both β-adrenergic receptors (Ferguson & Boutilier, 1988) and glucocorticoid receptors (high level of transcripts in RED-FLAGs own RBC RNA sequencing data) have been found in Atlantic salmon RBCs.

1.5 Main goals

This master's thesis is a part of the Norwegian Veterinary Institutes research project RED-FLAG, which aim to better understand how the salmonid red blood cells respond to stress and infections. If a viral infection and stress lead to measurable, and lasting, changes in the red blood cells, the cells can be effective sensors for the fish's health and welfare. By downscaling how much blood is needed for different analysis, the diagnostic work in the field of salmonid aquaculture can get easier and better.

There is not enough information about how the red blood cells respond to stress, and how the different stress hormones affect the cells. This master's thesis is going to focus on analysing red blood cells isolated from Atlantic salmon that have been exposed to acute and chronic stress in vivo or stress hormones ex vivo, and to see how these cells react to dsRNA and ssRNA when the cells are stimulated in the laboratory (ex vivo).

Currently, the best way to monitor if the fish is stressed is by measuring the concentration of stress hormones in blood plasma. One of the main goals of this thesis is to test of there are specific genes that gets upregulated or downregulated when the red blood cells are exposed to stress. In other words, one wants to find stress-target genes that can be used to better monitor stress-exposure of fish.

It is expected that an increase in concentration of stress hormones in blood affect the RBCs, as the RBCs have receptors for both adrenalin and cortisol. As adrenalin and cortisol are known to change the immune response against pathogens, the following hypothesis is that stress can affect the immune functions of the RBCs and how the RBCs react to virus.

2 Materials and methods

2.1 In vivo stress trial

The small stress trial described here was intended as a pilot experiment of 90 fish prior to a larger trial but was further reduced to 20 fish due to a viral outbreak at the facilities, affecting the tank of the planned experimental salmon. The trial took place at the Aquaculture Research Station in Kårvika, from 21st of March until 28th of March 2022. 20 Atlantic salmon, with an average weight of 730 grams when sampling, were transferred to three different quadratic 900L seawater tanks, with water temperature about 12 ℃, for three different experimental treatments (groups): seven fish for the control (group 1), seven for acute stress treatment (group 2) and six for chronic stress treatment (group 3). The fish were already present at the facility – all the fish were transferred to the experimental tanks the initial day of the trial and no sedatives were used during transfer.

The acute and chronic stress group were exposed to what one can call “normal handling stress”. First the water level was lowered to 1/5 of its original level, and the fish were chased with a hand-net for approximately 5 minutes before the fish got transferred to another 900L seawater tank. The fish were then transferred back to their original tank within 15 minutes of total handling time. It was also made sure that the fish were in the air for approximately 5 seconds. The oxygen level in the tank was measured and did not reach below 83% during the whole operation. For the chronic stress treatment group, this procedure was repeated twice on day -3 prior to sampling-day (d-3), three times on day -2 (d-2), twice on day -1 and once on day 0 (d0), at different times of the day to avoid that the fish could predict and expect the treatment. For the acute stress treatment group, the fish only got the treatment once, on day 0.

On day -3, one fish from the control-group and one fish from the acute stress group were sampled, just to practice the procedure. On day 1 after the final stress treatment (d1), three fish from every group were sampled. The rest of the fish, three fish in every group, were sampled on day 4 (d4).

An overview of the in vivo stress trial setup is shown in figure 7 below.

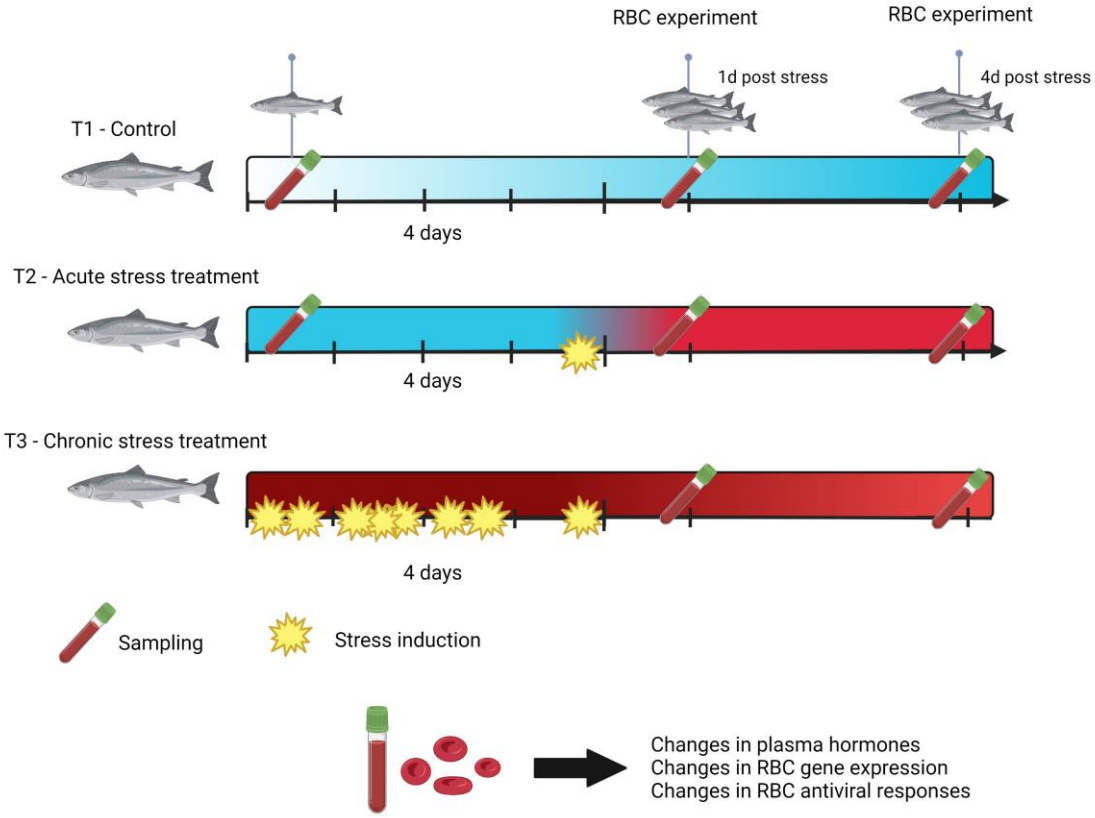


Figure 7: In vivo stress trial experiment setup. Tank 1 (T1) is the control-group (n=7), tank 2 (T2) is the acute stress treatment group (n=7) and tank 3 is the chronic stress treatment group (n=6). The chronic stress treatment group went through 8 stress inductions over a total of four days, and the acute stress treatment group got stress induced once. Blood was sampled one- and four-days post stress, three fish from each group at each sampling point. Figure created in BioRender.com

The fish were euthanized one by one by bath-immersion containing a lethal dose of the anaesthetic Benzoak vet. (200mg/L water). Blood was drawn from the caudal vein using 2mL BD Vacutainer lithium heparin tubes. The haemoglobin was then measured in 10 µL blood using a Hemocue Hb 201+ Analyzer, according to the manufacturers protocol (Hemocue). The blood was then stored on ice until further analysis.

Mucus from the skin was collected using a cell lifter and transferred to 2 mL eppendorf tubes.

Faeces were collected in 2 mL eppendorf tubes by either pressing on the sides of the abdomen, or by opening the abdomen of the fish and cutting the intestines and directly collecting it from here. These samples were not analysed any further.

The weight of the fish was noted before a piece of the following organs were sampled: gills (2nd gill arch), spleen, heart, head kidney and brain. The samples were collected in formalin and RNAlater in case one wanted to analyse these samples further.

The same day as the sampling of blood in Kårvika, it was further treated in the lab at UiT. 20µL blood was directly added in 400µL lysis buffer, and 500µL blood was centrifuged at 500g/5 min/4°C for collection of blood plasma. The plasma samples were stored at -80°C for further hormone measurements at VI in Ås.

An overview of the different sampling and sampling treatments in the stress trial is shown in figure 8 below.

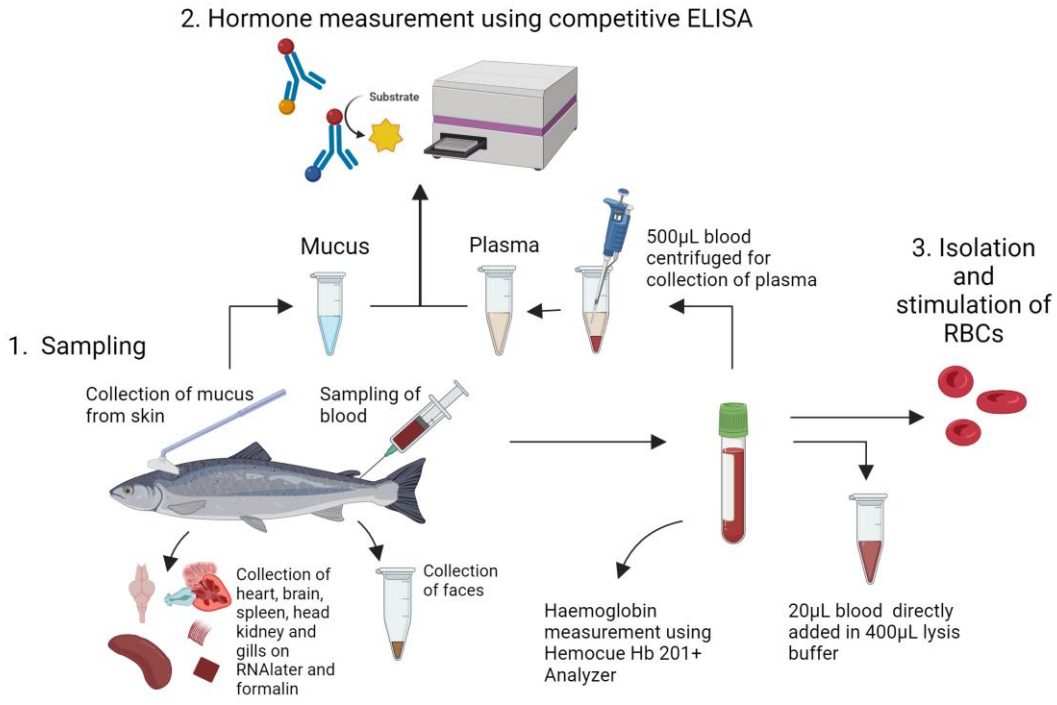


Figure 8: Illustration of the different sampling and sample treatments used in the in vivo stress trial (part 1). Collection of blood, mucus, organs, and faeces from Atlantic salmon (1), where plasma was separated from the blood cells and stress hormones (adrenalin and cortisol) were measured in plasma and mucus using competitive ELISA (2). The haemoglobin was measured in the blood using Hemocue Hb 201+ Analyzer, and RBCs were isolated from the remaining blood, and further stimulated with different agonists (3). Figure created in BioRender.com

At UiT, the red blood cells were isolated, stimulated with poly I:C and imiquimod, and harvested.

2.1.1 Isolation of red blood cells

In order to study only the RBCs, the cells need to be separated from the white blood cells (Figure 9). This was done using a protocol (Appendix A) where the RBCs are separated by using density gradient centrifugation with percoll. To practice this method prior to the trial, a downscaled pilot on blood (250 µL) from a couple of smaller Atlantic salmon was performed. Blood and organs were sampled, and RBCs were isolated using this described method.

Percoll consists of 15-30nm diameter colloidal silica particles coated with polycinulpyrrolidone (PVP). Because of the heterogeneity in particle size, sedimentation occurs at different rates after low-speed centrifugation, and cells with varying density will settle to a density band that matches their own individual density (Sigma-Aldrich, 2005). RBCs will settle at the bottom of the gradient due to high density. Through this procedure, one is able to remove dead cells and other debris which will settle at the top of the gradient. The white blood cells will be higher up in the gradient due to lower density compared to the RBCs and can also be collected.

In short, the blood was diluted 1:20 in distilled PBS (dPBS) and two gradients for each fish were prepared in a 51% Percoll-solution: 7,5 mL percoll and 5 mL diluted blood in 15 mL tubes. The gradients were centrifuged at 500 x g for 20min/4°C, acceleration 7 and break 1 (slow stop to avoid disturbing the gradient).

Percoll 51% was removed, the RBCs were resuspended in 5 mL dPBS, and the suspensions from the same fish were combined and transferred to a new 50 mL tube with 20 mL dPBS (30 mL total volume). The cells were washed by centrifuging the solution at 500g/10min/4°C, acceleration 9 and break 9. The supernatant was removed carefully, and the RBC pellet was resuspended again in 30 mL dPBS before another wash in the centrifuge using the same settings.

The viable cells were counted using the Countess procedure (Invitrogen, Eugene, Oregon, USA) before the second wash. This was done by diluting 10µL of the cell suspension in 90µL dPBS (1:10 dilution). 10µL tryphane blue and 10µL of the 1:10 cell dilution were mixed and placed in the countess slide for counting in the Countess machine.

After the second wash, the cells were diluted in dPBS for a final concentration of 20 million RBCs/mL and added to Leibovitz L-15 medium supplemented with 2% FCS and 50 µg/mL gentamicin. 1 mL of the solution was added to 12-well plates (5 wells pr fish), and the cells were incubated in an orbital shaker with the following settings: 90 rpm and 14-15℃ for 24 hours to ensure that the cells were in a resting (basal) state.

L-15 medium is formulated to promote cell growth, FCS is fetal calf serum containing cell nutrients, and gentamicin is antibiotics that prevents bacterial growth.

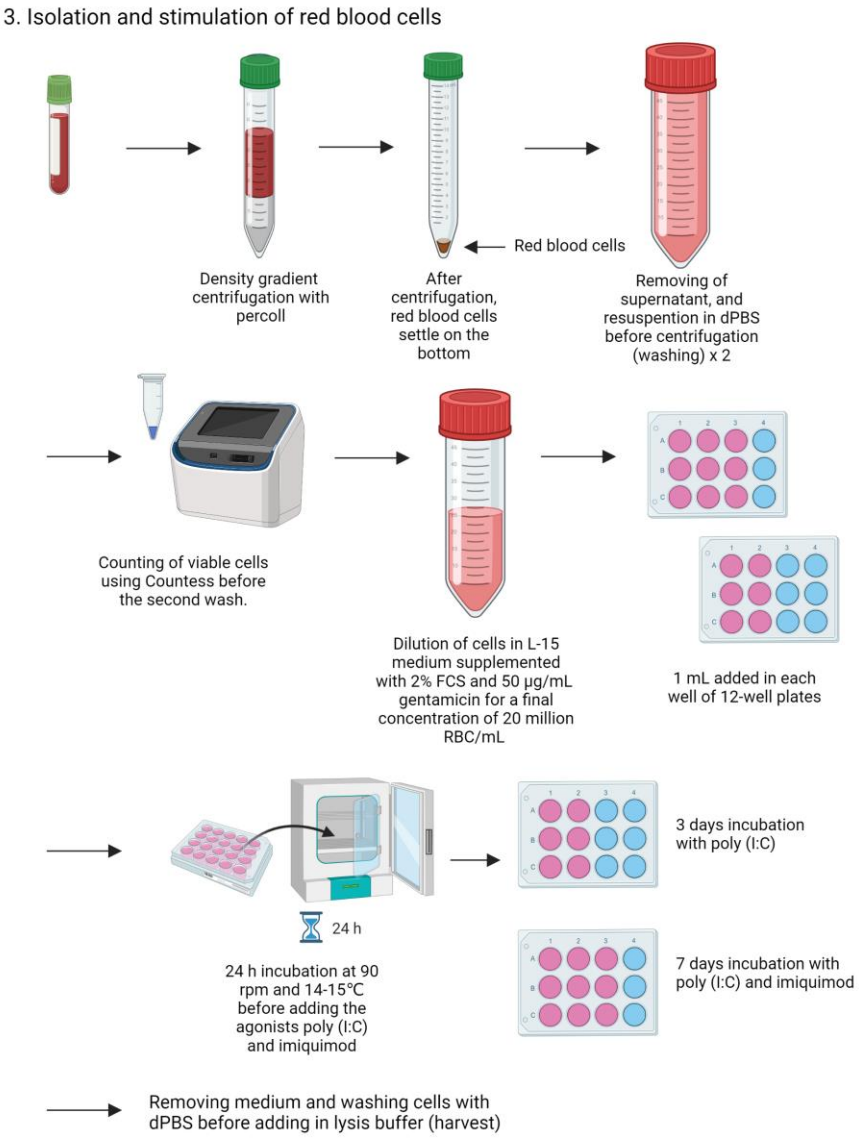


Figure 9: Illustration of the different methods used in the in vivo stress trial (part 2). Red blood cells from Atlantic salmon were isolated and stimulated with poly (I:C) and imiquimod before harvested (3). Figure created in BioRender.com.

2.1.2 Ex vivo stimulation and harvest of red blood cells

To test whether the RBCs would change their antiviral response after exposure to acute and chronic stress, the cells were stimulated ex vivo using the agonists imiquimod and poly(I:C).

After one day of incubation, 5µL 10 mg/mL poly (I:C) were added for a final concentration of 50µg/mL, and 1µL 500 µg/mL imiquimod were added for a final concentration of 0.5µg/mL. Poly (I:C) was added to two of the wells pr fish and imiquimod was added to one of the wells per fish. One of the wells per fish only contained RBCs as a negative control (see appendix B for plate setups).

RBCs stimulated with poly(I:C) were harvested at two time points: 3 days and 7 days post stimulation. The cells stimulated with imiquimod were harvested at 1 time point: 7 days post stimulation, as previous ex vivo trials prior to the in vivo trial showed no response to imiquimod after only 3 days stimulation.

The first step of the harvest was to centrifuge the samples at 500 x g for 10 minutes at 4°C. The L-15 medium was removed, and the blood pellet was resuspended in 1 mL dPBS and centrifuged with the same settings. dPBS was removed again, and the blood pellet was resuspended in 30µL dPBS. 50µL of this solution was transferred to 350µL MagNa Pure lysis buffer (total volume approximatly 400µL). This lysis buffer breaks open the cells and makes it possible to extract mRNA.

The samples were stored at –20°C until further RNA -isolation, cDNA synthesis and RT qPCR at VI.

2.2 Ex vivo stimulation trials to test stress target genes

To identify target genes specific to acute and chronic stress hormones, ex vivo stimulation models of RBCs with stress hormones and stress hormone receptor agonists were performed.

2.2.1 Effects of acute stress hormones on RBC antiviral responses

To test the effects of acute stress on antiviral responses and to investigate acute stress target genes, RBCs from three Atlantic salmon were isolated and stimulated with isoproteronol, epinephrine and poly (I:C).

The RBCs were isolated using the same method as for the RBCs in the in vivo stress trial, only that four gradients were made for each fish. The pellet was also washed in 50 mL dPBS, not 30 mL as for the in vivo stress trial samples. This because the in vivo stress trial was a downscaled process as no more than 100 million cells were needed (5 wells per fish with 1 mL cell suspension with approximatly 20 million cells).

Isolated RBCs from each fish were plated in 2 x 12-well plates (6 12-well plates total) (see appendix C for plate setups). In each well, there should be approx. 20 million cells per mL suspended in L-15 supplemented with 2% FCS and gentamicin. The plates were incubated at 14-15℃ with agitation at ~90 rpm for 24 hours before different concentrations (1, 5, 10, 50 and 100 µM) of isoproterenol and epinephrine were added in different wells, two wells of each concentration. 100 µM diemethyl sulfoxide (DMSO) was also added in two of the wells of each plate as a control. DMSO was used as a solvent for isoproterenol and epinephrine, as ethanol cannot be used (in high doses the cells shink and die, and lower concentrations will disturb intracellular signaling) (Kar et al., 2021)). After one day of incubation with the acute stress agonist and hormone, the cells in one well of each concentration of the agonist/hormone were stimulated with 50 µg/mL poly (I:C).

The cells were harvested 3 days post stimulation with poly (I:C) using the same method as for the samples in the in vivo stress trial.

2.2.2 Effects of chronic stress hormones on RBC antiviral responses

To test the effects of chronic stress on antiviral responses and to test potential chronic stress target genes, RBCs from three Atlantic salmon were isolated and stimulated with hydrocortisone and dexametasone. To test if chronic stress had any effect on antiviral immune responses, the cells were also stimulated with poly (I:C).

The RBCs in this trial were isolated using the same method as for the ex vivo trial for testing acute stress hormones.

Isolated RBCs from each fish were plated in 12-well plates (see appendix D for plate setups). In each well, there should be approx. 20 million cells per 1 mL suspended in L-15 supplemented with 2% FCS and gentamicin. The plates were incubated at 14-15℃ on an otbital shaker at 90 rpm for 24 hours before different concentrations of hydrocortisone (20, 50, 100 and 150 µM) were added in different wells for each fish – two wells for every concentration. A higher dose of dexametasone (100 µM) was mainly used as a positive control, as it has been shown in previous ex vivo trials to have an effect.

After one day of incubation, 50 µg/mL poly (I:C) was added into one of the wells of each concentration of hydrocortisone, one well with the positive control and one well with the negative control.

The cells were harvested 3 days post stimulation with poly (I:C) using the same method as for the samples in the in vivo stress trial.

2.2.3 Effects of acute- and chronic stress hormones on RBC antiviral responses

As both catecholamines and corticosteroids are released due to stressful events, stimulation with a combination of the acute stress-like β-adreneric receptor agonist isoproterenol and the chronic stress like anti-inflammatory glucocorticoids dexametasone and hydrocortisone were tested to find out the effect on antiviral responses of RBCs of Atlantic salmon.

The same RBCs isolated from the blood of the three Atlantic salmon used for testing the effects of chronic stress hormones were used in this trial.

After 1 day incubation at 14-15℃ and ~90 rpm different concentrations of hydrocortisone (50, 100 and 150 µM) were added in each well with 10 µM isoproterenol of a 12-well plate for each fish. Two wells for each concentration. 10 µM isoproterenol was used because the results from prior acute stress ex vivo experiments showed that this concentration had the best effect. 10 µM isoproterenol and 100 µM was also added to two of the wells. Only 10 µM isoproterenol was added to two of the wells alone.

100 µM diemethyl sulfoxide (DMSO) was also added in two of the wells as a control because isoproterenol needs to be diluted in this.

After 24 h incubation at 14-15℃ and ~90rpm, the cells were further stimulated with 50 µg/mL poly (I:C). The cells were harvested 3 days post stimulation with poly (I:C) with the same method as for the samples in the in vivo stress trial, acute stress ex vivo trial and chronic stress ex vivo trial.

Figure 10 below shows an overview of methods used for testing stress response genes and antiviral genes.

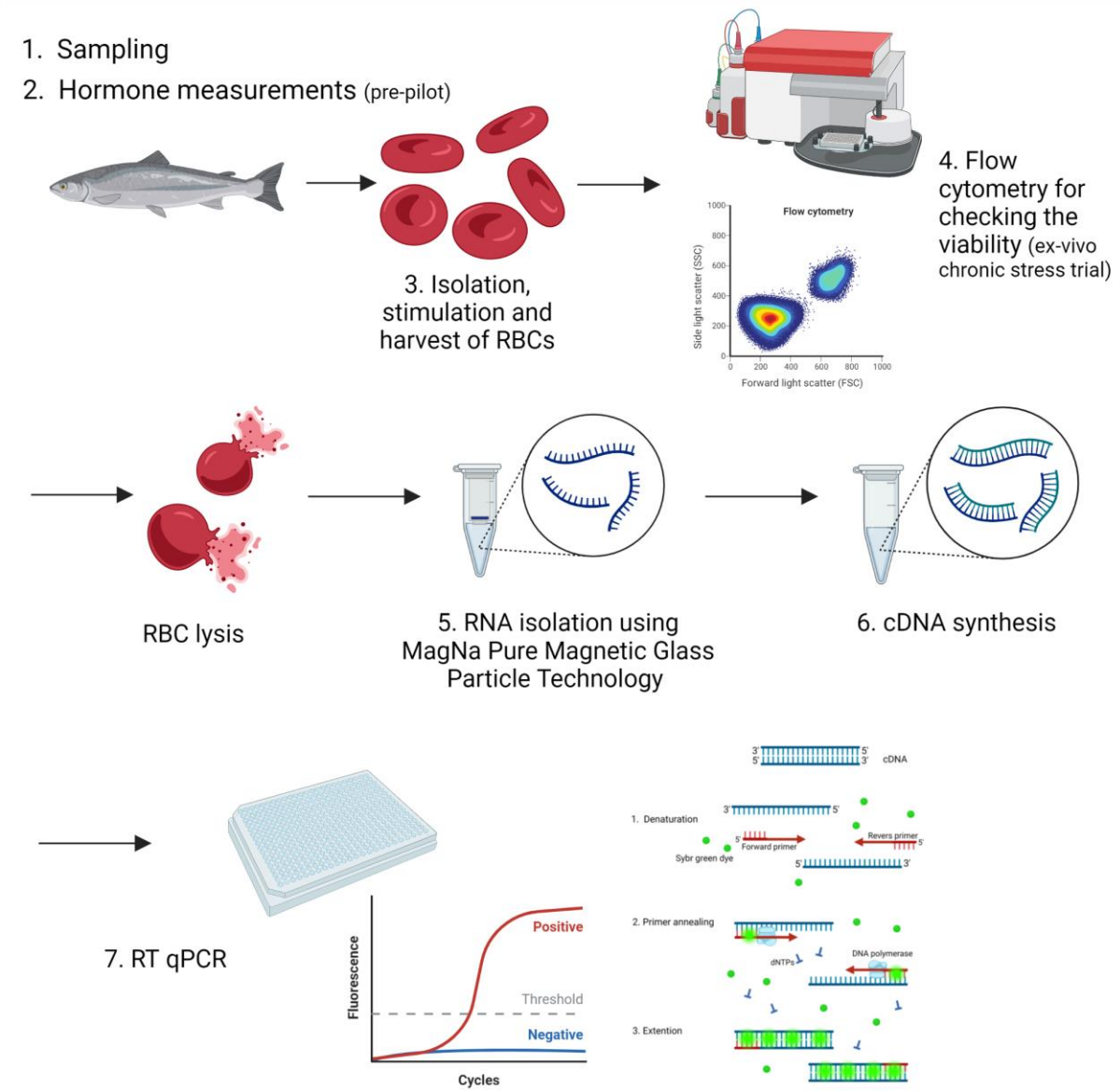


Figure 10: An overview of the methods used in this thesis. First the fish were sampled (1) and secondly, hormones were measured in plasma and mucus (only in vivo stress trial samples) (2). This is shown in detail in figure 7. Then RBC were isolated, stimulated, and harvested (3) (shown in detail in figure 8). The viability of some of the samples in the ex vivo chronic stress stimulation trial were measured using flow cytometry (4). Thereafter one has to lyse the RBCs before RNA isolation (5) and cDNA synthesis (6) to continue with RT qPCR (7) to test the expression of different genes in the cells. Figure created in BioRender.com.

2.3 Hormone measurements

To measure the concentration of different molecules in samples, one can use a technique called enzyme-linked immunosorbent assay, or ELISA for short. This technique relies on antibodies to detect a target molecule by using a highly specific antibody-antigen interactions.

There are different kinds of ELISA's: Direct ELISA, indirect ELISA, sandwich ELISA and competitive ELISA (Figure 11) (Abcam, 2018).

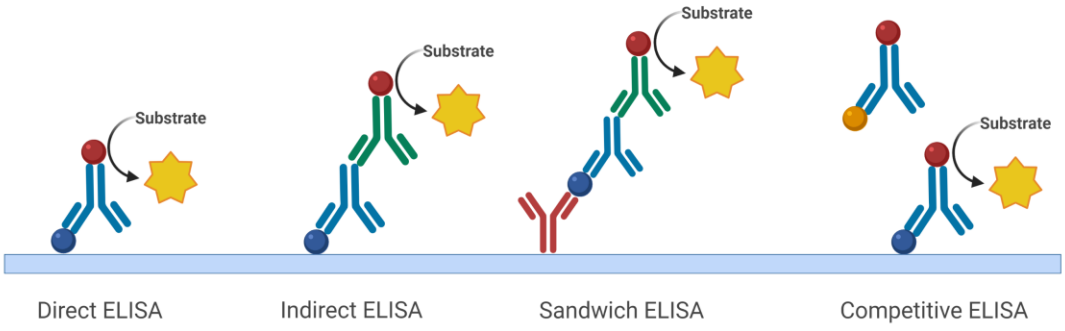


Figure 11: Illustration of direct, indirect, sandwich and competitive ELISA. Figure collected from BioRender.com

Direct ELISA uses an immobilized antigen that is attached to the surface of the wells of the plate, and the antigen is detected by binding an antibody, labelled with a detection molecule, that is specific for the antigen.

As for the indirect ELISA, the antigen is also immobilized to the surface of the well. But here, a primary antibody specific for the antigen binds to the target molecule, and a secondary molecule, labelled with a detection molecule, binds to the primary antibody for detection.

A sandwich ELISA uses two antibodies specific for different epitopes of the antigen, where one of the antibodies is coated on the surface of the wells and capture the antigen. The other antibody binds to the antigen again, and facilitate the detection of the antigen

In this setup, for measuring the concentration of cortisol and adrenalin in different fluids, competitive ELISA was used (Figure 12). The principle of this method is that the sample antigen competes with a reference antigen, that is already pre-coated on the wells' surface, for binding to a specific amount of labelled antibody. The more antigens there are in the sample,

the less reference antigen will be bound to the antibody, and less antigens will be detected. This gives a weaker signal.

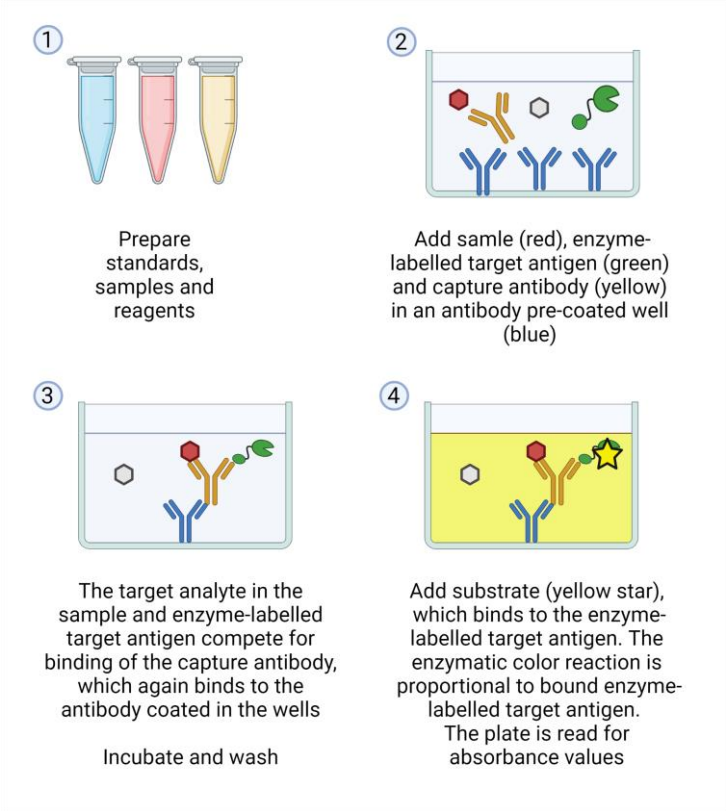


Figure 12: Competitive ELISA described in more detail. First (1), the standards, samples and reagents are prepared, before adding the samples (red), enzyme-labelled target antigen (green) and capture antibody (yellow) in an antibody pre-coated well (blue) (2). The target analyte in the sample and the enzyme-labelled target antigen compete for binding the secondary antibody coated in the well (3). Lastly the substrate are added, which binds to the enzyme – giving a yellow colour. The plate is read for absorbance values, as the enzymatic colour reaction is proportional to bound conjugate. Figure collected and created in BioRender.com.

2.3.1 Cortisol measurements in skin mucus and blood plasma samples from the in vivo stress trial

The concentration of cortisol was measured in mucus sampled from the fish skin, and in blood plasma using Cortisol ELISA kit (Enzo). The protocol was followed with one exception, that it less of the wash solution was used in each well for all the washes. As specified in the protocol, one should use 400µL of wash solution in every well for each wash. For this setup, only 250µL of the wash solution was used in each well, as the amount specified in the protocol would overflow the wells.

In short, a dilution series was performed for the standard diluent, the plasma samples were diluted 1:50 and the mucus samples were diluted 1:2 with the Assay buffer. Every component

in the kit and the diluted samples were added in the appropriate wells, and every sample were run in duplicates. After the capture antibody was added to the appropriate wells, the plate was incubated at room temperature on a plate shaker for two hours at ~500 rpm.

The content of the plate was emptied, and the wells were washed three times with the wash solution. After this, the blue Conjugate was added to the TA wells, and pNpp Substrate was added to each well. The plate was incubated for 1 hour without shaking, before the stop solution was added to every well and the plate was read immediately at optical density 405 nm using BioTek 800 TS Absorbance microplate reader (Agilent).

2.3.2 Adrenaline measurements in blood plasma samples from the in vivo stress trial

The concentration of adrenalin was measured in the blood plasma for each replicate, using Adrenalin Elisa Kit (Abcam, 2021). The protocol was followed with one exception, as it was used less of the wash solution in each well for all the washes. As specified in the protocol, one should use 350µL of wash solution in every well for each wash, but for this setup only 250µL of the wash solution was used in each well as the amount specified in the protocol would overflow the wells.

In short, the reagents were prepared, and the plate was washed twice with 1X Wash Solution before adding standards, samples, and control in the appropriate wells. The antibody solution was added in each well and the plate was incubated for 45 minutes at 37℃.

After incubation, the plate was washed again twice, and the conjugate was added in each well. The plate was then once more incubated at 37℃ for 30 minutes.

The solution in the wells was discarded, and the plate was washed 5 times. Substrate was added in each well, and the plate was set to incubate one last time for 15 minutes at 37℃. Stop solution was added to each well, and the plate was read immediately at optical density at 450 nm using BioTek 800 TS Absorbance microplate reader (Agilent).

2.4 Haemoglobin measurements

As mentioned, the haemoglobin in the blood from the fish in the in vivo stress trial were measured using Hemocue Hb 201+ Analyzer. This was done by placing 10µL blood in the microcuvette before placing the microcuvette into the analyser (Figure 13).

Figure 13: Illustration of measuring the haemoglobin using Hemocue Hb 201+ Analyzer. First one fills the microcuvette with 10 µL blood and places the microcuvette into the analyser. The analyser measure the haemoglobin levels (g/dL) and shows the levels on the display (Hemocue).

Haemoglobin is a protein found in red blood cells, which carries oxygen throughout the body (Evensen, 2023). Blood samples from healthy adult Atlantic salmon range from 8.9-10.4 g/dL haemoglobin (Sandnes et al., 2006).

2.5 Flow cytometry

To check if hydrocortisone had any effect on the viability of the red blood cells, the viability was tested in a BC Accuri C6 Plus Personal Flow Cytometer upon harvest.

Flow cytometry is a method to analyse multiple parameters of single cells in a solution such as size, complexity, phenotype, granularity, and viability or cell marker molecules using fluorescent markers or antibodies. The principle of the flow (Figure 14) is the passing of thousands of cells per second through a laser beam, which creates scattered and fluorescent light signals that can be detected and read by photodiodes or photomultiplier tubes. These signals are then converted into electronic signals that are analysed by a computer (McKinnon, 2018).

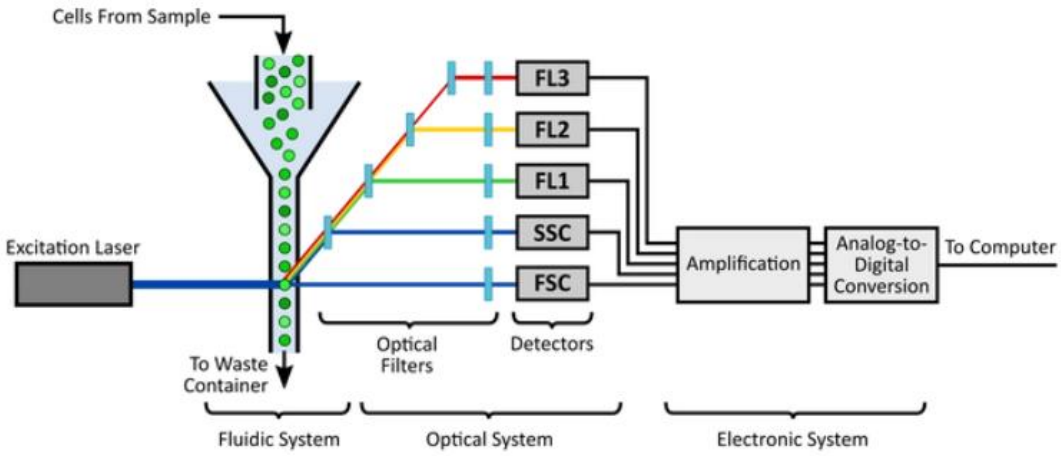


Figure 14: Schematic of a common flow cytometer with three key systems: the fluidic, the optical and electronic system. The transport of cells happens in the fluidic system, where cells pass individually through pressurized lines containing sheath fluid to the interrogation pint where the laser intersects with the sample. Illumination and light collection happen in the optical system, where the lasers ensure that cells in the interrogation point are excited with uniform light of specific wavelengths. Lenses and filters separate and direct the specific wavelengths of fluorescens and scatters laser light to the appropriate detectors. The detectors then convert the signals into photocurrent and passes it to the electronic system to be digitized and processed for further analysis (AAT Bioquest, 2019).

One of the detectors is in line with the laser and is used to measure Forward Scatter (FSC), and another detector is places perpendicular to the stream and is used to measure Side Scatter (SSC). Fluorescent labels are also used to detect different cells/components, and therefore fluorescent detectors are also in place. The cells of the sample pass through the laser, and scatter the light beams, while the fluorescently labelled cells/components are excited by the laser and emit light at longer wavelength than the light source. FSC detects the cell volume, and the SSC reflects the inner complexity of the particle – such as cytoplasmic granule

content or nuclear structure. An example of a plot one can get after performing flow cytometry in a sample with mononuclear cells from Atlantic salmon (neutrophils/granulocytes are not present, and most of the red blood cells are removed) is shown in figure 15. The R1-area is the red blood cells, R2 is lymphocyte-like cells and R3 are macrophage-like cells (Fernandez, 2022) .

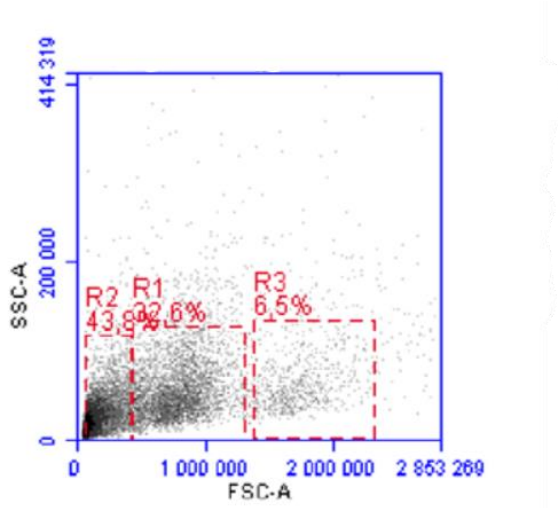


Figure 15: An example of a flow cytometry result from a mononuclear fraction of a blood sample from Atlantic salmon showing different cell types like red blood cells (R1), lymphocyte-like cells (R2) and macrophage-like cells (R3) (Fernandez, 2022). The X-axis shows the side scatter plot and reflects the inner complexity of the particle/cell. The Y-axis shows the forward scatter plot, which reflects the cell volume.

To enable exclusion of dead cells during flow cytometry, the cells need to be stained with a dye that bind specifically to dead cells, like Propidium Iodine (PI). PI binds to the dsDNA if the cellular membrane of a cell has been damaged (McKinnon, 2018; AAT Bioquest, 2019).

50 µL from the RBC samples were taken from the wells to be analysed and transferred into 1,5 mL eppendorf tubes. The samples were centrifuged at 500 x g for 5 minutes at 4°C. The L-15 medium was removed, and the blood pellet was resuspended in 100 µL dPBS. 1 µL PI was added before the viability of the red blood cells was tested in the flow cytometer according to the protocol (Appendix E).

2.6 mRNA-isolation

To test stress target genes and antiviral genes by RT-qPCR, the RNA from the RBCs needed to be isolated. This was done using "MagNa Pure 96 kit" (Roche, 2019), a procedure based on the MagNa Pure Magnetic Glass Particle (MGP) Technology (Figure 16). The first step of the process is lysis of the sample material, where nucleic acids are released, and nucleases are denatured. Further, the nucleic acids bind to the silica surface of the added MGP due to the chaotropic salt conditions (disrupts the hydrophobic effects) and the high ionic strength of the lysis buffer. Nucleic acids bound to MGP are magnetically separated from residual lysed sample, and unbound substances like proteins, cell debris and PCR inhibitors are removed after several washing steps. Lastly, purified nucleic acids are eluted from the MGP at high temperatures (Roche, 2018).

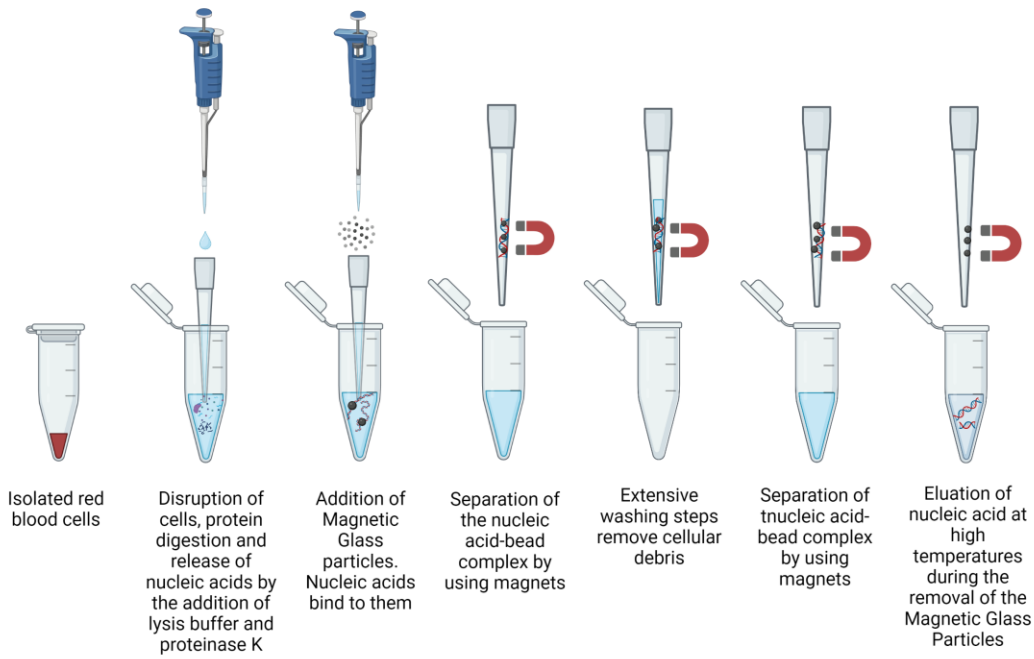


Figure 16: The principles of RNA isolation by using the MagNa Pure Magnetic Glass Particle (MGP) Technology. First the cells are lysed in lysis buffer. Secondly the magnetic glass particles are added, and nucleic acids bind to them. By using magnets, the nucleic acid-bead complex are separated, and the sample is washed several times to remove cellular debris. Lastly the nucleic acids are eluted at high temperatures. Figure created in BioRender.com.

In short, all the samples got homogenised by adding a 5 mm steel bead in each sample and mixing them thoroughly with TissueLyser II (Qiagen) for two minutes at 24 Hz. After a short centrifugation for one minute, 200µL of each sample was added to a well in a MagNa Pure 96

plate and placed in the MagNa Pure 96 machine. Every reagent, and other components, were added according to the protocol (Roche, 2019).

50µL elution of each sample got transferred to new tubes, and frozen at -80°C until further cDNA synthesis.

The mRNA concentration (ng/µl) and the purity of each sample were measured using MultiSkan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific) at 230nm, 260nm and 280nm according to the manufacturers protocol. At the wavelength of 260nm, one can measure the nucleic acid content in a sample. Wavelength of 280nm will absorb larger components such as aromatic amino acids. By measuring the ratio between A260 and A280, one can estimate the purity of the RNA, where a ratio of ~2.0 is considered "pure". The ratio between A260 and A230, is a secondary measure of nucleic acid purity, and the absorbance for "pure" nucleic acid at this ratio is often higher than the 260/280 value – about 2.0-2.2. If the ratio is lower, one can expect presence of contaminants like carbohydrates and phenols (Thermo Fisher Scientific, w.d.-a)

2.7 cDNA synthesis

Further, the mRNA template needs to be converted to complementary DNA (cDNA) via reverse transcription (Figure 17). cDNA is more stable and not as easily degraded by omnipresent RNases than mRNA (Farkas & Holland, 2009).

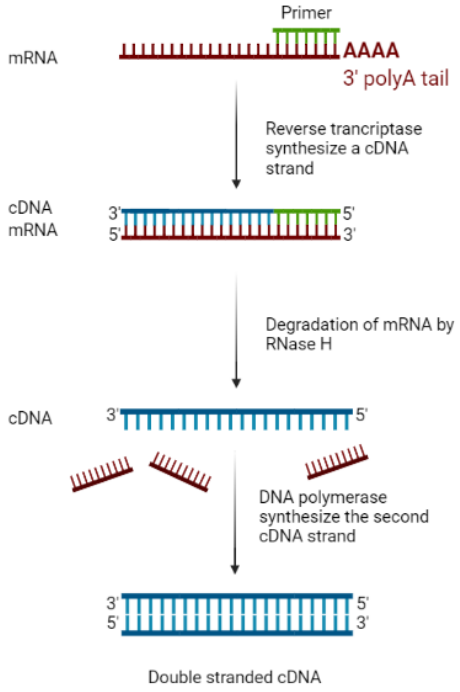


Figure 17: cDNA synthesis with mRNA as a template. First, a primer binds the mRNA, and the reverse transcriptase synthesizes a cDNA strand using mRNA as a template. RNase H degrades the mRNA, leaving only the cDNA. DNA polymerase synthesizes the second cDNA strand, resulting in a double stranded cDNA. Figure created in BioRender.com

cDNA synthesis was carried out following the protocol QuantiTect Reverse Transcription Kit (Qiagen, 2009). The first step was to get rid of the genomic DNA (gDNA) to get a more accurate gene expression result as primers can amplify DNA-sequences during RT-qPCR. This was done by taking 10µL 30 ng/µL of each of the RNA samples in pcr-strips and adding the following components (Table 1) for every reaction before a two-minute incubation at 42°C in the pcr-machine:

Table 1 1: Components for genomic DNA elimination reaction

Components	**Volume/reaction**	**Final [c]**
gDNA Wipeout buffer	2 µL	1X
Templat RNA	10 µL	1 µg
Rnase-free water	2 µL	---
Totale volum	**14 µL**	

After gDNA elimination, one needs to add dNTPs (dGTP, dCTP, DATP, DTTP), primers and reverse transcriptase (RT), which is a DNA polymerase. The RT-primer mix in the kit contains a mix of oligo-dT and random primers that enables cDNA synthesis from all regions of the RNA-template.

The reverse transcriptase enzyme has three main functions during the making of cDNA: RNA-dependent DNA-polymerase, exonuclease (RNase H) and DNA-dependent DNA polymerase. RNA-dependent DNA polymerase, synthesise a cDNA strand using mRNA as a template. RNase H degrades the mRNA, leaving only the cDNA. DNA polymerase synthesizes the second cDNA strand, resulting in a double stranded cDNA.

The RT-mix was prepared by mixing the following components for each reaction/sample: 1 µL Quantiscript Reverse Transcriptase, 4 µL Quantiscript RT buffer and 1 µL RT Primer mix. 6µL of the RT mix was added to each 14µL template RNA for a total volume of 20µL.

The samples were then incubated at 42℃ (30 minutes), 95℃ (3 minutes) and 4℃ (∞) in a PCR-machine.

The cDNA of each sample was diluted 1:6 by adding 100µL RNase-free water to get a final concentration of 2.5 ng/µL cDNA, and the samples were then stored at -20℃ until further RT qPCR.

2.8 RT-qPCR

Real time quantitative polymerase chain reaction, or RT-qPCR for short, is a technique to amplify, detect and quantify gene expression as the PCR process occurs. The target DNA is amplified using a solution containing DNA polymerase and nucleotides, in addition to primers that are complementary to the target DNA sequence one wants to study (Applied-Biosystems, 2008).

The first step of the process is heating the solution, in that way the dsDNA denatures, and the two strands get separated. When the solution cools, the primers anneal to the target sequence in the separate DNA strands, and the DNA polymerase forms a new strand by expanding the primers with complementary nucleotides creating a copy of the target DNA sequence (Figure 18). As this cycle is repeated, the number of target DNA sequences increases exponentially. If the target DNA sequence is not present, no amplification occurs (Thermo Fisher Scientific, w.d.-b)

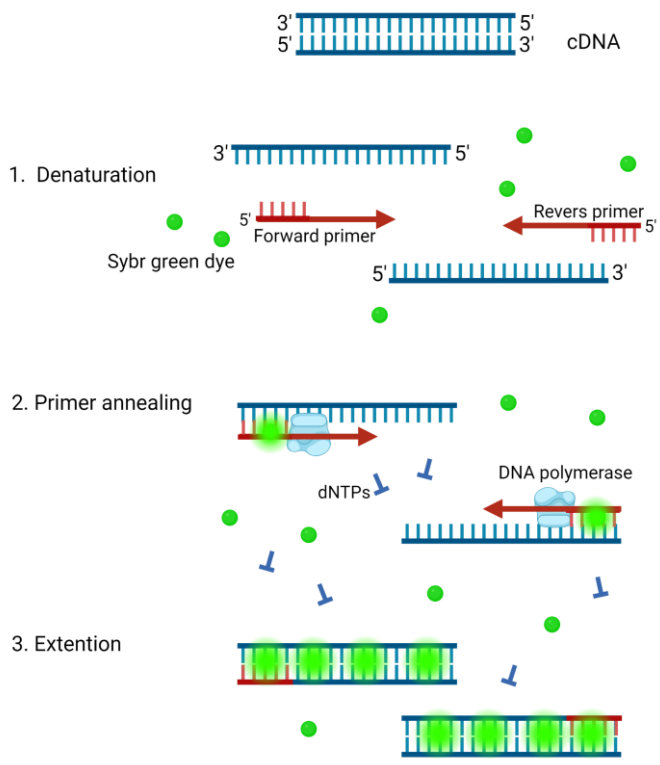


Figure 18: The concept of Real Time qPCR using sybr green dye. The first step is denaturation of the cDNA at 95℃. Then the primers (forward and reverse) bind to the cDNA and DNA polymerase starts to build the second strand of cDNA using dNTPs at 60℃. Sybr green binds to the minor groves of the dsDNA, giving a fluorescent signal that is detected by the PCR-machine. Figure created in BioRender.com.

To detect the target DNA sequence, different fluorescent chemistries are used, such as SYBR Green, that correlate PCR product concentration to fluorescence intensity. The dye binds the minor grooves of dsDNA (Applied-Biosystems, 2008). The reactions are characterized by which PCR cycle the target amplification is first detected as the fluorescens is measured after each cycle. This value is referred to as cycle threshold (C_t), the time at which fluorescence intensity is greater than the background fluorescence. This means that the greater the quantity of target DNA, the faster an increase in fluorescent signal will appear, resulting in a lower C_t value (Willey et al., 2017; Wong & Medrano, 2005)

The PCR can be divided into four phases (Figure 19): the linear ground phase, the exponential phase, the log-linear phase, and the plateau phase. The linear phase is in the beginning of the PCR, where the fluorescence emission has not reached above the background fluorescence. Further, at the exponential phase, the fluorescens is nearly at threshold, and is starting to increase rapidly above the background fluorescens. When the PCR reaches an optimal amplification period where the PCR product doubling after every PCR cycle, it has reached the log-linear phase. The plateau phase is when the reaction components become limited and the fluorescens intensity are no longer useful for data calculations (Wong & Medrano, 2005).

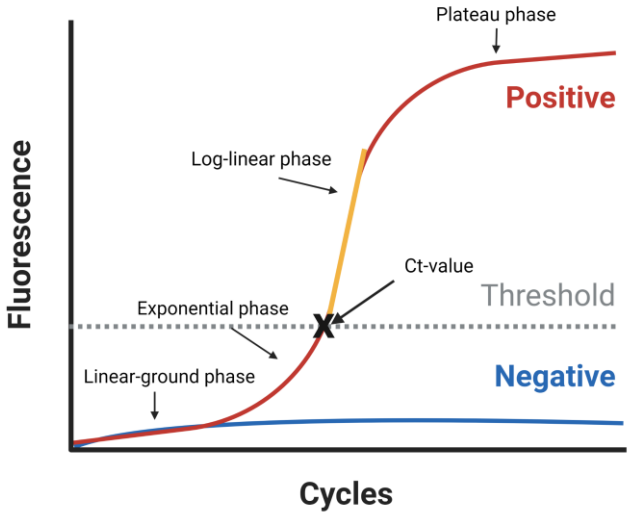


Figure 19: Phases of a PCR amplification curve. Blue curve is a negative control, whereas red is a positive sample. The threshold line is shown in grey. The linear-ground phase is at the very start of the PCR amplification curve, with very slow amplification of DNA. Further, the exponential phase is when the fluorescens is nearly at threshold and is starting to increase rapidly above the background fluorescens. When the PCR reaches an optimal amplification period where the PCR product doubling after every PCR cycle, it has reached the log-linear phase (yellow). The plateau phase is when the reaction components become limited and the fluorescens intensity are no longer useful for data calculations. The Ct-value is at the point where the fluorescens has reached above the threshold. Figure created in BioRender.com.

The real-time qPCR was done using the kit “BioRAD Sso Advanced universal SYBR” (cat.nr. 1725272) with precision blue dye and PCR machine BioRAD CDX 384. In short, for every qPCR reaction, 2.0 µL 2.5ng/µL cDNA, 5.0µL SsOAdvanced universal SYBR Green supermix (2x), 0.5µL 10µM primer F, 0.5 µl 10 µM primer R and 2.0µL RNase-free water was used for a total volume of 10µL. Every sample was run in duplicates and normalized against the housekeeping gene Ef1-α. A duplicate of a negative control with RNase free water instead of cDNA, and a duplicate without the RT-enzyme were also made for every primer set. This to ensure potential nonspecific binding as a result of contamination of SYBR Green, cDNA, primers, or water.

The samples were run in duplicates to see if the results could be replicated, which make the results more likely to be correct if the values of the duplicates are close enough to each other.

The plate with the samples was sealed, centrifuged to get the solutions completely in the bottom of the wells, and finally analysed in CFX384 Touch Real-Time Detection System (Bio-Rad laboratories) with the software program CFX Manager. The following program was used (Table 2):

Table 2: The program used for qPCR

1x	95℃	30 seconds
	95℃	15 seconds
40 x	60℃	30 seconds

2.8.1 Primers

The samples were tested against different antiviral, - and putative acute- and chronic stress response genes using RT-qPCR (Table 3).

A housekeeping or reference gene was employed as a control. These are genes that have a stable expression in the cells, and gives a basis comparable to genes of interest (Wong & Medrano, 2005). In this setup, the housekeeping gene Ef1-α was used. Ef1-α is involved in protein synthesis and acts as a translation elongation factor that is responsible for selection and binding of the cognate aminoacyl tRNA to the acceptor site (A-site) of the ribosome (Carvalho et al., 1984).

ISG15, Mx1 and IRF3 were the antiviral genes/primers of interest. As mentioned in the fish immunology part, IRF3, or interferon regulatory factor 3, is involved in the regulation of different immune responses, including regulation of IFN type 1 genes. These types of IFNs type 1 genes includes ISG15 and Mx1, which both help to eradicate viruses (Abbas et al., 2020; Bottrel et al., 1999; Robertsen, 2006).

The new primers for stress response genes were tested by Thomais Tsoulia, one of the supervisors of this thesis. This was done by first extracting RNA from random RBCs from Atlantic salmon, and then perform a dilution-series of it before running the primers in a qPCR. The length of the amplicon and the quality of the primers (one clear band) were tested using Agilent 2200 TapeStation following the manufacturers protocol (Agilent Technologies, 2015).

Tabell 3: Overview of the primers used in the trials of this thesis, with full name, sequence, accession no. and reference.

	Gene	Full name		Sequence (5' - 3')	Accession no.	Reference
Reference gene	Ef1-α	Elongation factor 1-alpha	F	TGCCCCTCCAGGATGTCTAC	BG933897	(Haatveit et al., 2017; Wessel et al., 2015)
			R	CACGGCCCACAGGTACTG		
Antiviral genes	Mx1	Myxovirus resistance 1	F	GATGCTGCACCTCAAGTCCTATTA	BT043723.1	(Haatveit et al., 2017; Wessel et al., 2015)
			R	CACCAGGTAGCGGATCACCAT		
	ISG15	Interferon -stimulated gene 15	F	ATATCTACTGAACATATATCTATCATGGAAACTC	EOG7V70Z2	(Haatveit et al., 2017; Wessel et al., 2015)
			R	CCTCTGCTTTGTTGTGGCCACTT		
	IRF3	Interferon regulatory factor 3	F	GCAGAGGGGATCTCAACCAC	FJ517643.1	(Kawai et al., 2007)
			R	ACCCTGAATAGCCTCTGTTGG		
Acute stress target genes	HSP90aa	Heat shock protein 90 kDa alpha member A1	F	CGAGGACATGAAGAAGAGGCAT	KC150878.1	(Beemelmanns et al., 2021)
			R	ACACTGTCACCTTCTCCACTTT		
	SOCS3	Suppressor of cytokine signalling 3	F	TGTGGGGACAGGGAGTTTG	XM_014180219.2	(Fasshauer et al., 2002)
			R	GGGTCCTTCTACACAGGTGC		
	Cahz	Carbonic anhydrase	F	TGGGGATATGGACCGTCTGA	BT058764.1	(F. B. Jensen, 2004)
			R	GCCAACTTGGAAGGAATGGC		
Chronic stress target genes	MAO-A	Monoamine oxidase	F	AGTGTGTTATCGGCCTTGGT	XM_014164473.2	(Manoli et al., 2005; Puhr et al., 2021)
			R	TGTCAAAGACACAAATCCTAGCAG		
	HSP70	Heat shock cognate 70 kDa protein	F	AGTGATCAACGACTCGACACG	BT045715.1	(Beemelmanns et al., 2021)
			R	CACTGCATTGGTTATAGTCTTG		
	GRt1	Glucocorticoid receptor-like, transcript variant X1	F	AGAGCCGTGGAAGGTACAGG	XM 014198677.2	
			R	CTGGCTCGATGGCCTTTAGT		
	CTNNB1	Catenin beta 1	F	CCTCCACCCAGTTTGATGG	NM 001173938.1	(Kawai et al., 2007)
			R	GTTCACCACCACCTGGTCC		
	FkBP1	FkBP prolyl isomerase 1	F	TGCTGAGCTTCAAAGGGGAG	BT048177.1	(Binder, 2009; Menke et al., 2012)
			R	AGAGAAGGTAGGTCTGCCTCA		

2.9 Data analysis

2.9.1 Calculate the hormone concentration by interpolating from the O.D value on the standard curve

The hormone measurements was analysed first by preparing a standard curve by plotting the mean O.D. of each standard solution (Y-axis) vs. the respective concentration of the standard solution (X-axis) for both adrenalin and cortisol. Thereby, the concentration of adrenalin/cortisol of the samples were interpolated from the standard curve.

2.9.2 Calculate the gene expression using the $2^{-\Delta\Delta Ct}$ method

The gene expression data from the RT qPCR-analysis got analysed further using GraphPad Prism, version 9.5.1. Relative gene expression was calculated using the Livak and Schmittgen (2001) $2^{-\Delta\Delta Ct}$ method in Excel. All the ct-values from the RT-qPCR were normalized against the reference gene Ef1-α (measured in the same samples) to get the ΔCT. These values were then corrected against the negative control in every group, which gives the ΔΔCT-value. By entering this value in the equation ($2^{-\Delta\Delta Ct}$), one gets the relative expression. An example of raw data from a RT qPCR and the calculation of relative expression is shown in appendix F.

If the relative expression is above 1, it is regarded as an upregulation of that gene. Below 1 is regarded as a downregulation/inhibition.

For this method to work optimally, it is necessary with 100% primer efficiency.

2.9.3 Statistically significant differences by using one way analysis of variance (one- way ANOVA)

As for finding out the statistically significant differences, one-way analysis of variance (ANOVA) was used between the mean of the relative expression of the negative control and the variable of interest in GraphPad Prism. The P-value was set to 0.05. A p-value lower than 0.05 means that there are a significant difference between the groups tested.

$$* P \leq 0.05, ** P \leq 0.01, *** P \leq 0.001, **** P \leq 0.0001$$

3 Results

3.1 In vivo stress trial

The overall observations of the fish during the experiment were that the more stress treatment episodes the chronic stress group got exposed to, the more “sluggish” and calm behaviour the fish had. At the final stress induction day, the fish in the chronic stress group did not seem bothered at all by the chasing and transfer between tanks. The fish in the acute stress group were more afraid and swam quickly away when trying to chase them with the hand-net during the one-time stress induction. The fish in the chronic stress group also had more wounds and red spots around pectoral-, pelvic- and anal fins (Figure 20) compared to the fish in the control group (Figure 21). The fish in the acute stress group also had higher amount of mucus on the skin during sampling compared to the controls and the fish in the chronic stress group, as the fish still had mucus left on the skin even after scraping.

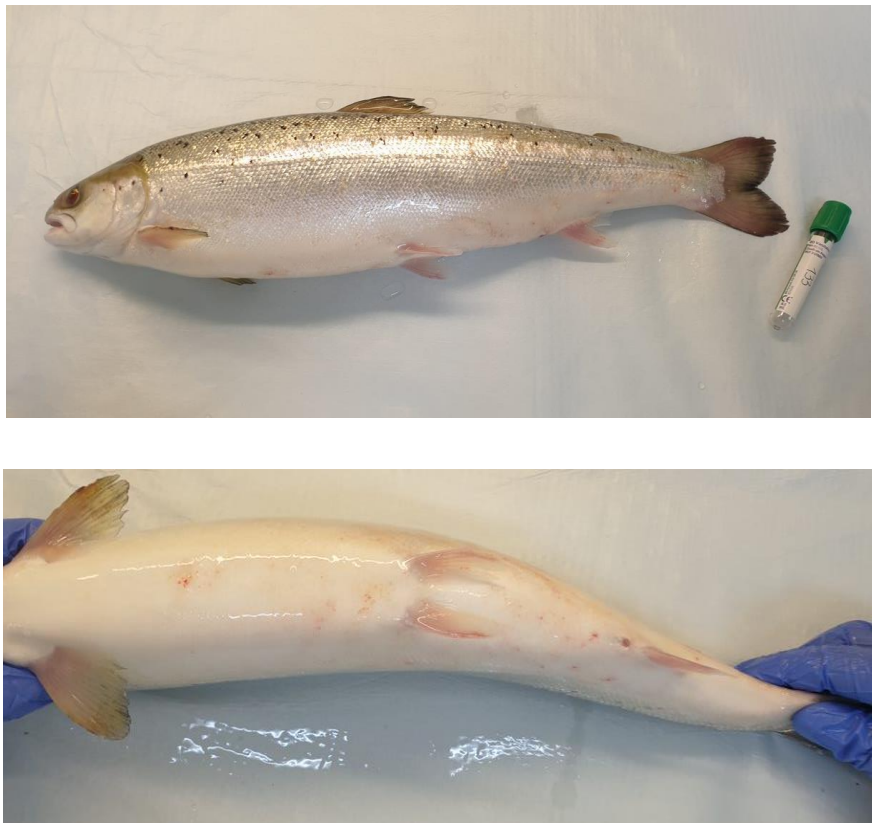


Figure 20: One of the fish from the chronic stress group after the first sampling. More visible wounds around the fins, and redness on the lower part of the body (abdomen). The fish had some bleeding in the eyes as well. Photo: Martine Aardal.

Figure 21: One of the fish from the control group after the first sampling. No visible wounds or redness in skin. Overall, a good-looking fish. Photo: Martine Aardal

3.1.1 Cortisol and adrenalin concentration in blood plasma and mucus, and haemoglobin measurement in the blood

The cortisol concentration was measured using competitive ELISA, in all the blood plasma and mucus samples from the in vivo stress trial (Figure 22). Adrenalin was only measured in the blood plasma, also using competitive ELISA.

Generally, the mean cortisol concentration in blood plasma measured was higher for the individuals that were sampled day one, than in individuals sampled day four. The acute stress group (T3) had the highest mean concentration of cortisol in blood plasma (148 ng/mL) on day one sampling. Further, the control group (T1) had the second highest mean concentration of cortisol in blood plasma at 74 ng/mL on day one sampling, while the chronic stress group (T3) had a mean cortisol concentration of 43 ng/mL.

The mean concentration of cortisol in the blood plasma for each group at day four sampling was 37 ng/mL for the control group, 65 ng/mL for the acute group and 34 ng/mL for the chronic group. Post stress levels of cortisol are known to vary from 20-500 ng/mL (Gamperl et al., 1994).

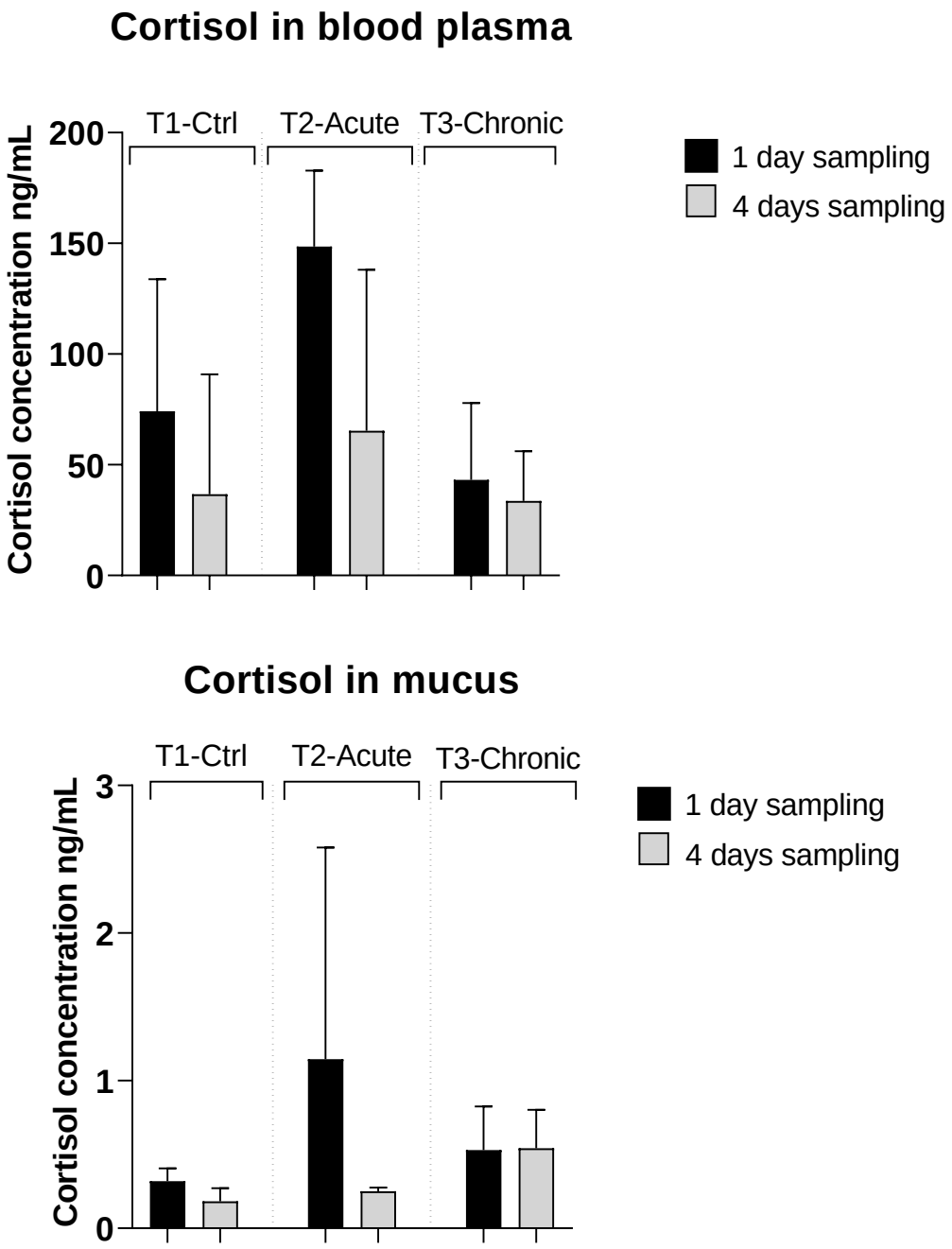


Figure 22: Mean cortisol concentration (ng/mL) in blood plasma (top) and in mucus (bottom) of Atlantic salmon (n=3/group) for every group (control (ctrl), acute stress, and chronic stress) after each sampling – one day sampling (black), and four days sampling (grey).

The standard deviation is high in all three groups, meaning large individual differences. For instance, one individual in the acute group sampled four days post stress had a cortisol concentration of 3,65 ng/mL, while another individual in the same group and sampled the same day had a concentration of 145.62 ng/mL.

There were no significant difference in cortisol levels for neither the acute stress group nor the chronic stress group, compared to the control group.

As for adrenalin in blood plasma (Figure 23 and 24), the mean concentration in every group was less variable, ranging between 0.21 ng/mL and 0,26 ng/mL.

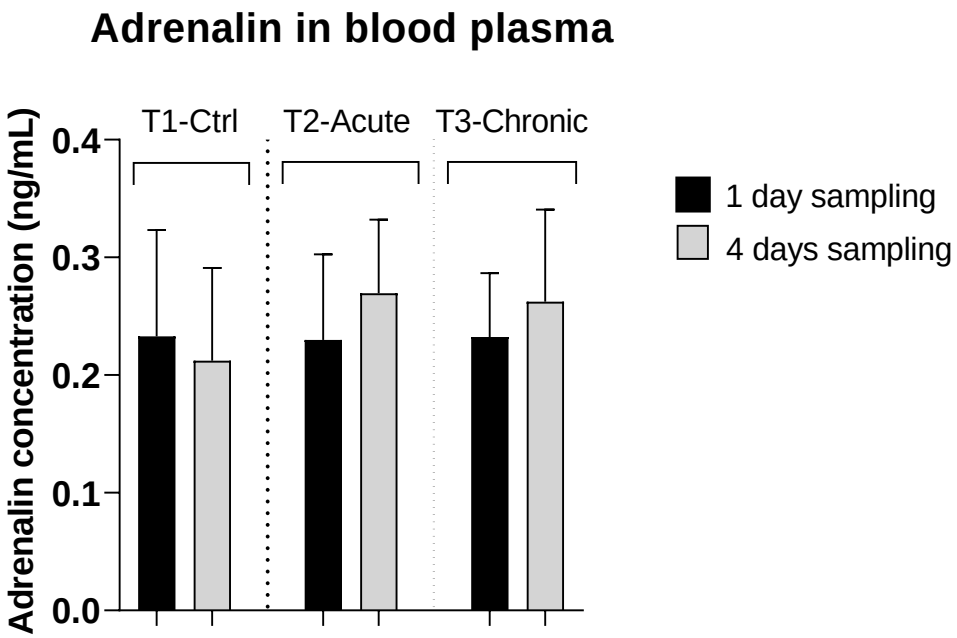


Figure 23: Mean adrenaline concentration (ng/mL) in blood plasma of Atlantic salmon (n=3/group) of every group (control (ctrl), acute stress, chronic stress) after each sampling – one day sampling (black), and four days sampling (grey).

There were no significant differences in adrenalin levels, neither for the acute stress group nor the chronic stress group.

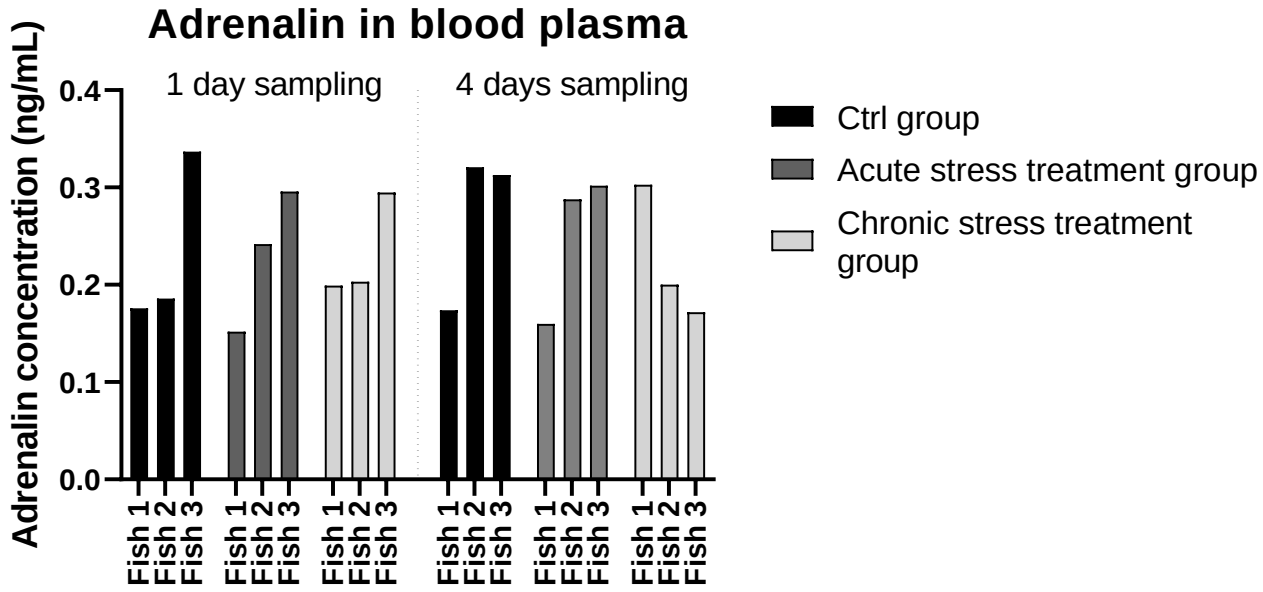


Figure 24: Adrenaline concentration (ng/mL) in blood plasma for each individual fish in every group (control (ctrl) (black), acute stress (dark grey), chronic stress (light grey)) after each sampling – one day sampling (left part of the graph), and four days sampling (right part of the graph).

The overall trend was that the adrenalin levels increased as the fish were sampled. The fish sampled first had the lowest level, the second fish sampled had a higher level and, lastly, the third fish had the highest level of adrenalin.

There were no significant differences in the haemoglobin levels (Hb) (Table 4) in neither the fish from the acute stress group nor the fish in the chronic stress group compared to the controls. However, there is a tendency towards an elevation of Hb in the acute stress group compared to the control group and the chronic stress group.

By running a Pearson correlation test in GraphPad Prism between the Hb level and cortisol level from the same fish, one finds that there is no correlation between these parameters ($r = -0.01$).

Table 4: Average haemoglobin (Hb) levels (g/dl) of the fish in each group (control (ctrl), acute and chronic) after each sampling (one day and four days post stress exposure).

	Hb (g/dl)	
	1 d sampling	**4 d sampling**
Ctrl	7,7	7,9
Acute	8,1	9,3
Chronic	7,1	8,1

3.2 Expression of antiviral genes in RBCs after ex vivo stimulation with poly (I:C) and imiquimod post exposure to stress in vivo.

RT-qPCR was only done for RBCs from the day one sampling because time was limited, and generally the cortisol-levels was higher in these samples. That way it made more sense to test these samples, as the main goal was to test the effects of stress hormones on the expression of antiviral genes.

Generally, there was a significant difference (marked with *) in the mean relative expression of the antiviral gene ISG15 in RBCs stimulated with 50µg/mL poly (I:C) for 3 days from fish in the control group compared to fish in the chronic stress group (Figure 25). There were no significant differences in the expression of ISG15 in the RBCs stimulated with poly (I:C) nor 0.5 µg/mL imiquimod for 7 days (Figure 26).

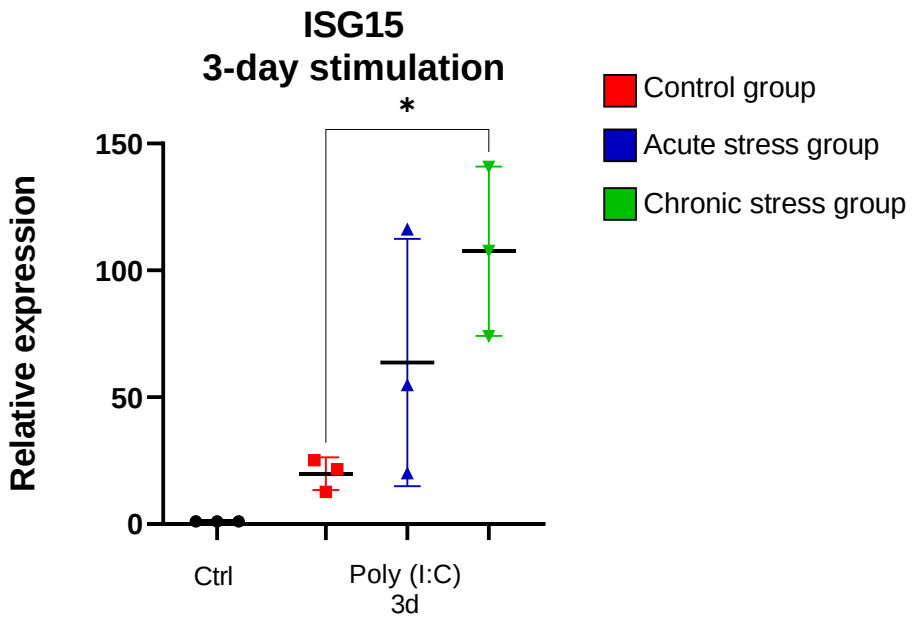


*Figure 25: Relative expression of ISG15 in RBCs from Atlantic salmon (n= 3/group) sampled at day one sampling in the in vivo stress trial, after a 3-day stimulation with 50µg/mL poly (I:C). The control (ctrl) is the mean relative expression of the gene in non-stimulated cells (one control/fish)- set to one (n=9). Red is the relative expression for fish in the control group (not stressed), blue is the acute stress group, and the green is the chronic stress group. * P ≤ 0.05*

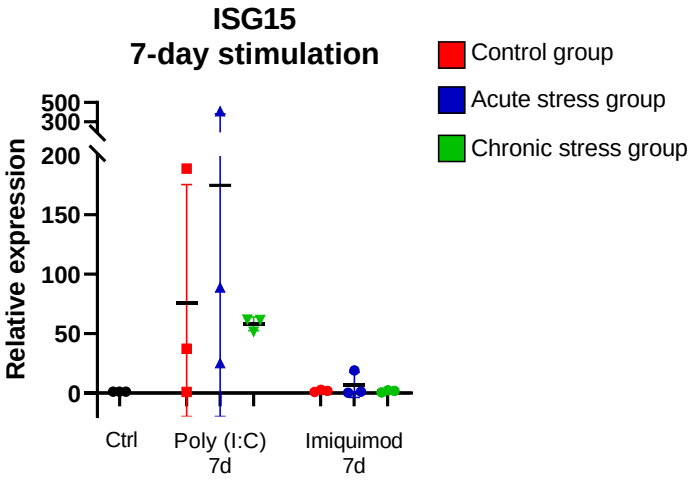


Figure 26: Relative expression of ISG15 in RBCs from Atlantic salmon (n=3/group & stimulation)) sampled at the day one sampling in the in vivo stress trial, after a 7-day stimulation with 50µg/mL poly (I:C) and 0.5 µg/mL imiquimod. The control (ctrl) is the mean relative expression of the gene in non-stimulated cells (one control/fish) - set to one (n=9). Red is the relative expression for fish in the control group (not stressed), blue is the acute stress group, and the green is the chronic stress group.

There were no significant differences between the groups when it comes to the relative expression of the antiviral gene Mx1 in RBCs after ex vivo stimulation with poly (I:C) and imiquimod (Figure 27 and 28).

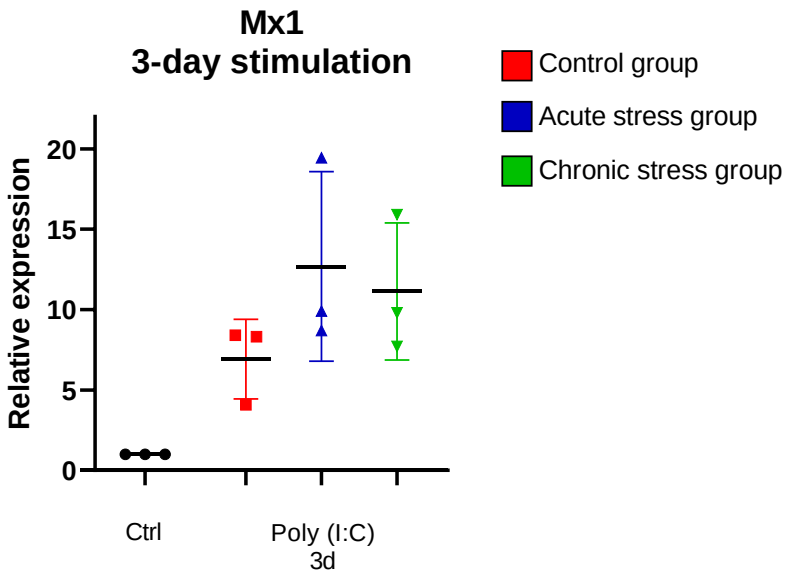


Figure 27: Relative expression of Mx1 in RBCs from Atlantic salmon (n=3/group) sampled at the day one sampling in the in vivo stress trial, after a 3-day stimulation with 50µg/mL poly (I:C The control (ctrl) is the mean relative expression of the gene in non-stimulated cells (one control/fish) - set to one (n=9). Red is fish in relative expression for fish in the control group (not stressed), blue is the acute stress group, and the green is the chronic stress group

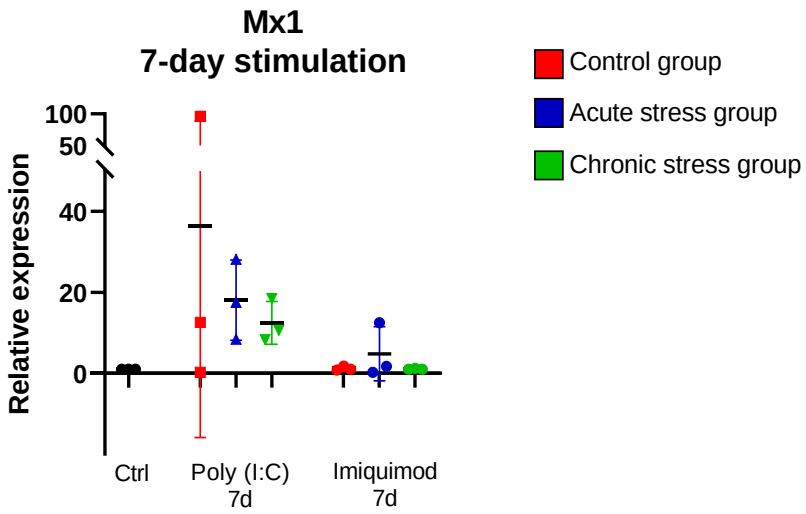


Figure 28: Relative expression of Mx1 in RBCs from Atlantic salmon (n=3/group & stimulation) sampled at the day one in the in vivo stress trial, after a 7-days stimulation with 50µg/mL poly (I:C) and 0.5 µg/mL imiquimod. The control (ctrl) is the mean relative expression of the gene in non-stimulated cells (one control/fish) - set to one (n=9). Red is fish in relative expression for fish in the control group (not stressed), blue is the acute stress group, and the green is the chronic stress group

3.3 Testing of stress target genes and antiviral genes in RBCs stimulated with acute- and chronic stress hormones and poly (I:C) ex vivo

Based on the results from the in vivo stress trial, and because of the large individual differences within the groups, it was decided to change the plan into stimulating RBCs from Atlantic salmon with acute and chronic stress hormones ex vivo. Then later stimulate the cells with poly (I:C) to see the effects stress hormones have on different antiviral genes.

3.3.1 Relative expression of antiviral genes in RBCs stimulated with acute stress hormones ex vivo

Red blood cells from three Atlantic salmon were stimulated with different concentrations of isoproterenol and epinephrine (1 µM, 5 µM, 10 µM, 50 µM, and 100 µM), and 50 µg/mL poly (I:C). The expression of the antiviral genes ISG15 (Figure 29), Mx1 and IRF3 (Figure 29) was tested with RT-qPCR.

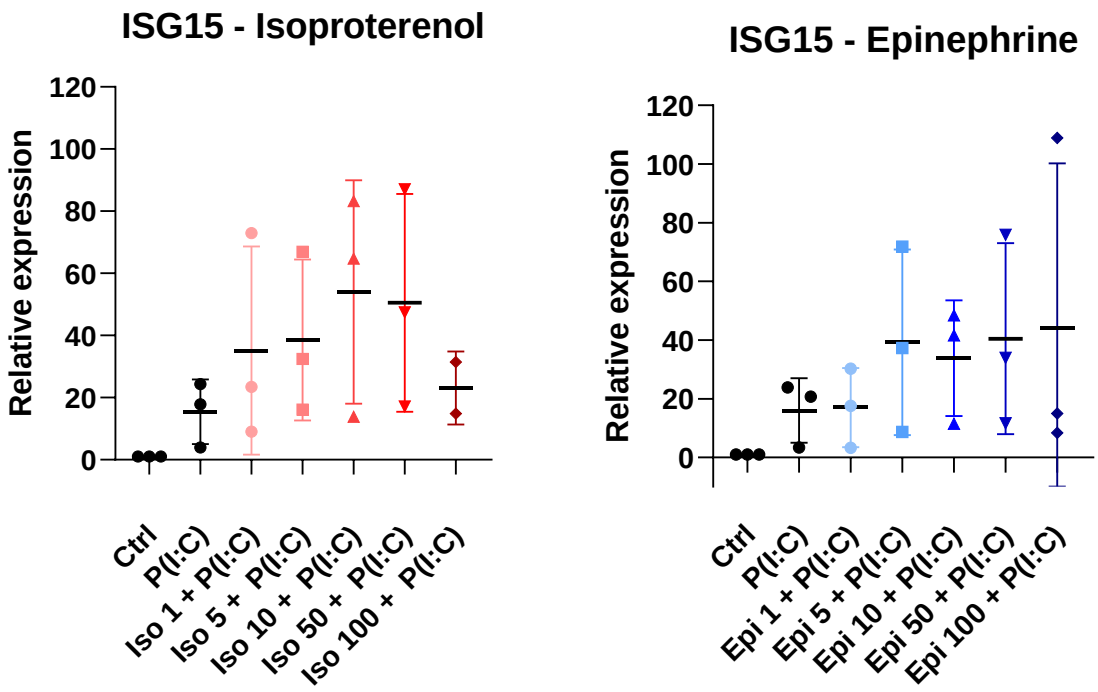


Figure 29: Relative expression of ISG15 in Atlantic salmon (n=3) RBCs pre stimulated with different concentrations (µM) of isoproterenol (Iso) (left) and epinephrine (Epi) (right) and thereafter 50 µg/mL poly (I:C). The control (ctrl) is the mean relative expression of the gene in non-stimulated cells (one control/fish)- set to one (n=3).

The relative expression of ISG15 shows a trend towards an increase in expression with increasing concentration of isoproterenol and reach a peak at 50 µM where the mean relative expression decreases again compared to the control with poly (I:C). As for epinephrine, there is a trend towards an increase in the mean relative expression with increasing concentration.

There are large individual differences between the three fish, making the standard deviation large for each concentration – and resulting in no significant differences. But even though there are no significant differences, one can see a "biological difference", where the expression in some individuals increases when the cells are stimulated with an acute stress hormone and poly (I:C) compared to the control.

Based on the results from the RT-qPCR of ISG15, the concentrations of isoproterenol and epinephrine that gave higher expression of the gene (10 µM and 50 µM for both isoproterenol and epinephrine), were chosen and further used in testing the expression of Mx1 and IRF3.

There was no effect of isoproterenol nor epinephrine on the poly (I:C) response of Mx1 and IRF3 (Figure 30)

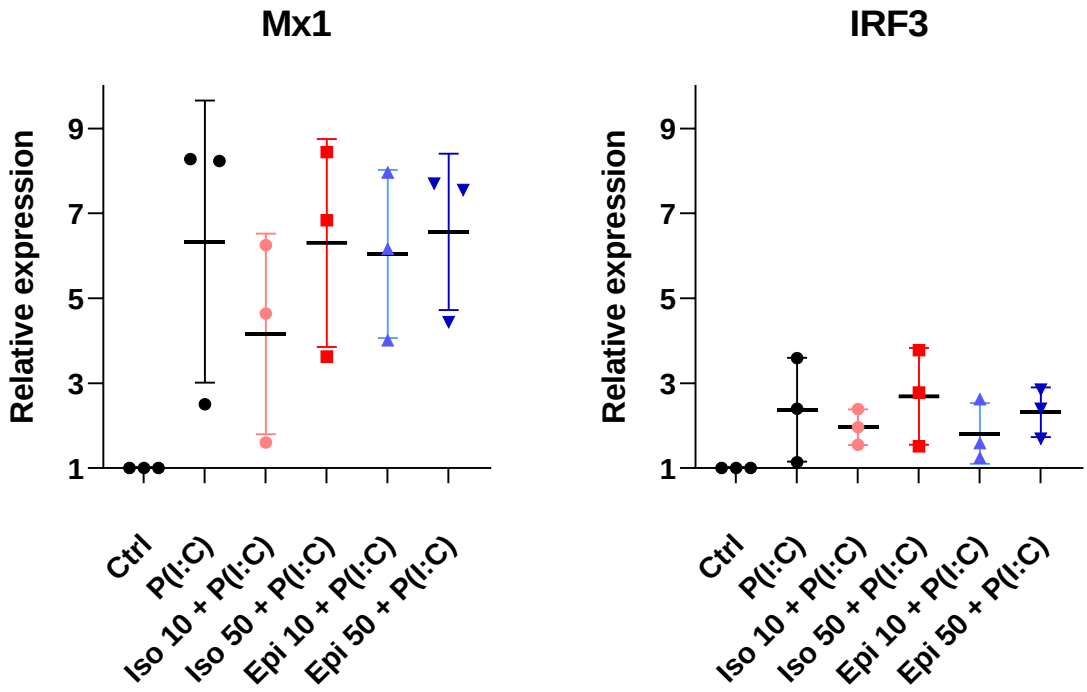


Figure 30: Relative expression of Mx1 (left) and IRF3 (right) in Atlantic salmon (n=3) RBCs pre stimulated with 10 µM and 50 µM isoproterenol and epinephrine, and thereafter 50 µg/mL poly (I:C). The control (ctrl) is the mean relative expression of the gene in non-stimulated cells (one control/fish) - set to one (n=3).

3.3.2 Identification of acute stress target genes in RBCs stimulated with acute stress hormones ex vivo

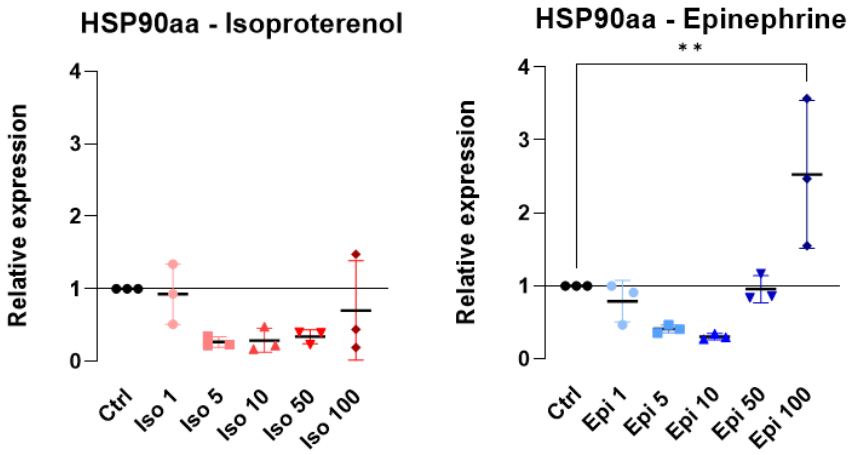
To identify potential acute stress target genes, like HSP90aa, SOCS3, and Cahz, RT-qPCRs were run for RBCs samples stimulated with isoproterenol and epinephrine.

The potential acute stress target genes, HSP90aa, SOCS3 and Cahz (Table 3) were chosen as different studies have shown an upregulation of these genes when exposed to acute stress, but in different cells other than red blood cells.

HSP90aa is a heat shock protein that, in a not stressed cell, function as a molecular chaperone assisting in folding of nascent polypeptides, protein folding, translocation of proteins and degradation of misfolded proteins. When the cells are exposed to a stressor, HSPs are upregulated, resulting in added protection to prevent damage from cellular stress associated

with uncontrolled protein unfolding (Iwama et al., 1998; Zügel & Kaufmann, 1999). A study has shown an upregulation of HSP90aa expression in Atlantic salmon hepatocytes exposed to warm and hypoxic conditions (Beemelmanns et al., 2021). Another study also showed enhanced synthesis of HSP90 in braincells of two species of gobiid fishes (*Gillichthys mirabilis* and *G. seta*) held at elevated temperatures (Zügel & Kaufmann, 1999).

Stimulation of Atlantic salmon RBCs with higher doses of epinephrine (100 µM), upregulated HSP90aa (2.5-fold) (Figure 31)



*Figur 31: Relative expression of the potential acute stress target gene HSP90aa in Atlantic salmon (n=3) RBCs stimulated with different concentrations (µM) of isoproterenol (left) and epinephrine (right). The control (ctrl) is the mean relative expression of the gene in non-stimulated cells (one control/fish) - set to one (n=3). ** P ≤ 0.01*

SOCS3, or suppressor of cytokine signaling-3, is important in regulating JAK/STAT signalling as it acts as a negative feedback signal inhibiting JAK/STAT activation and phosphorylation (Wunderlich et al., 2013). Studies have shown that expression of SOCS3 in human adipocytes are stimulated after exposure to isoproterenol, indicating that this gene is stimulated by β-adrenergic agents via activation of G_S-protein-adenylyl cyclase dependent pathway (Fasshauer et al., 2002).

The results from this ex vivo stimulation of Atlantic salmon RBCs, showed a downregulation of SOCS3 (0.5-fold) after stimulating the cells with a high dose of epinephrine (100 µM) (Figure 32).

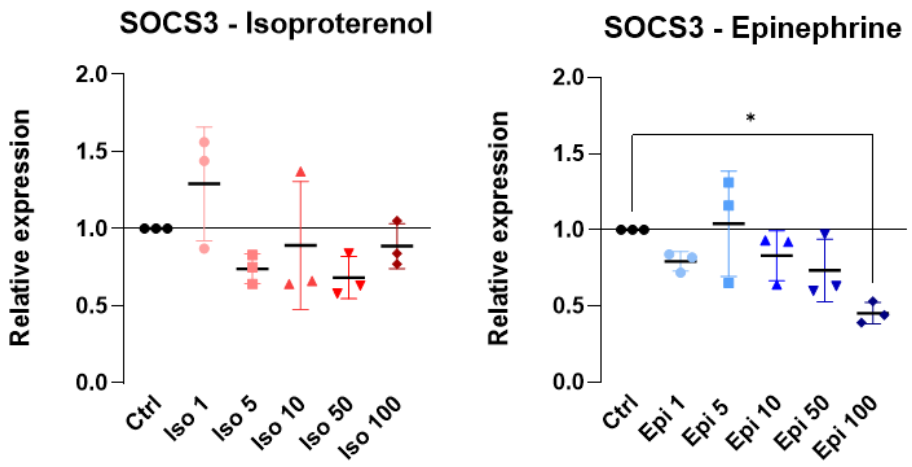


*Figure 32: Relative expression of the potential acute stress target gene SOCS3 in Atlantic salmon (n=3) RBCs stimulated with different concentrations (µM) of isoproterenol (left) and epinephrine (right). The control (ctrl) is the mean relative expression of the gene in non-stimulated cells (one control/fish) - set to one (n=3). * P ≤ 0.05.*

Lastly, Cahz, or carbonic anhydrase, is an enzyme involved in the primary function of RBCs as gas exchanger. The enzyme secures rapid CO_2 hydration and RBC acidification in tissue capillaries. In heavy exercise, for instance in acute stress responses, when the critical low capillary PO_2 is reached, lactic acidosis in muscle capillary blood further increases O_2 dissociation and facilitates O_2 delivery, making this enzyme important in acid-base homeostasis (Geers & Gros, 2000; F. B. Jensen, 2004).

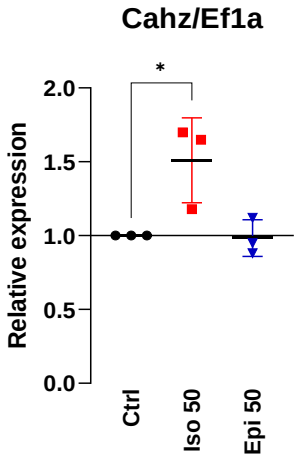


*Figure 33: Relative expression of potential acute stress target gene Cahz in Atlantic salmon (n=3) RBCs stimulated with 50 µM isoproterenol and epinephrine. The control (ctrl) is the mean relative expression of the gene in non-stimulated cells (one control/fish) - set to one (n=3). * P ≤ 0.05.*

Cahz was upregulated in Atlantic salmon RBCs in response to a higher dose (50 µM) of isoproterenol (1.5-fold) (Figure 33).

3.3.3 Relative expression of antiviral genes in RBCs stimulated with chronic stress hormones ex vivo

The effects of hydrocortisone on antiviral responses to poly (I:C) were tested in RBCs from three Atlantic salmon. By that, the expression of ISG15 and Mx1 antiviral genes was tested by RT-qPCR (Figure 34). Dexamethasone 100 µM was used as a positive control, as is has been shown to have effects in earlier ex vivo trials, and that way one can compare it to the effect of different concentrations of hydrocortisone.

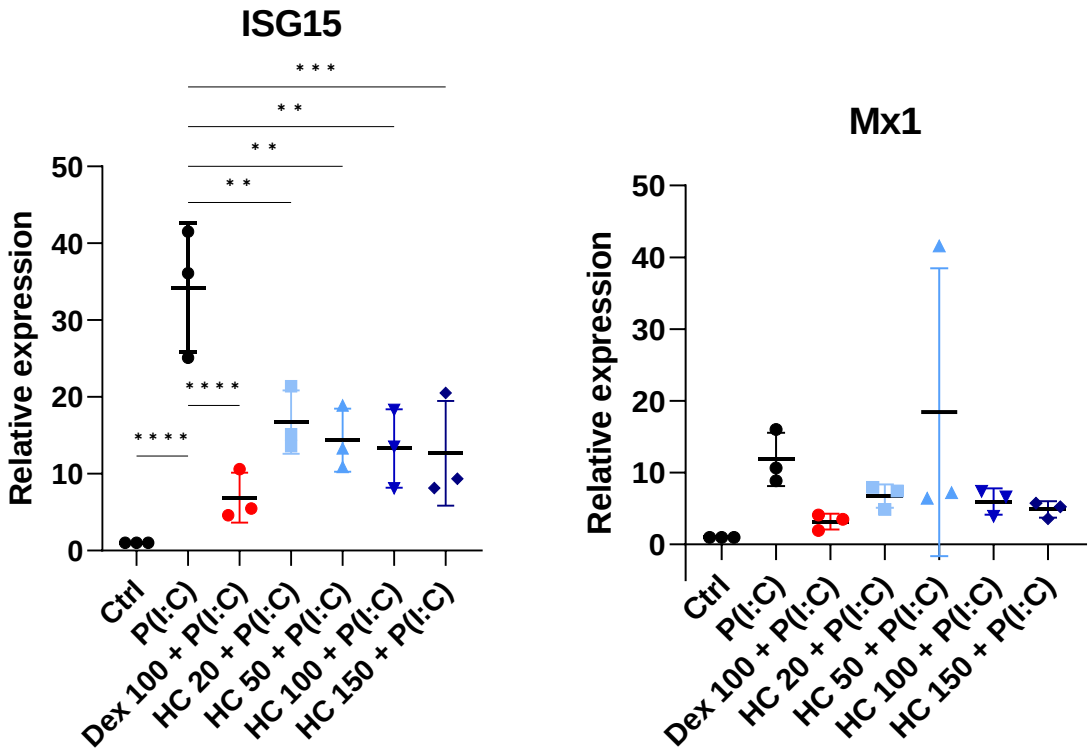


*Figure 34: Relative expression of ISG15 (left) and Mx1 (right) in Atlantic salmon (n=3) RBCs stimulated with different concentrations of hydrocortisone (20 µM, 50 µM, 100 µM, and 150 µM) prior to stimulation with 50µg/mL poly (I:C). Dexamethasone 100 µM was used as a positive control. The control (ctrl) is the mean relative expression of the gene in non-stimulated cells (one control/fish) - set to one (n=3). * P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001, **** P ≤ 0.0001.*

As expected, dexamethasone significantly downregulated the ISG15 response to poly (I:C). The same is observed with all doses of hydrocortisone tested, but the effect is less strong compared to the dexamethasone effect. As for the expression of Mx1, the effects are not significant, but they follow a similar trend as for ISG15.

Based upon this result, it was decided to continue with 50 µM hydrocortisone for further testing of other genes, as higher concentrations do not necessarily mean stronger effects. The

expression of the antiviral gene IRF3 was tested in these RBCs samples stimulated 50 µM hydrocortisone and poly (I:C) (Figure 35).

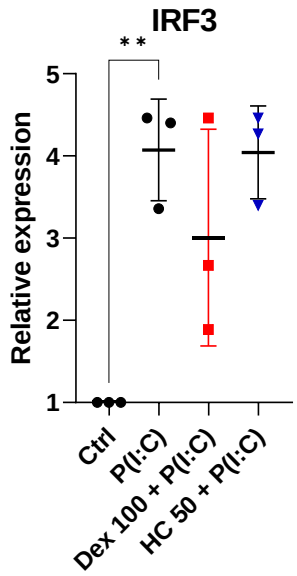


*Figure 35: Relative expression of the antiviral gene IRF3 in Atlantic salmon (n=3) RBCs stimulated with 50 µg/mL poly (I:C) with or without pre-treatment of 50 µM hydrocortisone or 100 mM dexamethasone as a control. The control (ctrl) is the mean relative expression of the gene in non-stimulated cells (one control/fish) - set to one (n=3). ** P< 0,01.*

There was a significant difference in relative expression of IRF3 between the negative control and the control stimulated with poly (I:C), but no significant difference between cells stimulated with chronic stress hormones compared to the control.

3.3.4 Identification of chronic stress target genes in RBCs stimulated with chronic stress hormones ex vivo

Further, the expression of potential chronic stress target genes like MAO-A, HSP70, GRt1, CTNNB1 (Figure 36) and FkBP1 (Figure 37) were tested in Atlantic salmon RBCs samples stimulated with hydrocortisone and dexamethasone.

The potential chronic stress target genes were also chosen based on different studies on different types of cells in different species, where they had shown an upregulation when exposed to chronic stress.

MAO-A, or monoamine oxidase A, is a mitochondrial enzyme involved in the catabolism of catecholamines, including norepinephrine (X. Sun et al., 2020). The gene is expressed in many cells in mammals, and it is shown that glucocorticoid receptors are needed for dexamethasone induced MAO-A expression (Manoli et al., 2005; Puhr et al., 2021)

HSP70, a heat shock protein functioning in a similar way as HSP90aa tested in the acute stress ex vivo trial, also shows an upregulation in in Atlantic salmon hepatocytes when exposed to warm and hypoxic conditions (Beemelmanns et al., 2021). Another study done on rainbow trout also shows an upregulation of this gene along with increasing cortisol levels caused by increasing stocking density (Yarahmadi et al., 2016)

CTNNB1 is a gene encoding a protein called beta-catenin that plays an important role in cell signalling and regulates cellular homeostasis (Zhuang et al., 2023) . This gene was chosen as it has been shown to be upregulated in peripheral blood cells from humans (medical students) exposed to chronic psychological stress (Kawai et al., 2007).

GRt1, glucocorticoid receptor-like transcript variant X1, is a variant of the target receptor that cortisol/dexamethasone binds to when initiating chronic stress responses. In mammals, glucocorticoids are shown to repress their own receptor gene (Ramamoorthy & Cidlowski, 2013) Primers for this gene was designed by this thesis' supervisors to check the effect of stress on its regulation, especially after addition of dexamethasone and hydrocortisone.

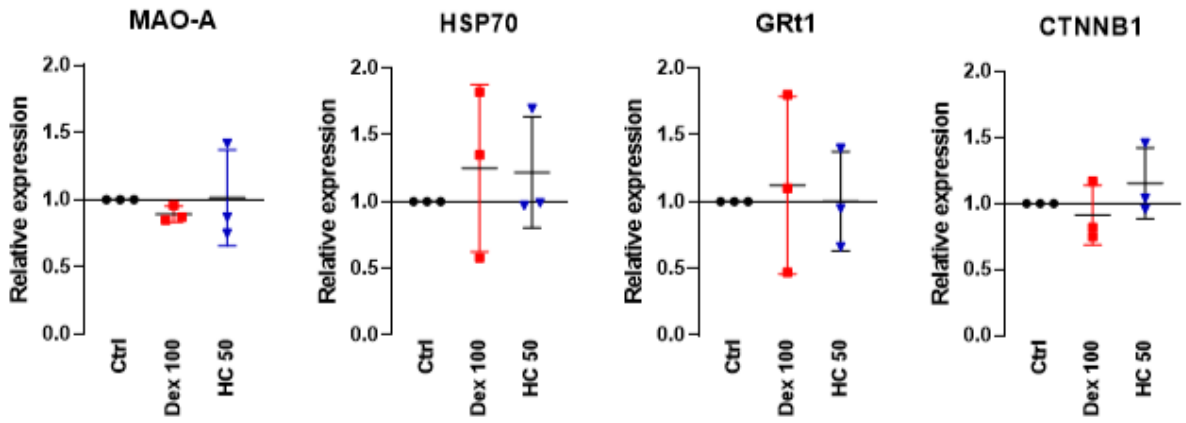


Figure 36: Relative expression of potential chronic stress target genes MAO-A, HSP70, GRt1 and CTNNB1 in Atlantic salmon (n=3) RBCs stimulated with 50 µM hydrocortisone and 100 µM dexamethasone. The control (ctrl) is the mean relative expression of the gene in non-stimulated cells (one control/fish) - set to one (n=3).

FkBP1 were chosen as it has shown to be a reliable marker of glucocorticoid receptor activation in human peripheral blood cells (Menke et al., 2012). FKBP proteins belong to the immunophilin family, which are proteins with roles in folding and transport of other proteins. These proteins have also been shown to bind to immunosuppressive drugs, and to be linked to infectious disease, cancer and neurodegenerative diseases in mammals (Kolos et al., 2018). Biological functions in Atlantic salmon are uncharacterized.

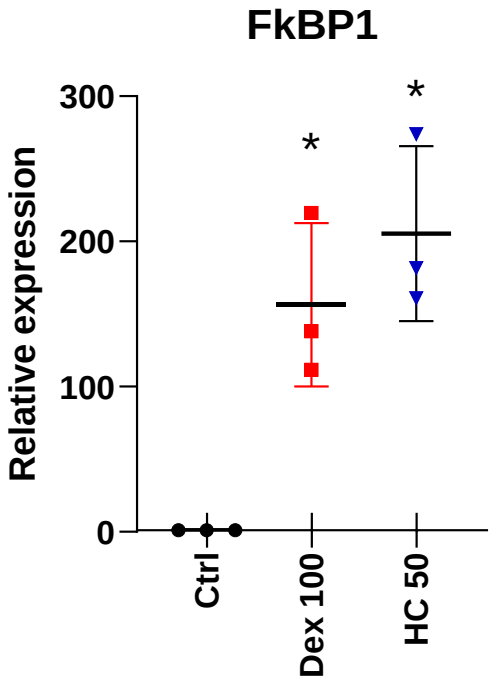


*Figure 37: Relative expression of FkBP1 in Atlantic salmon (n=3) RBCs stimulated with 50 µM hydrocortisone (HC) and 100 µM dexamethasone (Dex). The control (ctrl) is the mean relative expression of the gene in non-stimulated cells (one control/fish) - set to one (n=3). * P< 0,05.*

The relative expression of MAO-A, HSP70, GRt1 and CTNNB1 was not significantly changed compared to the control. On the other hand, FkBP1 had a great increase in relative expression, with a 156-fold upregulation for dexamethasone 100 mM and 205-fold for hydrocortisone 50 µM – having a significant difference in expression compared to the control.

3.3.5 Relative expression of the antiviral gene ISG15 in RBCs stimulated with a combination of chronic stress hormones and acute stress hormones ex vivo

In vivo, there are usually both chronic and acute stress hormones working simultaneously when the individual experience stress. For that reason, we aimed to study responses to hormone combinations.

The poly (I:C) (50µg/mL) response of the antiviral gene ISG15 in Atlantic salmon RBCs pre-stimulated with both the chronic stress hormone hydrocortisone (50, 100 and 150µM) and the acute stress hormone agonist isoproterenol (10µM) was tested using RT-qPCR (Figure 38).

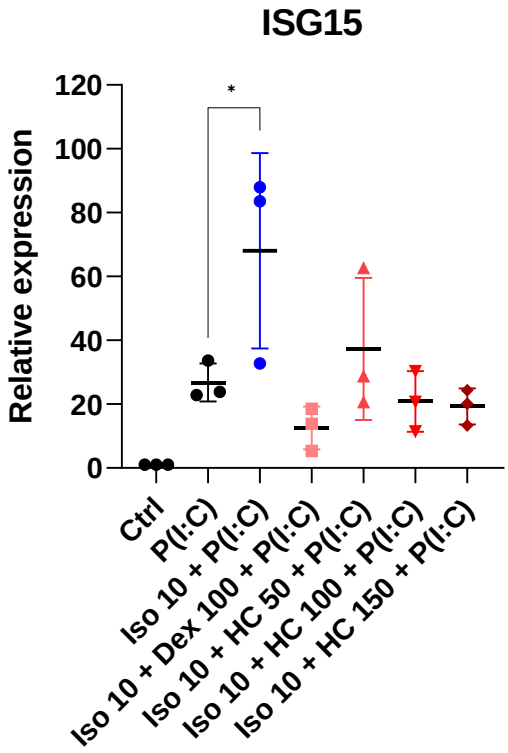


*Figure 38: Relative expression of ISG15 in Atlantic salmon n=3) RBCs stimulated with different concentrations of hydrocortisone (HC) (50 µM, 100 µM, and 150 µM), 10 µM isoproterenol (Iso) and 50µg/mL poly (I:C). Dexamethasone (Dex) 100 µM was used as a positive control. The control (ctrl) is the mean relative expression of the gene in non-stimulated cells (one control/fish) - set to one (n=3). * P ≤ 0.05*

In this experiment, we obtained a significant difference between the relative expression of ISG15 in cells stimulated only with poly (I:C) compared to cells stimulated with 10 µM isoproterenol and poly (I:C).

When the cells are stimulated both with isoproterenol and dexamethasone, the inducing effect of isoproterenol on the poly (I:C) response of ISG15 is lost compared to the cells only stimulated with isoproterenol.

3.3.6 Viability of RBCs stimulated with hydrocortisone ex vivo

Since hydrocortisone had not been properly tested on Atlantic salmon RBCs, the viability of cells stimulated with higher doses of hydrocortisone was checked. The viability was also checked in cells that had been stimulated with dexamethasone and isoproterenol. An example of the histogram given by the flow cytometer by counting living and dead cells is shown in figure 39.

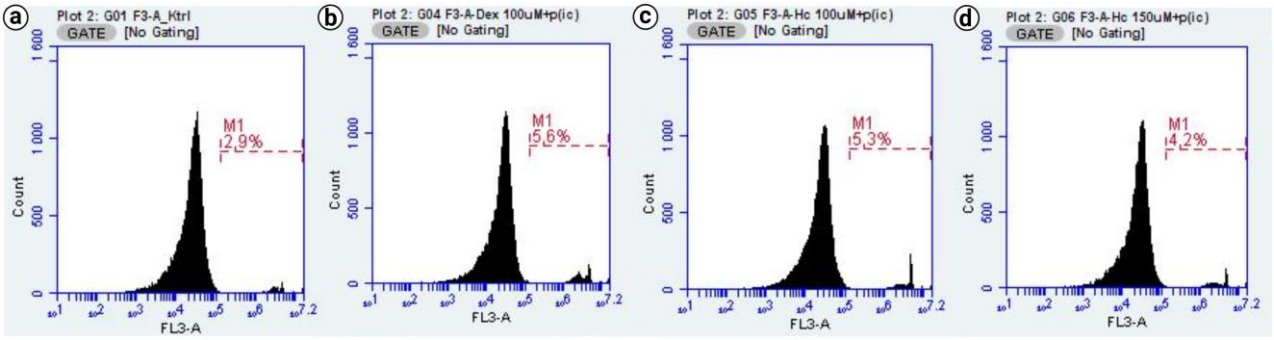


Figure 39: Representative histogram of the count of the living (left peak) and the dead RBC (right peak) from one experiment with different treatments. From left to right is the control (a), sample with cells stimulated with 100µM dexamethasone and 50µg/mL poly (I:C) (b), 100 µM hydrocortisone and Poly (I:C) (c), and lastly 150 µM hydrocortisone and Poly (I:C (d)). Out of the 20 000 cells counted in each sample, the percentage of dead cells were calculated, being 2,9%, 5,6%, 5,3% and 4,2 % respectively.

In the representative figure for one fish/experiment, the control-sample had 2,9% dead cells out of the total number of 20 000 cells counted, and the sample with cells stimulated with 100 µM dexamethasone had 5,6% dead cells. Cells stimulated with the higher doses of hydrocortisone and poly (I:C) had a percentage of dead cells at 5.3% and 4.3 % for 100 µM and 150 µM hydrocortisone respectively.

When taking the mean of the percentage of dead cells in all three fish/experiments for every stimulation (Figure 40), there was a decrease in viability, meaning more dead cells (%), for all groups tested except the cells stimulated with only poly (I:C). There was only a significant difference in viability between the control and the RBCs stimulated with 100 µM dexamethasone alone, and in combination with poly (I:C). The viability also had a tendency

for being higher for cells stimulated with isoproterenol in combination with hydrocortisone/dexamethasone compared to the same dose of hydrocortisone/dexamethasone alone.

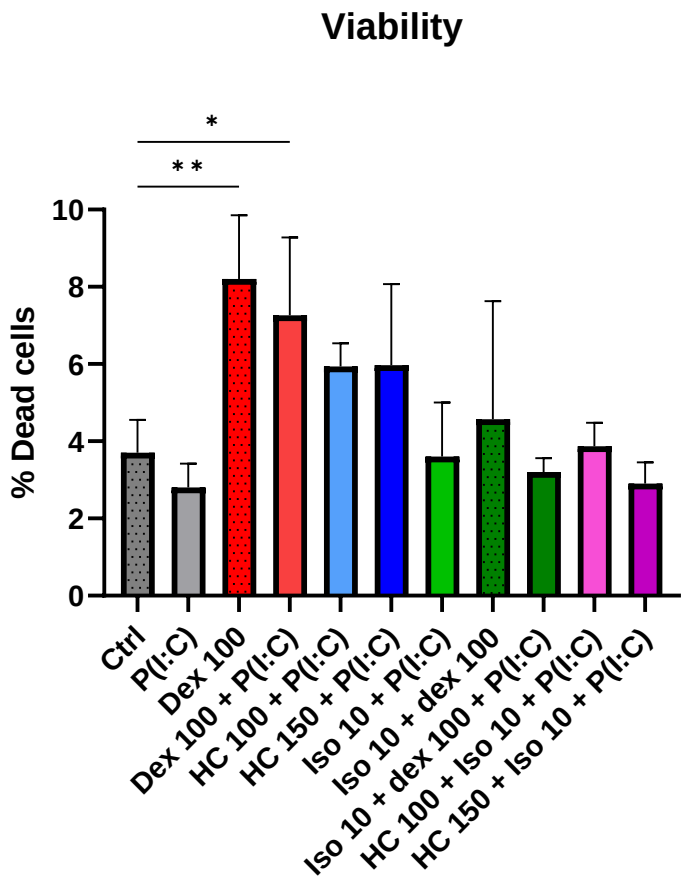


*Figure 40: The mean viability of the RBCs isolated from three Atlantic salmon (3 separate experiments) after stimulation with higher doses of hydrocortisone (100 and 150 µM), 50µg/mL poly (I:C), 10 µM isoproterenol and 100 µM dexamethasone. The control (ctrl) is the mean percentage of dead cells in non-stimulated cells (one control/fish or experiment) (n=3). * P ≤ 0.05, ** P ≤ 0.01*

3.4 Testing the expression of the potential chronic stress target gene FkBP1 in RBC samples from in vivo stress trial

Since FkBP1 showed some potential being a good stress target gene, it was decided to test the expression of the gene in the samples from the in vivo stress trial.

There were no significant difference in the relative expression of FkBP1 between the groups (Figure 41).

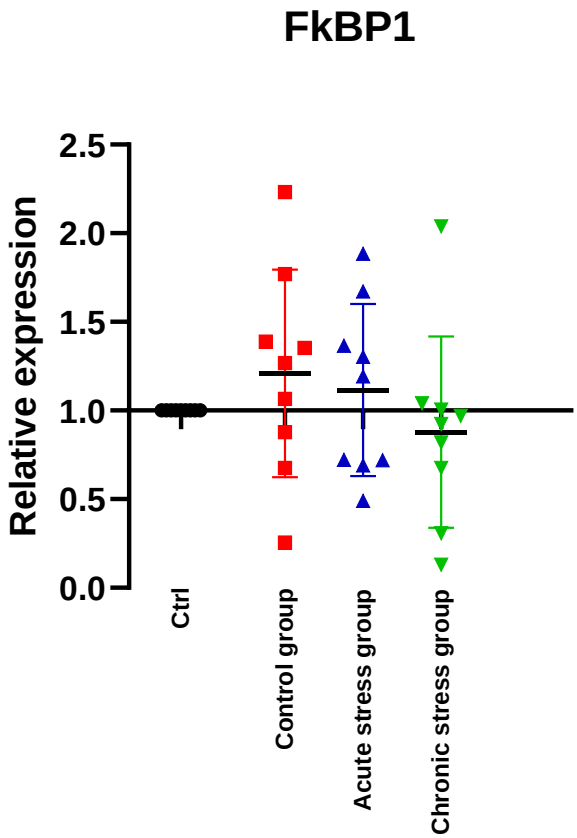


Figure 41: Relative expression of the potential chronic stress target gene FkBP1 in Atlantic salmon RBCs sampled on day one in the in vivo stress trial (three fish pr group, each in three parallel wells with different stimulation treatments: 1+3 days and 1+7 days incubation with 50µg/mL poly (I:C), and 1+7 days incubation with 0.5 µg/mL imiquimod). The control (ctrl) is the mean relative expression of the gene in non-stimulated cells (one control/fish and parallel) - set to one (n=9).

4 Discussion

The main goal of this thesis was to get a better understanding of how salmonid red blood cells respond to stress and infections. This thesis focuses largely on Atlantic salmon RBCs antiviral responses to "normal" handling stress and testing different potential stress-target genes to help monitor long term effects of stress.

4.1 Carrying out in vivo stress trials

The in vivo stress trial was carried out with permission from the Norwegian Food Authority (Mattilsynet) in accordance with the regulation of experimental animals (Forskrift om bruk av dyr i forsøk) (Lovdata, 2015). The purpose of this regulation is to limit the use of animals for scientific and educational purposes, promote good welfare and respect for animals used for such purposes, but also help to ensure that the animals are not subject to unnecessary stress loads. The regulation promotes the principle of "the three Rs". The first R is "Replacement", which means that one should consider methods that allow a given goal to be achieved without carrying out experiments or scientific procedures on animals. To be able to monitor the stress level in fish exposed to "natural" stress, it is hard to avoid using whole living animals. But other measures were done to reduce the number of individuals and to refine the methods used.

The second R is "Reduction", meaning that one should reduce the number of animals used in experiments as much as possible, but at the same time get satisfactory results. To find the sample size necessary to meet the desired statistical constraints, a sample size calculator was used. The main plan for this thesis was to have a pilot-trial, with enough fish (90 smaller fish) to get satisfactory results, but due to the fish that was supposed to be used got infected with salmon gill poxvirus (SGPV) and had to be euthanized, the number of fish was drastically reduced to 20 larger fish. This pilot was planned prior to a larger in vivo trial, in that way one could reduce the number of samplings and treatments, and therefore also the number of fish used.

The reduction of the number of fish meant that one had to downscale the in vivo trial originally planned. The initial plan was to have five sampling points, one sampling on day -3 (the day the trial started), another on day 0 (the last day of stress induction), and on day 1, 4 and 7 (1-, 4- and 7-days post stress induction for the acute and chronic stress group). With

five timepoints, vs two in the downscaled trial, one could get a better picture of the trend in hormone levels and the antiviral responses of the RBCs.

Six fish from each group at each timepoint were supposed to sampled, double the number of fish as the plan now was. This way the results were not as satisfactory or representative as originally was hoped for, as the results were unstable without many significant differences.

Initially, the sedative drug Aqui-S was also supposed to be used during sampling to reduce stress after the fish being controllingly exposed to stress. Disturbing effects of this was going to be tested after the first sampling timepoint. As the trial now only had two sampling timepoints, there was no possibility to test and use Aqui-S, and the stress the fish experienced during sampling could have had an impact on the results.

Lastly, the third R is “Refinement”, where one considers methods that reduce the suffering of the animals (NC3Rs, w.d). After the in vivo trial, ex vivo trials was done to replace living animals, and thereafter reducing animal suffering. The fish in the in vivo trial were also “only” exposed to normal handling stress that did not exceed the stress the fish experience in the industry. Even though experimental stress is not without strain and reduced welfare, the stress induction was short-lasting and in the range of normal handling. Mortality and harm was not expected, and if the fish for some reason got many wounds and injuries, the fish would be euthanized.

Knowledge from this smaller trial was still considered useful to help us find a way to imitate the responses with hormones in cell models, and in that way cell models may replace animal experiments.

4.2 Did the fish get long lasting changes in stress hormone levels?

The fish did not have long lasting significant changes in stress hormone levels, neither for cortisol nor adrenaline.

It was especially "obvious" that the fish in the chronic stress group adapted to being exposed to stress over the eight times they were handled in the experiment, as on the last day of stress inductions, the fish seemed sluggish and not bothered by the chasing. On the other hand, the fish in the acute stress group seemed more bothered and swam quickly away to avoid the hand-net during chasing. The cortisol concentration in blood plasma supported this "hypothesis" as the fish in the chronic stress group had a lower cortisol concentration than the fish in the acute stress group. It was only fish in the acute group that had a cortisol level above 100 ng/ml at the day 1 sampling, which we here considered as a threshold for a stress response.

The fish did not get properly acclimatized before the trial started, meaning the fish got transferred to the experiment tanks the same day as the trial started, without the use of any sedatives. This could mean that the fish already got stressed. The fact that the fish was "old", and may be used to being handled, the fish could have had a lower stress response than if one used younger and smaller fish that are not "used" to being handled. If one used more fish and had more sampling timepoints, one could possibly easier se the effect of not acclimatizing the fish prior to start of the trial.

To physiologically cope with prolonged exposure to stress, adaptive responses are initiated as prolonged or cumulative increases in cortisol secretion reduces benefits afforded by enhanced stress reactivity and will eventually become maladaptive. To keep the impact of stress in check, a process called habituation is initiated. This process reduces the HPI-axis responses when exposed to homotopic stressors (Herman, 2013) and is traditionally considered a nonassociative form of learning for conservation of energy and resources by dampening the responses to stressors that are not life threatening (Grissom & Bhatnagar, 2009). It seems like the physiological capacity of habituation in fish depends on the type of stressor, the severity and the species involved. Some data indicate that fish are able to habituate, like for instance Madaro et al. (2016) showed that Atlantic salmon had a reduced HPI response upon exposure to repeated stressor (5-min chasing, twice a day) for seven days. This is similar to what was

found in this thesis in vivo stress trial. A study done on the two costal fish species in the Bothnanian Sea Eurasian perch (*Perca fluviatilis*) and roach (*Rutilus rutilus*), also showed habituation in fish exposed to motorboat noise repeatedly for 11 days compared to the group only exposed to noise once for 30 min (Johansson et al., 2016). On the other hand, there are also studies showing that some species do not have the same ability to induce habituation, like for instance Senegalese sole (*Solea senegalensis*) exposed to short-term repeated chasing stress over the course of three days (Conde-Sieira et al., 2018).

In previous stress trials done by Fast et al (2008), the cortisol level after short-term acute stress (15s handling stress) had increased at 1 h after stress and remained elevated up to 3 h. By 6 h, the levels had returned to pre-stress levels. Another study also found that the highest values of cortisol in plasma were at 45 min post-acute stress (Madaro et al., 2023). Fast et al also found that long term stress over a 4-week period did not result in chronically elevated level of cortisol. This is the same with the result for this in vivo stress trial. The acute stress group had an elevated cortisol concentration in the plasma, whereas the chronic stress group had a lower concentration of cortisol, some even lower than the control. As cortisol release follows adrenalin release after acute stress, it is safe to say that the fish in the acute stress group was indeed stressed. The decrease of the cortisol level again at 4-days sampling shows that the fish was not chronically stressed. If one had as many fish and as many sampling timepoints as originally planned, one would get a better idea and understanding of the trend in stress hormone levels.

In this study, the cortisol concentration in the mucus was very low for all groups, compared to the concentration in blood plasma. Measurement of cortisol level in mucus could be a minimal/non-invasive method to assess stress, as it does not require wounding (as with blood sampling) or killing the fish. It is shown that skin mucus cortisol levels reflect acute stress responses in fish, and correlates positively to plasma cortisol levels (Bertotto et al., 2010; De Mercado et al., 2018). These studies shows that the cortisol levels in skin mucus increase significantly just in the moment of stress. Carbajal et al (2019) also showed that the cortisol levels in mucus decrease significantly at 24 hours compared to the cortisol concentration in blood plasma. In other words, skin mucus does not give long-lasting, measurable changes in cortisol levels after stress exposure, as also demonstrated in this thesis' results.

The adrenaline concentration in blood plasma indicates if the fish was stressed during sampling as the levels increase and decrease rapidly. In studies done by Skrynska et al (2018) on Gilthead Sea Beam (*Sparus aurata*) exposed to stress induced by air exposure for 3 minutes, it was shown that plasma catecholamines had a significant increase 15 minutes post-stress induction, returning to baseline values 30 minutes post-stress.

The adrenalin concentration for our samples was still quite low, at 1,8 nmol/L (0,337 ng/mL) for the fish with the highest concentration, compared to the levels measured in the study by Skrynska et al, where the fish had levels above 100 nmol/L. Another study (Floysand et al., 1992) also show plasma adrenalin levels above 200 nmol/L, where Atlantic salmon experienced stress in form of ±3 h period of pre-slaughter crowding and handling. After an 0.5 h period of the fish struggling out of the water led to even higher levels of adrenalin plasma at 480 nmol/L. This could mean that the sampling itself went smoothly, as the levels was so low. But it also means that the long-lasting effects of acute stress cannot be assessed by measuring adrenalin concentration in blood plasma.

A trend was that the first fish of each sampling of each tank had a lower adrenalin concentration than fish two and fish three. As adrenalin levels in the blood increases rapidly, one could imagine that the netting of the fish prior to sampling stressed the fish as one continued sampling. It also took some time for the fish to get euthanized as well. As a study has shown that repeated use of sedatives do not increase in cumulative stress or inflammation (Chance et al., 2018), one should consider using some sedative to calm down the fish prior to sampling and administer it as discreetly as possible, as originally planned in the larger trial. One should also use a higher dose of sedative to euthanize the fish as quickly as possible, ensuring better welfare for the fish.

As mentioned in the materials and methods section, it has been shown that fish exposed to acute stress have an elevated haemoglobin level. A study done by Lund et al (2017) on PRV-infected Atlantic salmon, showed that the haemoglobin levels dropped after infection. If one considers an infection as a stressor, catecholamines are secreted as a response. Adrenalin binds to adrenoreceptors, AC is activated, and cAMP is produced. This in turn activates Na^+/H^+ exchange, so that Na^+ enters the cell along its electrochemical gradient and H+ exits the cell. The pH and O_2 affinity increase rapidly, especially under hypoxic conditions. There

is a net uptake of Na^+ and Cl^-, followed by osmotically obliged water, causing the RBCs to swell and the haemoglobin level increases (F. B. Jensen, 2004).

This was not the case for the fish in the in vivo stress trial, as the haemoglobin levels did not significantly increase for the fish in the acute stress group. Measuring haemoglobin level is therefore not a good method for assessing the long-lasting effects of stress.

4.3 Can stress in vivo inhibit or enhance the antiviral response in Atlantic salmon RBCs?

The expression of the antiviral genes ISG15 and MX1 in response to poly (I:C) and imiquimod was tested in RBCs sampled one day post stress induction, as this were the samples with the highest cortisol-levels.

The fish in the acute stress group had a cortisol level above 100 ng/mL, which is considered as elevated (Gamperl et al., 1994). The cortisol levels in this group was also higher than the control group, but the chronic group had a mean cortisol level lower than the control group. By comparing the levels of cortisol in blood plasma from fish in the chronic group to fish in the acute group, it is easier to understand the results from the qPCR. Based on the background information about cortisol, one knows that this constant release of this hormone typically leads to inhibition of immune responses.

But due to the low and unstable hormone levels upon measurement, it is overall difficult to conclude the effects of stress in vivo on antiviral responses ex vivo. As the cortisol level of the acute stress group decreased at 4-days sampling, one could say that the fish were not chronically stressed.

As poly (I:C), which mimics an infection with dsRNA-virus, binds to the endosomal TLR3 and initiates expression of different antiviral genes through signalling pathways, it is expected to see a change in the expression of these genes when cells are exposed to this substance. As the chronic stress group, with the lowest measured cortisol levels in blood plasma, did have the highest relative expression of ISG15, and the acute stress group had a lower expression in response to a 3-days stimulation of poly (I:C), one could hypothesize that higher cortisol-levels results in inhibition of antiviral responses. The effect after a 7-days stimulation was not significant. The standard deviation between the fish in the control group and the acute stress

group was quite high, and this makes it difficult to make any conclusions for this stimulation. More replicates are needed for more representative results. This is the same for the expression of Ma1.

The cells stimulated with imiquimod, which mimics an infection with a ssRNA virus, for 7 days did not upregulate neither ISG15 nor Mx1. Imiquimod is therefor probably not going to be used in later trials. Imiquimod 0.5 µM was decided to be used in this trial, as it had shown (in another pilot trial done prior to this one) to have a slight effect on the antiviral responses in RBCs after 7-days stimulation and giving the highest expression of antiviral genes at this concentration. The agonist has shown to have potent antiviral activity in both human and Atlantic salmon, as it for instance can induce both Mx1 and ISG15 in salmon – supporting the presence of a TLR7-like receptor in teleost fish liver and head kidney (Kileng et al., 2008). TLR7 mRNA has also been found in RNA sequences of Atlantic salmon RBCs, but in rather low amounts (6-8 transcripts). Given the results from this trial, one could argue that imiquimod is not a good target for stimulating antiviral responses by its binding to TLR7 in RBCs. It could be that the fish has a different variant of TLR7 compared to mammals, that do react more strongly to imiquimod.

After the RBCs was isolated, the cells were incubated in L-15 medium to ensure that the cells went in a resting (basal) state. One also expect hormones from plasma to be washed away from the cells. This would isolate the effects on RBC that were long-lasting beyond the effect on present hormones. The effects that were studied were lost during the three days of absence of stress hormones, and thereby cannot be considered very long-lasting.

4.4 Does ex vivo stimulation with the acute stress hormones isoproterenol and epinephrine change the antiviral response in RBCs?

The antiviral responses to poly (I:C) in Atlantic salmon RBCs pre-stimulated with the acute stress hormones isoproterenol (synthetic) and epinephrine were tested. For this, the expression of the antiviral genes ISG15, MX1 and IRF3 were analysed by RT-qPCR.

According to the literature, acute stress often results in an enhancement of immune functions. For instance, a study done on mice showed an upregulation of immune genes in the group exposed to 6 hours of restraint stress compared to the non-stressed control group (Tang et al., 2022). For both isoproterenol and epinephrine, there was an increase in the poly (I:C) mediated expression of ISG15 with increasing concentrations of the agonist. But for the higher doses of isoproterenol, the effect is lost – making the higher doses not as effective or having the opposite effect. A cell will not have an infinite number of receptors for the agonist/hormone, and a higher dose of the agonist/hormone does not necessarily mean greater effects.

It has been shown that β-adrenergic receptors respond very potently to isoproterenol and are less sensitive to epinephrine (Hayward et al., 2004). The results of the relative expression of ISG15 can indicate this as well, as cells stimulated with isoproterenol had the highest relative effect for some concentrations. Either way, since epinephrine did give an effect, one can use the hormone in ex vivo experiments. Epinephrine is cheaper, and better to use since it is the physiological acute stress hormone – and isoproterenol is just a synthetic agonist that bind to the same β-adrenergic receptor.

4.5 Can we identify any acute-stress target genes?

In searching for biomarkers, in this case stress hormone target genes, one can identify the secondary effect of stress. As the hormone levels in vivo did not seem to be long-lasting, it would be useful to find other long-lasting markers of stress.

The expression of potential acute stress target genes like SOCS3, HSP90aa and Cahz in RBCs were tested after stimulation with the acute stress hormones isoproterenol (synthetic) and epinephrine.

There was a significantly upregulation of Cahz in response to isoproterenol, with a slight relative induction of 1,5. High doses of epinephrine slightly upregulated HSP90aa and downregulated SOCS3.

As mentioned in the result section, carbonic anhydrase, or Cahz, secures a rapid CO2 hydration and RBC acidification in tissue capillaries (Geers & Gros, 2000; F. B. Jensen, 2004). When the fish are exposed to acute stress, fight and flight responses are activated, leading to heavy exercise and often critical low capillary PO_2 and lactic acidosis in muscle capillary blood which further increases O_2 dissociation and facilitates O_2 delivery. As the RBCs were studied ex vivo, outside the living organism itself, one could argue that the Cahz response was low, as the red blood cells were not exposed to such low PO_2 or lactic acidosis and was not needed for speeding up a $CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$ reaction to ensure an equilibrium balance in acid-base levels (homeostasis). If one considers this, the gene could potentially be a biomarker for acute stress in vivo, but further testing needs to be done.

As for HSP90aa, a heat shock protein, it is shown that the gene is upregulated in times of stress in other cells than RBCs, including Atlantic salmon hepatocytes (Beemelmanns et al., 2021). HSPs are involved in protection from cellular stress as it prevents damage associated with uncontrolled protein unfolding. In Beemelmanns study, the fish were exposed to heat shock and hypoxia, where the temperature was increased from 12 to 20 °C, increasing it 1°C per week, and a decrease of air saturation from 100% to 70%. With this method, one could argue that the fish was exposed to chronic stress rather than acute stress, but the cortisol levels were not measured, so one cannot say anything for sure. Another study, also done by

Beemelmanns et al. but not published, have shown that Atlantic salmon exposed to an incremental temperature elevation to 22°C did have an increase in plasma cortisol levels. A suggestion would therefore be to test the expression of this gene when RBCs are stimulated with hydrocortisone, a chronic stress hormone.

Further, SOCS3, or suppressor of cytokine signaling-3, is important in regulating cytokine-activated JAK/STAT signalling pathways as it acts as a negative feedback signal inhibiting JAK/STAT activation and phosphorylation (Wunderlich et al., 2013), just as mentioned in the results section. Studies have shown that expression of SOCS3 in human adipocytes is stimulated after exposure to isoproterenol, indicating that this gene is stimulated by β-adrenergic agents via activation of G_S-protein-adenylyl cyclase dependent pathway as the increase in expression was blocked after pre-treatment β-adrenergic receptor antagonist (Fasshauer et al., 2002). The explanation of the low SOCS3 expression could be as easy as Atlantic salmon RBCs β-adrenergic signalling does not stimulate the SOCS3 expression in the same way either due to differences between human and salmon gene regulation, or differences in transcription factors between different cell types.

4.6 Does ex vivo stimulation with the chronic stress hormone hydrocortisone change the antiviral response in RBCs?

The antiviral responses to poly (I:C) in RBCs from three Atlantic salmon stimulated with different concentrations of the chronic stress hormone hydrocortisone were tested by measuring the expression of the antiviral genes ISG15, MX1 and IRF3 doing RT-qPCR. The results show an inhibition of immune responses as cells are exposed to hydrocortisone. This also agrees with the literature.

During chronic stress, caused by a constant release of glucocorticoids, the individual experience an allostatic overload with a maladaptation that cannot be compensated. Cortisol is originally anti-inflammatory which contains the immune response, but chronic elevations can lead to the immune system to be “resistant”. An accumulation of the stress hormone leads to an increase in inflammatory cytokines – giving a systematic inflammation. This represents dysregulation of the immune system and increases the risk of infections and diseases (Morey et al., 2015).

4.7 Can we identify any chronic-stress target genes?

Out of the five potential stress target genes tested in RBCs stimulated with the chronic stress hormone hydrocortisone, only one of them had a significant difference in relative expression compared to the control – the FkBP1 gene.

4.7.1 The potential of FkBP1 being a good chronic stress biomarker

The FkbP1gene, or FkBP prolyl isomerase 1, has shown to be a reliable marker of glucocorticoid receptor (GR) activation in human/mammalian peripheral blood cells. In these cells, the protein this gene encodes works as a heat shock protein 90 associated co-chaperone of the GR and its expression is induced by glucocorticoids via intronic steroid response elements as part of an intracellular short negative feedback loop for the GR activity. Meaning, when there are high enough concentrations of cortisol that binds to GR, the activity of the receptor is inhibited as a result of negative feedback loop to ensure stability and homeostasis, the FkBP1 gene expression increases (Menke et al., 2012). As this ex vivo trial with hydrocortisone stimulation of Atlantic salmon RBCs shows a great increase in the expression of this gene, one could say that the gene also is a reliable marker for the GR activation in these cells.

The protein encodes is a member of the immunophilin protein family in mammalian/human cells, which plays an important role in the immunoregulation and basic cellular processes involving protein folding and trafficking. The protein binds to immunosuppressants like FK506 and rapamycin (NIH, 2023).

FK506 works as a immunosuppressant through disruption of signalling events mediated by the calcium-dependent serine/threonine protein phosphatase, calcineurin (CaN) (Dumont, 2000). CaN activates the nuclear factor of activated T cells (NFAT) family of transcription factors. This Ca^{2+}-CaN-NFAT-signalling pathway is important for regulating several T—cell functions, like initiating the expression of cytokines, chemokines and their receptors, and master regulators for T helper-cell differentiation (Mencarelli et al., 2018). Rapamycin also affect T-cells by inhibiting IL-2-dependent T-cell proliferation (Wiederrecht et al., 1995).

An upregulation of this gene indicates that chronic stress suppress immune functions of Atlantic salmon RBCs. The antiviral gene ISG15 was also downregulated for RBCs stimulated with hydrocortisone and poly (I:C) compared to the control stimulated only with

poly(I:C). Both of these findings reinforces the hypothesis that chronic stress supress the immune functions in RBCs.

FkBP1 expression in samples from the in vivo stress trial was not significant. Even though the difference in expression was not significant compared to the control, there still is an upregulation of the gene for the fish in the acute stress treatment group sampled one day post last stress induction. This was the group with the highest cortisol level. The chronic stress response was not that high in the in vivo stress trial, but nevertheless, the gene still shows some potential being a good biomarker for chronic stress.

4.7.2 Alternative biomarker genes

The other four genes tested, MAO-A, HSP70, CTNNB1 and GRt1, did not show as good potential for being a biomarker for chronic stress as the FkBP1 gene

As mentioned in the results section, MAO-A, or monoamine oxidase A, is a mitochondrial enzyme involved in the catabolism of catecholamines, including norepinephrine (X. Sun et al., 2020). The gene is expressed in many cells in mammals, and it is shown that glucocorticoid receptors are needed for dexamethasone induced MAO-A expression targeting (Manoli et al., 2005; Puhr et al., 2021). The low expression of this gene in Atlantic salmon RBCs shows that this potentially does not has to be the case for these types of cells.

The heat shock protein, HSP70, which functions the same way as HSP90aa tested in the acute stress ex vivo trial, also shows an upregulation in in Atlantic salmon hepatocytes when exposed to warm and hypoxic conditions (Beemelmanns et al., 2021). Another study done on rainbow trout (*Onchorhynchus mykiss*) also shows an upregulation of this gene with increasing cortisol levels with increasing stocking density (Yarahmadi et al., 2016). Here again, the reason for the low expression could as easily be that the gene in RBCs of Atlantic salmon are not easily affected by chronic stress.

CTNNB1 is a gene encoding a protein called beta-catenin that plays an important role in cell signalling and regulation of cellular homeostasis (Zhuang et al., 2023). This gene has shown to be upregulated in peripheral blood cells from medical students exposed to chronic psychological stress (Kawai et al., 2007). As for MAO-A and HSP70, the expression of this gene is not easily affected by chronic stress hormones.

GRt1, glucocorticoid receptor-like transcript variant X1, is a receptor that cortisol/dexamethasone binds to, initiating chronic stress responses. When the cells are exposed to agonists over a longer time period, the cells could upregulate the gene that encodes the receptor for that agonist (Melroy-Greif et al., 2016). The expression GRt1 that encodes a glucocorticoid receptor, did not increase. On that note, one can suggest that increase in glucocorticoids does not increase the number of glucocorticoid receptors in the cell, and therefore the gene is not a good biomarker for chronic stress. One could test higher concentrations of hydrocortisone/dexamethasone over a longer time period, just to make sure.

4.8 Does stimulation with acute and chronic stress hormones effect the viability of the red blood cells?

The viability of the cells decreased when stimulated with a chronic stress hormone, but the viability was rescued when the cells were stimulated with both an acute and chronic stress hormone. Isoproterenol alone did not affect the viability in any significant way.

Cells can respond to stress in different ways ranging from the activation of survival pathways to the initiation of cell death resulting in elimination of damaged cells. The initial response to stressful stimulus is to help the cell defend itself against and recover from the insult, for instance, increase heat shock responses to mediate an increase in chaperone protein activity which enhances the protein folding capacity of the cell. This counteracts the stress impacts and promote cell survival. If the mechanisms of different defences and pro-survival strategies are unsuccessful, then cell death programs are activated to eliminate the damaged cells (Fulda et al., 2010). This could be the reason for the decrease in viability for cells stimulated with a chronic stress hormone, whereas the acute stress stimulated cells still were protected against the impact of stress. Even though the expression of HSP90aa did not increase for the cells stimulated with isoproterenol, there are still other HSP genes that could have been upregulated. It could seem like acute stress hormones counteracts the chronic stress hormone’s ability to activate programmed cell death, as the viability is rescued.

4.9 What happens when one stimulates RBCs with both acute and chronic stress hormones?

When stimulating RBCs with both with the acute stress agonist isoproterenol and the chronic stress hormone hydrocortisone, relevant since the hormones occur in the blood at the same time physiologically, one sees that the poly (I:C)-mediated gene expression of ISG15 is inhibited compared to the expression in cells only stimulated with isoproterenol. Thus, cortisol has a stronger impact on the antiviral response than isoproterenol, meaning chronic stress responses beat acute stress responses in this setting. From this we can hypothesize that when the fish are chronically stressed with a continuous release of cortisol, the fish will have an inhibition of antiviral responses in red blood cells even when exposed to acute stress hormones that are expected to enhance antiviral responses.

4.10 Limitations of the methods used

Generally, for all the analysis done in this thesis, there are only a limited number of replicates used (n=3). To get a more representative result, more individuals are needed, as the low number of replicates resulted in large standard deviations and no significant differences between the controls and the stimulated groups. The result in this thesis still gives an indicator of future results what to focus on in future trials, and what to include and what not to include – for instance that imiquimod did not give a response in the antiviral genes and is not necessary to use again in later trials.

The relative expression of antiviral genes and potential stress target genes was only quantified by the use of RT-qPCR. This method gives a good indicator whether the genes are upregulated or downregulated, but it still has its limitations. Even though the genes are transcribed to mRNA, it does not necessarily mean that mRNA is translated to protein. RT-qPCR does not say anything about the amount of end-product (protein), and the regulation of the process from mRNA to protein and the variable protein half-life makes it that is not a 100% correlation between gene expression and protein expression (Y. S. Liu et al., 2016). For further work, proteomic analysis could be used to get a better understanding of the actual protein expression

RT-qPCR generates results of high level of sensitivity and specificity, making it vulnerable to significant variance within the datasets. Small differences could make great impact as the target sequence is two-fold amplified. One typically analyses differences in fold change between a control group and a treated group, and that is the reason to at least be aware of this.

There are also a possible downside of using the Livak and Schmittgen $2^{-\Delta\Delta Ct}$ method as it assumes a 100% primer efficiency (amplification equal to 2) for both primer pairs of the gene of interest and the reference gene. This seems to almost never be the case (Pfaffl, 2001).

Overall, errors will occur in any experimental setup – and will lead to significant variation within the dataset. Good planning and a well-designed experimental setup will try to minimise systemic errors, but, even then, random errors will occur and are difficult to exclude.

4.11 Future work

The fish in Norwegian aquaculture are exposed to a lot of stress, everything from pumping, handling, vaccination, delousing treatments, crowding and suboptimal water quality. As chronic stress inhibits immune responses, making the fish more suspectable to diseases, it would be great to be able to take low invasive blood samples in the field to monitor the stress level. If the blood samples shows that the fish already are chronic stressed, the fish health professionals could advice the owners to avoid expose the fish to additional stressors and just simply let the fish be alone for a period of time, as the fish would more likely get infected with other pathogens post handling.

As shown in this thesis, hormone measurements are not stable long-lasting stress markers, and to get more understanding of stress responses, and by potentially finding long-lasting stress biomarkers, one can help fish health professionals and owners to get a better indication of the fish health and welfare at the location, as stress often is a good indicator of whether the welfare is good or not. This could potentially give a “heads up” for upcoming incidents for increased mortality, and in that way the owners could intervene earlier to avoid this happening. Results from this thesis is showing that the FkBP1 gene has some potential to being a good chronic stress biomarker, making this reality more likely.

The results from the in vivo trial shows how difficult it could be to get stable results and shows how important it is to have enough replicates to get more representative results. Overall, it seems like the stressor our fish were exposed to was not strong enough, or not unexpected enough, to stress the fish. This leading to habituation for the chronic stress group. There are other methods one can use to run stress trials, and maybe one should consider using stronger stressors to avoid habituation. The stressors the fish are exposed to in the field are harsher than what we exposed our fish to in the in vivo trial, for instance the thermal delousing method explained in the introduction and extreme crowding. These are stressors that could be tested further. One could also consider doing experiments in the field by taking blood samples of the fish both prior handling and post handling. For instance, check the cortisol level of fish some time before a vaccination, straight after the fish have been vaccinated, and then days and weeks after, as a vaccination includes pumping, crowding, and a puncture with a needle.

One should consider optimizing sampling methods. As a pilot prior to the in vivo trial showed that it is possible to isolate red blood cells from smaller fish (less than 100 g), and since one only needs small amounts of blood (250µL), the sampling can potentially be completed with no lethal outcome in larger fish. Through this, one has the possibility to sample the same fish several times over a longer period, and potentially making the results more informative and reliable. Other blood sampling methods other than drawing it from the caudal vein has been used in other studies. For instance, one can take blood directly from the heart ventricle from anaesthetised fish (Witeska et al., 2022). But this method would not be repeatable, and one could also argue if this is in terms of good welfare.

If one also could get a device that could test cortisol levels and gene expression of chronic stress biomarkers, which is easy to use this in the field, it would be easier for the owners to access the fish welfare without having to first draw blood, then send it to a laboratory for further analysis – which could take days until one gets the results back. For this, better training and routines for blood sampling is needed, in that way fish health professionals don’t need to come in for sampling every time. For instance, if one could take blood samples while the fish are also being counted for lice.

By doing ex vivo stimulations, one avoids experimenting on whole living animals, which is good for the fish welfare overall, but one also get to see the effects of the hormones in a more controlled system and thus obtain more stable results. Not only did the stimulations indicate that chronic stress leads to inhibition of antiviral responses, and the opposite for acute stress, but it also showed that the cell viability decrease with chronic stress hormones, and the viability is rescued in the presence of acute stress hormones. One could test other genes that are known to be regulated by cortisol in cells prone to cortisol induced apoptosis/cell death. In human and mice, the NFkB inhibitor α (I*κ*B-*α*), FkBP51 and BCL2-like 11 (apoptosis facilitator) (BIM) are few of many genes showing some potential being good chronic stress biomarkers (Scmidt et al., 2004) and are expressed in Atlantic salmon RBCs according to the unpublished RNASeq data in the RED FLAG group.

As this thesis have indicated, Atlantic salmon RBCs have many interesting functions other than just carrying and delivering oxygen. Their immunological properties are interesting to study, and as these types of cells are the most abundant in the body, they are definitely worth looking more into.

5 Conclusion

- A downscaled pilot prior to the in vivo trial showed that it is possible to isolate red blood cells from small amounts of blood (250µl), potentially making it easier to monitor the fish health in the field with further research.

- From the in vivo trial, there was a trend towards increased cortisol levels for the acute stress group, but not the chronic stress group. The fish in the chronic stress group seemed to be adapted to the handling. The adrenalin level increased for each fish sampled, indicating that the fish got more stressed as sampling continued with more chasing of the fish when trying to catch it.

- Hydrocortisone and epinephrine regulated the poly (I:C) response in Atlantic salmon RBCs in an opposite manner ex vivo, hydrocortisone inhibited the response and epinephrine enhanced the response. This was in line with the results from stimulation

with isoproterenol and dexamethasone tested earlier. When stress was induced in vivo, this effect could not be confirmed as the hormone levels were too low upon measurement.

- When combining acute and chronic stress hormones ex vivo, the chronic stress response in the RBC from Atlantic salmon was stronger, as the antiviral responses still were inhibited even though it is shown that acute stress hormones alone enhance these responses.

- Ex vivo stimulation of Atlantic salmon RBC with the acute stress hormone epinephrine gave a slight, but significant, upregulation of HSP90aa, a heat shock protein important in repairing damages to the cell caused by stress. Isoproterenol also gave a slight upregulation of Cahz, an enzyme that primarily function to ensure acid-base homeostasis in times of acute stress.

- Stimulation of Atlantic salmon RBCs with the chronic stress hormone hydrocortisone gave an upregulation of the gene FkBP1 ex vivo. This gene encodes a factor that may inhibit antiviral functions.

- The chronic stress hormone hydrocortisone and dexamethasone reduced viability of RBCs, indicating that chronic stress can activate programmed cell death. When hydrocortisone was combined with isoproterenol viability was rescued.

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Appendix

A. Isolation and culturing in red blood cells from Atlantic salmon

Atlantic salmon is euthanized by benzokain treatment (1/1000 from stock), and blood is sampled from the caudal vain into heparinized blood tubes.

Material

- Sterile DPBS buffer (10x)
- Sterile DPBS buffer (1x)
- Sterile dH_2O
- Percoll
- 15mL sterile tubes
- 1,5 mL sterile Eppendorf tubes
- Pipettes and tips (10µL, 200µL and 1000µL)
- L-15 growth medium; supplemented with 2% FCS, 50 µg/mL gentamicin
- Cell counter, counting slides & trypan blue
- Orbital shaker
- 12-well plates
- 500mL glass sterile bottle
- Ice in a styropor box

Method

Notes:

- Always keep samples and reagents **on ice**
- Prepare **Percoll solution 51%** in a sterile glass bottle and store it wrapped with aluminum foil in the fridge
  For 100mL: 51mL percoll, 10mL 10x PBS, 39mL dH2O
  7,5 mL percoll per fish: 7.5 18= 135 mL per day of sampling (go for 300 mL- 2 days plus for some extra)*
  For 350mL percoll 51%:
  - 178,5mL percoll
  - 35mL 10x PBS
  - 136,5mL dH_2O

1. Add 20µL blood directly in lysis buffer.
2. Centrifuge 500µL blood at 300g/5 min/4°C. Collect the plasma and the blood pellet for further analysis
3. Dilute 500µL blood in 10mL dPBS (1:20) in a 15 mL tube
4. For each fish, prepare gradient in two-15 mL tube (**7,5 mL percoll – 5 mL diluted blood)**
5. Centrifuge 500g/20 min/4°C. Remember **low break on the centrifuge** (accel. 7 and break 0-1)
6. Evaluate the gradient. White cell band should be visible and without RBC. (May need to repeat steps #2-4 in a new gradient)

7. Collect white cells in 1.5mL Eppendorf tubes and centrifuge at 500g/10 min/4°C

Note: White cells will be washed twice with dPBS. After second washing, remove dPBS and add 400uL lysis buffer

8. Pipette off percoll and keep the RBC from the bottom of the gradient.
9. Resuspend RBC pellet with 5mL dPBS. Transfer the suspensions of the same fish in a new 50mL tube and add 20mL dPBS (30mL total amount in new tubes)
10. 1st wash: Centrifuge 500g/10 min/4°C (accel.9 and break 9)
11. Pipette off supernatant carefully
12. Resuspend RBC in 30mL dPBS
13. Sample 10µL of the cell suspension for counting. Dilute it with 90µL dPBS (1:10 dilution)
14. Count cells using countess: 10µL trypan blue and 10µL of 1:10 dilution; 10µL of the mix are placed in the countess slide for counting

Tabel 1: How to calculate the volume of L15 to add for a total of 20 mill RBCs/mL.

	Cells total	mL L15 medium to add for 20 mill/mL	Viability/other notes telletall
mal	**A= n x 30 x 10**	**A : 20 = B mL medium**	**n = count of living cells from Countess**
Example	330 mills	16.5 mL	$1.1*10^6$ / 89% viable cells
F			
F			
F			

15. 2nd wash: Centrifuge 500g/10 min/4°C (accel.9 and break 9)

A third wash may be necessary. Cells should not attach too strongly to the plastic.

16. Pipette off supernatant carefully
17. Resuspend the pellet in L-15 medium, 2% FCS, 50 µg/mL gentamicin to 20 mill/mL
    (Gentamicin 50ug/mL: 500mL L-15, 500uL gentamicin)
    (2%FCS: 500mL L-15, 10mL FCS)
18. Add 1 mL/well in a 12- well plate per fish
19. Incubate in a shaker overnight at 15°C and speed ~80 rpm.

B. Plate setups for stimulation of RBCs with poly (I:C) and imiquimod in samples from in vivo stress trial

Table 2: Plate setup for 3 days post stimulation harvest of Atlantic salmon RBCs. Different colour corresponds to different biological replicate (3) for each group 1 day and 4 days post stress. The control, with no agonist, to the right and 50 µg/mL poly (I:C) to the left

Fish 1 – no agonist	Fish 1 - Poly I:C 50µg/mL		
Fish 2 – no agonist	Fish 2 - Poly I:C 50µg/mL		
Fish 3 – no agonist	Fish 3 - Poly I:C 50µg/mL		

Table 3: Plate setup for 7 days post stimulation harvest of Atlantic salmon RBCs. Different colour corresponds to different biological replicate (3) for each group 1 day and 4 days post stress. The control, with no agonist, to the right, 50 µg/mL poly (I:C) in the middle and 0.5 to the left µg/mL to the left.

Fish 1 – no agonist	Poly I:C 50µg/mL	Imiq. 0.5 µg/mL	
Fish 2 – no agonist	Poly I:C 50µg/mL	Imiq. 0.5 µg/mL	
Fish 3 – no agonist	Poly I:C 50µg/mL	Imiq. 0.5 µg/mL	

C. Plate setups for stimulation of RBCs with acute stress hormones and poly (I:C) in ex vivo trial

Table 4: Plate setup for stimulation of Atlantic salmon RBCs with different concentrations of isoproterenol (iso) and 50 µg/mL poly (I:C). Poly (I:C) was added 24 h post stimulation with isoproterenol. 100 µM DMSO was used as a negative control.

DMSO 100 µM	DMSO 100 µM + poly(I:C) 50 µg/mL	Iso 1 µM	Iso 1 µM + poly(I:C) 50 µg/mL
Iso 5 µM	Iso 10 µM	Iso 50 µM	Iso 100 µM
Iso 5 µM + poly(I:C) 50 µg/mL	Iso 10 µM + poly(I:C) 50 µg/mL	Iso 50 µM + poly(I:C) 50 µg/mL	Iso 100 µM + poly(I:C) 50 µg/mL

Table 5: Plate setup for stimulation of Atlantic salmon RBCs with different concentrations of epinephrine (epi) and 50 µg/mL poly (I:C). Poly (I:C) was added 24 h post stimulation with isoproterenol. 100 µM DMSO was used as a negative control.

DMSO 100 µM	DMSO 100 µM + poly(I:C) 50 µg/mL	Epin. 1 µM	Epin. 1 µM + poly(I:C) 50 µg/mL
Epin 5 µM	Epin 10 µM	Epin 50 µM	Epin 100 µM
Epin 5 µM + poly(I:C) 50 µg/mL	Epin 10 µM + poly(I:C) 50 µg/mL	Epin 50 µM + poly(I:C) 50 µg/mL	Epin 100 µM + poly(I:C) 50 µg/mL

D. Plate setups for stimulation of RBCs with chronic stress hormones and poly (I:C) in ex vivo trial

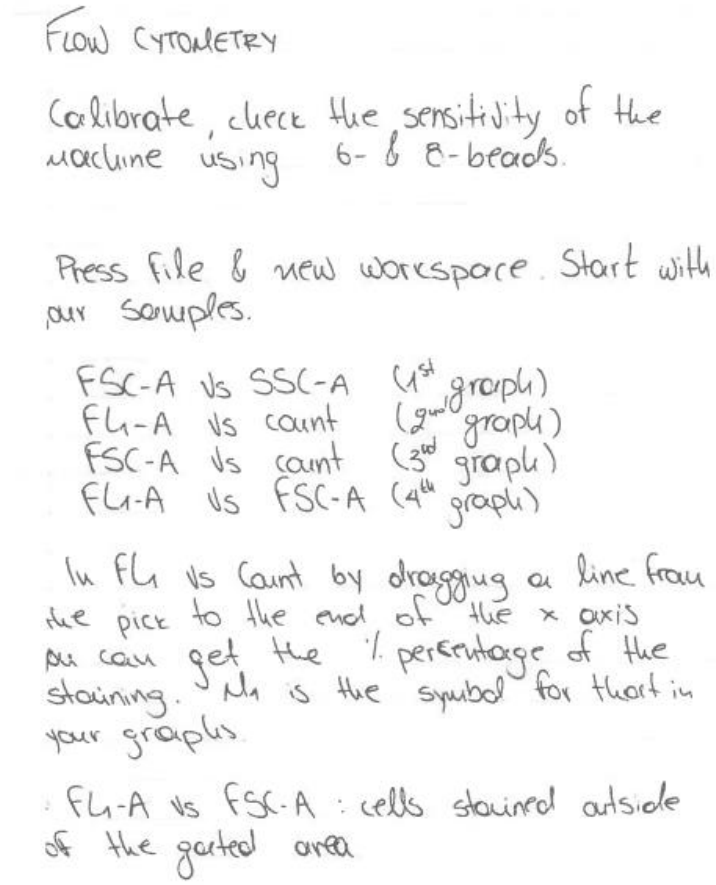
Table 6: Plate setup for stimulation of Atlantic salmon RBCs with different concentrations of hydrocortisone (HC), with a blank as a negative control and 100 µM dexamethasone (dex) as a positive control. Poly (I:C) was added 24 h post stimulation with hydrocortisone and dexamethasone.

	+ poly(I:C)	Dex 100 µM	Dex 100 µM + poly(I:C)
HC 20 µM	HC 50 µM	HC 100 µM	HC 150 µM
HC 20 µM + poly(I:C)	HC 50 µM + poly(I:C)	HC 100 µM + poly(I:C)	HC 150 µM + poly(I:C)

Table 7: Plate setup for stimulation of Atlantic salmon RBCs with different concentrations of hydrocortisone (HC) in combination with 10 µM isoproterenol and 50 µg/mL poly (I:C). 100 µM DMSO was used as a negative control, and 100 µM dexamethasone (dex) was used as a positive control. Poly (I:C) was added 24 h post stimulation with hydrocortisone, dexamethasone, and isoproterenol.

DMSO 100 µM	DMSO µM + poly(I:C)	ISO 10 µM	ISO 10 µM + poly(I:C)
ISO 10 µM + Dex 100 µM	ISO 10 µM + HC 50 µM	ISO 10 µM + HC 100 µM	ISO 10 µM + HC 150 µM
ISO 10 µM + Dex 100 µM + poly(I:C)	ISO 10 µM + HC 50 µM + poly(I:C)	ISO 10 µM + HC 100 µM + poly(I:C)	ISO 10 µM + HC 150 µM + poly(I:C)

E. Flow cytometry



FLOW CYTOMETRY

Calibrate, check the sensitivity of the machine using 6- & 8-beads.

Press file & new workspace. Start with our samples.

FSC-A Vs SSC-A (1st graph)
FL1-A Vs count (2nd graph)
FSC-A Vs count (3rd graph)
FL1-A Vs FSC-A (4th graph)

In FL1 Vs Count by dragging a line from the pick to the end of the x axis we can get the % percentage of the staining. M1 is the symbol for that in your graphs.

FL1-A Vs FSC-A : cells stained outside of the gated area

Figur 1: How to perform flow cytometry using BC Accuri C6 Plus Personal Flow Cytometer. Notes from Thomais Tsoulia.

F. Example of raw data from RT qPCR and the calculations of relative expression

Tabel 8: Example of raw data from RT qPCR done on Atlantic salmon RBCs from the in vivo stress trial. This is data from expression of Mx1 corrected against the reference gene Ef1-α in RBCs from one fish in the control group (not stressed) at one day sampling (111) stimulated with poly (I:C) (P) for 3 and 7 days and imiquimod (I) for 7 days. The expression in non-stimulated cells are also showed (K). Every stimulation was run in duplicates, and the mean Cq was calculated. dCt was calculated by taking Cq of the target and subtracting it by the Cq of the reference gene. Further the ddCT was calculated taking dCt of the sample and subtracting it by the dCt of the ctrl (non-stimulated cells incubated the same number of days as the target sample). Lastly to calculate the relative expression one use the formula 2^ - ddCt.

Sample	Target	Cq	Mean Cq	Reference gene	Cq	Mean Cq	dCt (Cq target - Cq reference gene)	ddCt (dCt-dCt ctrl)	2^-ddCt
111K-3	Mx1	27,85		EF1a	22,03				
111K-3	Mx1	27,71	27,78	EF1a	21,62	21,82	27,78 – 21,82 = 5,96	5,96 – dCt (ctrl 3 days) = 0,00	2^0 = 1,00
111P-3	Mx1	24,88		EF1a	21,96				
111P-3	Mx1	24,64	24,76	EF1a	21,75	21,86	2,90	5,96 – 2,9 = -3,06	2^-(-3,06) = 8,32
111K-7	Mx1	29,89		EF1a	22,47				
111K-7	Mx1	30,04	29,96	EF1a	22,19	22,33	7,63	7,63 – dCt (ctrl 7 days) = 0,00	1,00
111P-7	Mx1	23,40		EF1a	22,85				
111P-7	Mx1	23,79	23,60	EF1a	22,26	22,56	1,04	7,63 - 1,04 -6,59	96,22
111I-7	Mx1	29,90		EF1a	21,91				
111I-7	Mx1	30,00	29,95	EF1a	21,98	21,94	8,01	0,38	0,77