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The immune gene expression profile of sterile and fertile Atlantic salmon (Salmo salar L.) during smoltification and post transportation stress

A comparative overview

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Contents

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Abstract

The escape of farmed salmon may result in unwanted interbreeding with wild salmon populations. The development of a sterile salmon can prevent the negative effect of unwanted genetic introgression as well as provide the industry with solutions to drawbacks associated with sexual maturation. A sterile fish will also improve the competitive strength of the Norwegian breeding companies as the fish delivered cannot be used for breeding stocks. Currently the only sterile fish commercially available is the triploid salmon which is not performing optimal as it is sensitive to suboptimal environments and prone to deformities and is at present banned by the authorities for use in Norwegian aquaculture. Therefore, it is necessary to find new methods to produce sterile fish for commercial use without compromising fish welfare.

Through the project "Salmosterile" at Nofima AS there has been developed a new method for sterilization based on preventing the formation of the germ cell line during early development. Removal of the maternally provided 'Dead end', which is a critical factor for formation and maintenance of the germ cell line, by antisense oligonucleotides result in a germ cell free and sterile fish. Before this sterilization method can be transferred to large scale commercial production of sterile salmon, it is important that the effect of germ cell loss is evaluated in consideration of the fish's different physiological systems. It is, amongst other systems, important to ensure that the immune systems of the new sterile fish are robust and can handle stress equivalent to that of the fertile fish.

To evaluate parts of the immune system and endocrine regulation during known challenging physiologically processes, sterile and fertile fish was subjected/exposed to a protocol for photoperiod induced smoltification followed by a simulated smolt transport. During the study, expression of 8 gene markers of innate immune response and have been investigated by qPCR: IL1ß, CATH-2, HSP70, sIgM, TCR1α, IL10, IFN-γ, TNF1α, and MHC I, to see if the loss of germ cells had any significant influence on the gene expression. Also, possible differences in plasma cortisol, which is central to development of hypoosmoregulatory capacity and physiological responses to stress, between fertile and sterile Atlantic salmon were investigated. The results of this experiment showed that the loss of germ cells displayed little to no significant differences between the sterile- and the fertile salmon during this project. This is promising for the future production and commercial use of sterile A. salmon

produced by knockdown of the maternal factor ‘Dead End’ and thus displays an important next step for the aquaculture farming industry enabling continuous growth in a more sustainable manner.

1 Introduction

1.1 Background

In 2020 the registered first-hand value for Norwegian farmed salmon was set to 69 billion Norwegian kroners (Regjeringen, 2021). Aquaculture is one of Norway's most important and fastest developing industries and exports today. However, the development of the industry have stagnated due to environmental challenges that needs to be solved for the continuous sustainable growth (Regjeringen 2015; Vetrinærinstituettet 2021)

The genetic influence escaped farmed salmon have on the wild salmon stocks is identified as one of the most severe threats to natural salmon populations (Forseth et al., 2017). Farmed salmon that escape from aquaculture facilities can have environmental consequences, bringing disease and, or transfer genetic material not selected for optimal performance under natural conditions. (Forseth et al., 2017). Individual incidents can account for massive escape events, as one fish cage can hold up to 200 000 individuals of A. salmon (Michelsen et al., 2019). Norway is obligated to ensure overall management of the wild salmon stocks and thus, must ensure that the aquaculture does not contribute to permanent genetic changes in the wild salmon stocks. To ensure the management of wild A. salmon stocks, the future sea farming will have to be based on production of sterile farmed salmon (Tveiten et al., 2015).

The production of a sterile A. salmon may also be beneficial for the aquaculture industry. The breeding industry will avoid exporting their genetic material and, thus, give the Norwegian salmon production a competitive favourable advantage (Pike, 1993).

For the farming industry sexual maturation of A. salmon results in economic loss and fish welfare issues. Early maturation may lead to low somatic growth, low immune function, low quality of the meat and lower hypo-osmoregulation ability and elevated death rates amongst the maturing fish (Taranger et al., 2010). Sexual maturation is especially challenging in recirculating aquaculture systems (RAS) where use of high temperature and continues light to stimulate fast growth and maturation, especially in males (Fjelldal et al., 2011). Research found that c. 75% of juvenile males matured during freshwater -RAS production, which have a negative impact on smoltification, osmoregulatory capacity and growth rate (Summerfelt et al., 2012; Imsland et al., 2014; Fjelldal et al., 2011; Melo et al., 2014; Fjelldal, et al., 2018). Maturation at this scale accounts for large financial losses for the producers and may increase in the future, as the industry is likely to continue the expansion into modern land-based

aquaculture production. The production of a well-functioning sterile salmon will reduce the consequences of escapees (Cotter et al., 2000) and solve the challenges the industry faces with early maturation, both with fish welfare/health and economical loss with better quality meat and less fish death.

Triploid salmon is the only source, on an industry scale, of sterile fish to have three pairs of its chromosomes versus the normal two following a temperature or pressure treatment of the eggs right after fertilization (Maxime, 2008.) (Fraser et al., 2012) Hansen et al, 2012.). The triploid salmon has been shown to have some problems such as skeletal and heart deformities, cataracts, low growth and sensitivity to sub-optimal warmer water temperatures and low oxygen levels (O´Flynn et al., 1997; Hansen et al., 2015; Sambraus et al., 2017). This evidently results in a poor welfare for the triploid salmon during production cycles, and the use of triploid A. salmon in Norwegian aquaculture is now put on hold by the authorities (Norwegian Food Safety Authorities). Production of a more robust sterile A. salmon will be an effective measure for reducing the genetic influence on wild stocks and can contribute to avoiding the fish's health issues related to sexual maturation (Cotter et al., 2000; Veterinærinstituttet, 2018).

1.2 Production of Germ Cell Free A. salmon

An alternative sterilization method (to that of triploidization) for production of sterile A. salmon is to remove the germ cell-line to stop the development of gametes (eggs and sperm). The primordial germ cells(PCG) in teleost fish is defined/established early in the embryogenesis (Gross-Thebing, et al., 2017; Raz, 2003). The PCG's are the precursors of all gametes (germ cells) that migrates to the gonads where they differentiate into egg or sperm cells under maturation (Raz, 2003; Slanchev et al., 2005). One of the critical genes for PGC development of the germ cell line is that coding for the Dead-End protein (Dnd) (Weidinger, et al., 2003). Dnd is maternally (germ-plasm) deposited in the eggs as mRNA. In the period after fertilization the Dnd mRNA will get translated locally within the PCG and stimulate migration of the PCGs to the gonads where they later will develop into gametes (Slanchev et al., 2005). By inhibiting the translation of Dnd the PCGs don't migrate, and they enter an apoptosis or reverse to somatic cells (Gross-Thebing, et al., 2017). Thereby the primordial germ cells will not form, and the fish will be sterile without the possibility of developing gametes/germ cells in its gonads (Wedinger et al., 2003; Slanchev et al., 2005; Wong & Zoar, 2015). This type of sterilization can be done either by "knockout" (KO) or 'knockdown'

(KD) technologies of the Dnd gene. Dead end KO with the use of the CRISPR/Cas-9-system resulted in a mutation in the Dnd gene and, thus, sterility by loss of the germ cell line (Wargelius et al., 2016). However, gene editing by CRISPER/Cas9 method is subjected to legislations of genetically modified organism (GMO) The use of GMO rises ethical, jurisdictional and practical questions that will are problematic for the aquacultural industry (Wong & Zoar, 2015) both for producers and consumers alike. Therefore, it is a need for a non-GMO sterile salmon which performs in the same way as commercial fertile salmon. Another, likely less controversial, method for sterilization is Dnd KD by GapmeR (Gap) antisense oligonucleotide (ASO) technology (Tveiten et al. 2022). The Gap ASO initiate sequence specific degradation of the Dnd mRNA by activating the cell endogenous enzyme RNAaseH . (Pauli et al., 2015). Since Dnd protein synthesis will be inhibited only temporarily, this sterilisation method will probably not be subjected to laws of GMO since there is no changes done to the fish DNA (Pauli et al., 2015). This will also make it more desirable to use such a sterilization method in the aquaculture industry. The Gap mediated, Dnd KD dependent, sterilization method developed by Tveiten et al (2022) resulted in germ cell ablation, and sterilization, in ca. 90% of the treated individuals. Further research has confirmed the permanent removal of the germ cell line (Tveiten et al., 2022)

Before this, or other methods for sterilization, can be used in commercial production of sterile farmed salmon, it is important investigate how production performance and welfare is maintained. A prerequisite for the use of sterile salmon is that the production performance under aquaculture conditions is the same as for normal fertile fish. 1.3 The Immune System of Teleost Fish

A successful salmon production is dependent on fish having a well-developed immune system to ensure good abilities to fight pathogens and infectious diseases. Death or mandated slaughter of farmed fish due to disease is one of the biggest commercial losses in fish farming to date (Krasnov et al., 2020).

The immune system of teleost consists of the innate- and the adaptive immune system. The innate immunity is considered the first line of defence against pathogens. It is consistent of physical barriers like skin, gills and digestive tracts dividing the body from the external environment as well as immune cells, antimicrobial molecules and pattern recognition receptors (PRRs) within the body (Uribe et al., 2011). To recognize foreign cells and particles the innate immune system is equipped with pattern recognition receptors (PRR) that

can alert the immune system to mobilize (Magnadóttir, 2010). The most studied PRR's are the toll like receptors (TLR). Eleven unique TLR's are identified in teleost's (TLR 1,2,3,5,7,8,9,14,21,22 and 23) and can recognize viral genetic material both on the cell surface and intra -cellular (Workenhe et al., 2010). PRRs recognize pathogen-associate molecular patterns (PAMPs) and stimulates to expression of cytokines. Cytokines modulate the immune response and mediate inflammation by signalling and recruiting other cells within the immune system (Abbas, et al., 2020, p.267). Fish interferons (IFN) and interleukins (IL) are the most studied cytokines (Uribe et al., 2011). IFN have an important role in the cell mediated defence against virus infection in teleost (Zou & Secombes, 2011). Cytokines regulate the phagocytes' ability to destroy pathogens and regulates the antigen presenting cells (APCs) migration to lymphoid tissue to present antigen to T-cell receptors (TCR) to further activate the adaptive immune systems response such as activating T and B-cells (Wang & Secombes, 2013).

An essential part of the innate immune system of teleost is the phagocytosis of foreign particles that may enter the body. This is done by monocytes, macrophages and neutrophil cells. In addition, there is also found natural killer cells (NK) like cytotoxic cells in fish (Fischer et al., 2013). Dendritic like cells (DC) has also been found in rainbow trout (Bassity and Clark, 2012) and together with phagocyting like B-cells also found in fish which connect the innate and the adaptive immune system (Li et al., 2006, Bassity and Clark, 2012).

The adaptive immune response is activated by the innate immune response and is especially important for combatting recurrent infections by the formation of memory cells, receptors and Immunoglobulin (Ig). This allows for fast and effective eliminations of specific fish pathogens the immune system has been exposed to earlier (Rauta et al., 2012).

The immune system of teleost's differs from that of vertebrae in their lack of bone marrow and lymph nodes (Press & Evensen, 1999). Instead, the tasks are performed by the head and trunk kidney, thymus, spleen, and mucosal lymphoid tissue in the skin, intestine and gills (Press and Evensen, 1999; Morrison and Nowak, 2002; Zapata et al., 2006; Haugarvoll et al., 2008). The head kidney is one of the teleost's primary lymphoid organs where B-cell maturation happens as well as phagocytosis, antigen processing, immunoglobulin M (IgM) production, and immunological memory in melanomacrophage centres.(Kaattari og Irwin, 1985; Irwin og Kaattari, 1986; Hansen og Zapata, 1998; Rauta et al., 2012). B- and T cells constitute the most important cells in the adaptive immune defence. They are activated by

either foreign antigen binding to the major histocompatibility complex (MHC I and II) that is presented on all the cell surfaces of the fish's own cells, or by antigen binding to immunoglobulin (Ig), free or membrane bound (Abbas et al., 2020, p.). The head kidney and the spleen also work as secondary lymphoid organs where the mature B-cells migrate and get activated by antigen (Zwollo et al., 2005). It is believed that B-cells differentiate to plasma cells or immature plasma cells (plastmablasts) in the head kidney of teleost. The maturation of the T-cell happens in the thymus (Hansen og Zapata, 1998), and they accumulate in the spleen and mucosal lymphoid tissues. The mucosal lymphoid tissues in the gills have shown to associate with tasks done by the secondary lymphoid organs as gills are exposed antigens trough constant contact and filtration of the water and displays both innate and adaptive immune cells (Koppang et al., 2010, Salinas, 2015). Interactions between the reproductive axis and the immune system

The hypothalamus -pituitary, gonad axis (HPG-axis) is an essential part of the endocrine system that regulates the reproduction development in fish. The peptide hormone GnRH (gonadotrophin releasing hormone) is released from the hypothalamus to the pituitary gland and stimulates secretion of follicle stimulation hormone (FSH) and luteinizing hormone (LH). These hormones bind to receptors on the gonads and leads to the secretion of sex steroid hormones (Yaron & Levavi-Sivan, 2011). In female fish testosterone (T), estradiol-17-beta (E2) and different progestogens (PG) are typically found in plasma during different stages of the reproductive cycle (Yaron & Levavi-Sivan, 2011). In males, T, 11- ketotestosteron (11-KT) and PG is secreted (Yaron & Levavi-Sivan, 2011). The GnRH and the sex hormones(steroids) are highly involved in the development and maturation of the immune system in fish. GnRH and corresponding receptors are locally developed by immune cells as well as in the HPG-axis, indicating that GnRH acts autocrine as well as exogenous stimulating immune responses. During embryo development it is believed that the sex hormones play an important role in developing the immune system and gender differences deriving immune responses in mammals (Tanriverdi et al., 2003). The receptors of sex hormones are expressed in primary lymphatic organs and peripheral immune cells. Estrogen is believed/has shown to amplify a humoral immune response in mammals, whilst testosterone is believed/has shown amplifies suppression of T-cell activity in the immune system (Tanriverdi et al., 2003). In studies on A. salmon sterilized by Dnd KO it was suggested that production of androgen and estrogen in the gonads was germ cell dependent (Kleppe et al., 2017). However, Tveiten et al. (2022) found that germ cell free male A. salmon

is able to produce sex steroids during early stages of puberty, albeit at somewhat lower levels than in intact fertile fish. It is known that the immune system and reproductive system interacts, but if the loss of germ cells affect the gene expression of immune genes remains (mostly) unknown.

1.3 The hypothalamus–pituitary-inter renal axis (HPI-axis) and endocrine regulation of smoltification and stress

1.3.1 The HPI-axis

The cascade of hormonal changes leading to cortisol release in the inter-renal cells of the head kidney is regulated by the hypothalamus-pituitary gland –inter renal -axis, The HPI- axis is activated both during smoltification and during stress by release of corticotrophin releasing factor (CRF) from the hypothalamus. CRF subsequently stimulate adrenocorticotrophic hormone (ACTH) release from the pituitary, which then is the main factor stimulating cortisol release from inter-renal cells in the head kidney. Smoltification

1.3.2 Smoltification

The A. salmon is an anadromous species and migrate to the sea as juvenile. Before the juvenile fish can migrate into the sea it goes through smoltification, a morphological, physiological and behavioural change induced by an increasing photoperiod and driven by different endocrine systems to adapt to life in the sea. (Hoar, 1988; Folmar & Dickhoff, 1980; McCormick & Saunders, 1987; McCormick et al., 1998; McCormick, 2012). Activation of the HPI-axis (together with the growth hormone axis) is stimulated by increasing day length and is of paramount importance for development of hypo-osmoregulatory capacity and sea water adaption, where plasma cortisol may increase up to 30-50 ng/ml towards the end of smoltification process (Heggberget et al. 1992; Hoar, 1988; McCormick 2012).

Growth hormone (GH) and cortisol are considered to be the main sea water adapting hormones in A. salmon (Heggerberget et al., 1992). The increase in these hormones initiates changes in metabolism, growth, and sea water tolerance for the salmon that is essential for the survival rate upon transferred to sear (McCormick, 2012).

1.3.3 Stress in Teleost's

Stress challenge the homeostasis of the body and denotes the organism's ability to maintain the equilibrium of different biological processes (Bonga, 1997; McEwen, 1998; Van Weerd & Komen, 1998). During aquaculture production the fish is exposed to a lot of different

stressors such as handling during moving, sampling, treatments, transport, environmental factors, different diseases and microbes throughout the whole production cycle. The stress responses in fish can be divided in three reaction phases, primary, secondary and tertiary (Bonga, 1997; McEwen, 1998; Selye, 1946). The primary phase is the instant reaction of an outer stressor when expressed in the activation of a “fight or flight” mode/reaction (Adamo, 2014). During this response the sympathetic nervous system (SNS) is activated which sends signals from the hypothalamus to chromaffin tissue in the head kidney to quickly release catecholamines (adrenalin and noradrenaline) to the cardinal vein bloodstreamto quickly mobilize metabolic processes for energy bursts trough glycogenolysis (Barton, 2002; Reid et al., 1998; Bonga, 1997; Chrousos and Gold, 1992). The other main neuroendocrine response during the primary phase is the activation of the HPI-axis (see above), and dependent on the severity and duration of the stressor, plasma cortisol increases substantially within 3-4 minutes, and may reach levels of 200-400 ng/ml. (Melo et al., 2014; Mommsen et al., 1999). The main task of cortisol during a stress response is to mobilize energy through redirection of metabolism trough gluconeogenesis. Catecholamines ensures the oxygen supply is sufficient by increasing the blood flow and perfusion trough the gills (Bonga, 1997; Chrousos and Gold, 1992).

Primary and secondary stress responses are adaptive stressors and are associated with acute stress (Tort, 2011). It is the duration of the stressors that determine if the stressor is hazardous for the fish. The acute stress of primary and secondary responses is not believed to be health hazardous as it is a short-term exposure to stressors. Tertiary stress responses are associates with chronical stress, reduces immunity, reduced appetite and growth, and inhibited reproduction (Tort, 2011; Barton & Iwama, 1991).

1.3.4 Influence of smoltification and stress on the immune system

Some studies implies that the immune system is weakened during the smoltification but cannot solely conclude that the smoltification is the only reason (Maule et al., 1987, Melingen et al., 1995, Melingen et al., 2002), and information is still lacking about how the smoltification process, and related neuroendocrine changes (e.g., cortisol), influence the immune system in A. salmon. There has been done studies that show changes in multiple immune genes for salmon during smoltification, generally the results are associated with repression of the immune system (Johansen et al., 2016; Krasnov et al., 2020; Johansson et al., 2016). It is well known that stress and elevated cortisol relates directly to suppression of

the immune system (Tort, 2011). Elevated concentrations of corticosteroids after stress have shown to reduce the immune status by affecting the number of lymphocytes and the capacity of antibody production (Van Weerd & Komen, 1998). There has been shown reduction in lymphocytes in association with increased cortisol levels in coho salmon (Melingen et al., 2002). Also, Apoptosis of B-cells in the head kidney and blood, as well as a down regulation of the immune system is shown in correlation with increased cortisol levels due to chronical stress responses in carp (Weyts et al., 1998, Verburg-van Kemenade et al., 1999). In Trout there had been registers increased levels of cortisol simultaneously as abnormally low values of lymphocytes (Pickering, 1984). There is no conclusive answer to whether cortisol has a significant negative effect on the immune status of the fish during smoltification or not and further studies are needed. Therefore, the cortisol levels during smoltification and stress will be included in this experiment to detect any possible differences in the sterile fish compared to fertile.

1.4 Selection of immune genes

Based on the literature cited above I have chosen to look at genes associated with innate immunity and early immune response because as we have sampled juvenile fish (200-300g). If not all, but some of these genes are also found to be influenced both by smoltification and stress (Johansen et al., 2016; Johansson et al., 2016; Tort 2011; Krasnov et al., 2021; Culbert et al., 2022; Karlsen et al., 2018, Iversen et al., 2005; Poltronieri et al., 2007; Krasnov et al., 2020; Jensen et al., 2019).Innate immunity provides an early first line of defence and is important for survival in the sea as well as its capacity to initiate the adaptive immune response (Salinas 2015).

1.4.1 Cytokines

Cytokines are part of the innate immune system and an important part of the first line of defence against pathogens. Cytokines are proteins that are produced and secreted by many different cell types to mediate inflammatory responses and immune reactions by activating or recruiting immune cells. Cytokines are in principle the mediator for communication between cells within the immune system (Abbas, et al., 2020, p.267).

IL-1b, TNF1a and IFNg are pro-inflammatory cytokines secreted by different immune cells (macrophages, T cells) (Secombes et al., 2011; Abbas et al., 2020, p.298; Sakai et al., 2020); Zou & Secombes, 2011). These activates other immune cells like lymphocytes and phagocytic cells and are important for inflammatory response and Th differentiation (Zou et

al., 2016; Sakai et al,. 2020; Abbas et al., 2020, p. 42) On the other hand, IL-10 is an anti-inflammatory cytokine secreted by macrophages and regulatory T cells and inhibits the pro-inflammatory response for tissue repair (Abbas et al., 2020, p.298; Secombes et al,. 2011).

Since these cytokines are the early responders of innate immune defence and decides the fate of future response, we considered these cytokines in our study.

1.4.2 Major Histocompatibility Complex I (MHC I) and TCR1α

The major histocompatibility complex (MHC) is membrane bound proteins on APC's that display peptide antigen to T-cell receptors (TCR) (Abbas et al., 2020, p. 284). By this the MHC complex is important for the activation of the adaptive immune system. The MHC pattern variations in fish are similar to mammals with class I and II MHC, although with seemingly higher allelic diversification and no loci linkage (Uribe et al., 2011; Yamaguchi & Dijkstra, 2019). TCR alpha and beta is membrane bound antigen receptors on T lymphocytes and is presented on all CD8+ and CD4+ cytotoxic T-cells respectively. TCR1α is a known TCR in A. salmon (Bilal et al., 2018) The class II MHC molecules are mainly expressed on dendritic cells, macrophages, B-lymphocytes (APC's) that can internalise extracellular proteins and s present to TCR on CD4+ T-cells that activate macrophage killing of the phagocytosed microbe or B-cell antibody secretion that can bind and neutralize the foreign protein in the extracellular fluids (Abbas et al., 2020, p.67-70).

TCR1α is a known TCR in A. salmon (Bilal et al., 2018) and MHC I and TCR has been studied in regards to smoltification and transport with a downregulation in expression in smolts and after sea water transportation (Johansson et al., 2016). MHC I and TCR1α is two of the genes chosen to be studied in this experiment to research if the sterile salmon has the same expression and possible ability of antigen presenting as fertile salmon and if changes in its expression was related to smoltification and/or stress.

1.4.3 Immunoglobulin

Immunoglobulins (Ig) also known as antibodies, is part of the B-cell regulated, humoral adaptive immune system, but are important in both the adaptive and innate immune defence system of fish (Salinas et al., 2021). Free Ig's are important in the defence against microorganisms in tissue, blood and mucus, or work as membrane bound antigen receptor (BCR) on the B-cell. Ig's are produced by B-cells that differentiate into plasma-cells or plasma blasts (Salinas et al., 2011; Salina et al., 2021. IgM, IgT and IgD are the three Ig's

known to exist in teleost's (Hansen et al., 2005; Hikima et al., 2011; Salinas et al., 2011). The three immunoglobulins discovered in fish slightly differ in functions, but they are all commonly associated with the immune defence in the mucosal layers and blood/serum. IgM is the most dominant type of Ig found in the teleost bodily fluids and neutralize virus and bacteria pathogens and activate the compliment system (Salinas et al., 2021). Since the fishes' mucosal layers in the gills are constantly exposed to the outer environment and pathogens it is important to research the sterile salmon's expression of secretory IgM contra the fertile one to ensure its ability to upheld homeostasis and the first line of defences over the gill's is equal. We will investigate the expression of secretory IgM during this experiment.

1.4.4 Antimicrobial peptide

Antimicrobial peptides are essential mediators of the innate immune response in all vertebrates and invertebrate and the CATH-2 antimicrobial peptide has been identified as a mediator of the early innate immune response in fish (Katzenback, 2015) (McGrath et al., 2022). CATH-2 are a part of the family of broad-spectrum antimicrobial peptides that work as effector molecules of the innate immune system (Bridle et al., 2011) Antimicrobial peptides have the ability to break down bacterial walls (Uribe, et al., 2011) CATH-2 works antimicrobial by interacting with the membrane of invading pathogens trough electrostatics. Thus, ruining the membrane of the pathogens. CATH-2 also stimulates cytokines, and differentiation and maturation of immune cells (McGrath et al., 2022) CATH-2 was included in this study to see if sterile salmon differ from fertile salmon regarding possibility for early antimicrobial responses in the innate immune system and if changes in its expression was related to smoltification and/or stress.

1.4.5 Heat shock protein

In addition to neuroendocrine responses to stressors there is also cellular reposes to stress. Cells respond to stressors by upregulating its gene expression of stress proteins called heat-shock proteins (HSP) (Roberts et al., 2010). The transcription of these genes increases in the early phases of stress in teleost's, and are up regulated by heat shock but also other forms of cellular stressors such as toxins, hypertrophy, hypoxia, acidosis, microbial damage, infections and neoplasia (Wei et al., 1995; Ciocca & Calderwood, 2005; Welch, 1993; Deane & Woo, 2011). HSP is important for the repair of damaged proteins. It also plays a role in regulating immune responses, inflammation, and apoptosis (Jacquiersarlin et al., 1994; Wieten et al., 2007; Deane & Woo, 2011). In this experiment the gene expression of HSP70 was included to

see if sterile salmon differed from fertile salmon with regard to cellular stress response and if changes in its expression was related to smoltification and/or stress.

1.5 Objectives

Till date, no immune status study has been performed for the germ-cell free sterile salmon. The research data that are available about the immune status of sterile salmon is mainly done with triploid salmon where germ cells are present. Therefore, it is very important to study the immune status of the germ cell free sterile fish in relation to the normal fertile fish. This master thesis is a part of the first studies exploring the immune status of germ cell free sterile A. salmon during the juvenile stage. The germ cell free sterile salmon used in this study is the first batch produced through microinjection method and has been investigated further based on two research questions.

1. To investigate whether sterilization through the ablation of germ cells affects the immune status during the smoltification process. To answer this research question, important immune and stress related genes expression and cortisol levels were studied during the start, middle and end of the smoltification process.

2. To investigate whether the germ cell ablation affects coping/handling of transportation stress compared to normal fertile salmon smolts. To answer this research question, important immune and stress related genes expression and cortisol levels were studied pre and post transportation stress.

This study will broaden the knowledge about immune status/profile of germ cell free sterile salmon during smoltification and transportation stress leading to possible benefits for further commercial production of sterile fish in the future.
This study will also give information about the overall/general changes in the immune system related to smoltification and stress in A. salmon.

2 Materials and Methods

2.1 The Experimental Fish

The fish, experimental set up and sample material described in this study originate from two previous projects ("Salmosterile" (NRC, project number 221648) and "Evaluation of health, welfare and growth in sterile salmon after deletion of embryonic germ cells" (FHF, project number 901459). Thus, the current author did not take part in the actual sampling of the fish but was provided with preserved tissue samples and blood plasma/plasma extracts for their further analysis. For the same reason, this author cannot be held accountable for the original experimental set up and sample handling which is described in Jenssen (2020), Aspen (2020) both unpublished MSc thesis's and Tveiten et al. (2022)

Sterilization through Gapmer mediated knock down of the *dnd* mRNA was carried out by microinjection at the one-cell stage at Nofima in late October 2016. Injected and non-injected control (giving rise to fertile fish) eggs were incubated at c. 6˚C until the "eyed stage" (c. 330-day degrees) when they were transferred to Tromsø Aquaculture research station (HiT) at Kårvika (21st of December) for further incubation at 6-7 ˚C until start feeding at c. 900-day degrees post fertilization. Sterile and fertile fish were start fed and raised under standard hatchery conditions under continuous light (24-hour light, 24L), water temperature of (6-10 ˚C) and feeding conditions (pellet size and daily rations by disc feeders) adjusted according to fish size and ambient water temperatures until the current experiment commenced in autumn 2018. During this experiment, the fish were kept in freshwater in 500 L circular tanks and fed with Olympic 3.0 mm (see further details below). Smoltification was induced according to established protocols (Fjelldal et al., 2011; Strand et al., 2018), where the different groups were subjected to manipulated photoperiod of short daylight (6-hour light: 18-hour dark) for 8 weeks with reduced temperature of 5-6 ˚C followed by a six-week period of continuous light (24L) and a water temperature of 10 ˚C (± 0.5 ˚C) staring at the 24.10. (Fig 1). The experiments were approved by the Norwegian Food Safety Authority.

2.2 Experiment design and sampling

The experimental design made it possible to answer the two main research questions: What are the influences of sterility on immune gene expression i) during smoltification and ii) after transportation stress and acute sea water exposure.

Each of the two experiments consisted of two groups, one sterile group and one fertile control group. From previous studies of the same fish, germ cell ablation and sterility, sex and

maturational status have been determined (Karlsen 2020; Tveiten et al., 2022) and for the current study only samples from immature individuals were selected for further analyses. A sub population (50-60%) of males within both groups initiated sexual maturation but, these individuals were not subject for this study. During sampling by for "Salmosterile", each group were further subdivided into two subgroups: male and females. A total of one hundred and twenty fish samples from "Salmosterile" were randomly chosen from the available material to be used for both the experiments in this thesis (90 fish for experiment 1 and 45 fish for experiment 2). Sterile (germ cell free) fish were transferred to tank number 1, 5 and 6 with 20 fish /tank. Fertile salmon were transferred to tanks 2, 3 and 4. Both the experiments were carried out in triplicate tanks. Each tank was kept in the same room and were maintained in a similar water volume of 500 Liters (l), oxygen levels (> 80 %) and water flow (40 l/min). From these tanks a random selection of five immature individual samples was selected from each tank, where maturation status, gender, and sterility were known to conduct our experiment and answer our research questions.

Experiment 1: In order to look at the gene expression during smoltification, samples were taken at three time-points T1 (0 weeks), T2 (3 weeks) and T3 (6 weeks) after exposure to 24 hours of light, respectively. T1 was taken at the start of the smoltification (week 0). T2 was taken during smoltification (week 3 i.e., three weeks after the start of the smoltification). T3 was taken at the end of the smoltification (week 6 i.e., six weeks after the start of the smoltification). Tank setup sampled fish number and group details are shown in the figure 1.

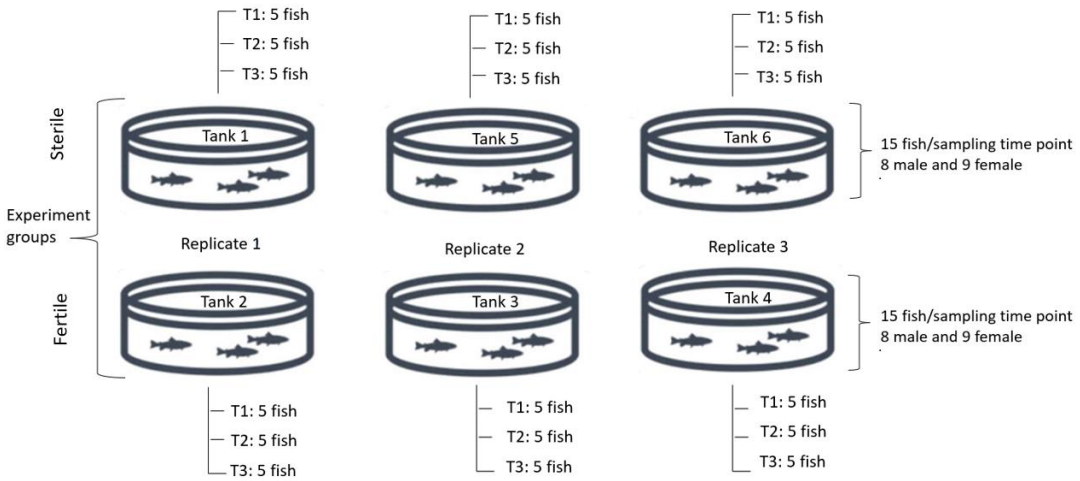
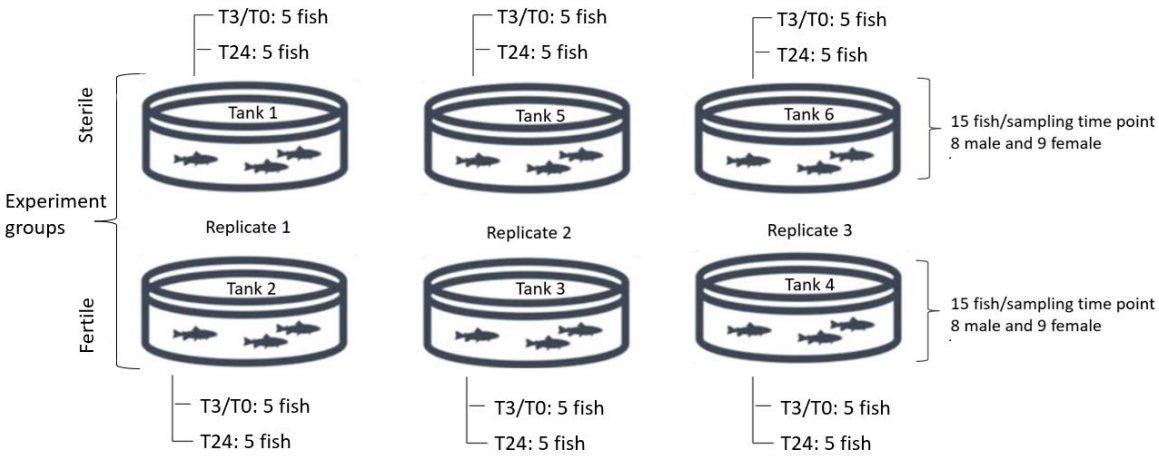


*Figure 1 1**: The tank setup for the smoltification experiment**. Figure 2 shows the tanks set up for the sterile and fertile groups and the number of fish sampled from each tank at each timepoints. (Modified after Aspen (2020) Jenssen (2020)*

Experiment 2: Thirty fish, 15 sterile and 15 fertile, (50% of each sex) T3 were further used for the transportation stress experiment, see figure 2. These fish where stressed by simulating a smolt-transportation where the fish was transferred to a transportation tank with a fish density of 112g/l water and moved it interchanging 15 minutes of transport with 15 minutes of pause, for 2 hours. After transport, the fish was transferred back to its original tank that now contained full strength (34 ‰) sea water (SW) and were sampled 22 hours later (2-hour transportation + 22-hour exposure to (T24) (Aspen 2020). Oxygenation was necessary during transport and was closely monitored and varied from 68-130 % saturation (Aspen 2020). Thirty fish (15 fertile, 15 sterile) were randomly chosen before imitated transportation stress (T0). Here, T0 is the zero time-point and state of the fish before stress and is the same fish used at T3 sampling time-point of the smoltification experiment i.e. experiment 1 as shown in figure 1. T0 is used as a baseline cortisol level for the stress study.



Figur 2 2: ***The tank setup for the transportation stress experiment****. Figure 3 shows the tanks set up for the sterile and fertile groups and the number of fish sampled from each tank at each timepoints. (Modified from Jenssen (2020) and Aspen (2020))*

Tissue samples were collected from both the experiments. Before removing organs and / or blood from the fish, fish were euthanized with an overdose of Benzoak (Felleskatalogen, 2020) (50 ml / 50 l seawater). All fish lost equilibrium and were completely immobilized within 1-2 minutes after being netted out of the tank. All blood samples were drawn within 4-5 minutes after netting (Jenssen, 2020; Aspen, 2020)

Blood was collected using BD Vacutainer containing sodium heparin from all the fish of both groups at the different time points and further centrifuged at 4500 rpm for 10 minutes for

plasma collection. Plasma was further stored at – 20 ºC to perform Radioimmunoassay (RIA) for cortisol quantification.

Tissue samples were obtained from triplicate groups where tank/replicate 1, 5 and 6 contained fertile fish and tank/replicate 2, 3 and 4 contained fertile fish. Samples were taken from 5 fish per tank/replicate with a total of 15 fish from each group and approximately 50% of each sex at each time-point (Figure 2 and 3). All tissue was stored in RNA-later and first kept at 4 C for 24 hours and then stored at – 20C˚ until further processing. In this study, we only used G- and HK tissue samples for downstream processing and gene expression studies.

2.3 RNA Isolation

RNA was isolated from gill and head kidney using the RNeasy Mini Kit (Quiagen) as instructed in the accompanying protocol and illustrated in appendix I (Quiagen, Hilden, Germany). All use of chemicals was carried out in fume hood with associated safety equipment. Tissue samples approximately 30 mg were chopped into smaller pieces with a scalpel blade before homogenization. Tissue samples were homogenized in eppendorf tubes supplemented with one iron ball and 600 ul RLT buffer using TissueLyser II (Quiagen, Hilden, Germany) for 1 minutes at a frequency of 28 1/s.
For head kidney samples, β-Mercaptoethanol (10 µl β-ME per ml buffer RLT) was used and for gill samples, dithiothreitol was used in the buffer solution (10 µl DTT per ml buffer RLT). DTT made a very viscous solution when homogenizing head kidney tissue. This was presumed due to the amount of protein found in the head kidney tissue. Using buffer with β-ME worked better and was not as viscous and gave less probability of clogging the column. Thus, β-ME buffer was used for the head kidney tissue. RNA isolation of gill tissue was also done using the RNeasy Mini Kit but with additional step with proteinase K for a better RNA yield. For the gill sample, after homogenization, 2 ml of the formed lysate was pipetted to a new micro-centrifuge tube. 1.2 ml RNase-free water and 20 µl proteinase was added to the centrifuge tube and incubated at 55 C˚ for 15 min. The solution was then centrifuged for 3 minutes at 8000 rpm. The supernatant was pipetted out to a new centrifuge tube together with 900 µl ethanol (100%) and mixed. 700 µl of the solution was pipetted into the RNeasy mini spin column placed in a 2 ml collection tube before being centrifuged for 15 seconds at 8000 rpm. From here, both gill and head kidney samples were processed the same way as mentioned in RNeasy Mini Kit protocol shown in appendix I.

For RNA elution, 30 µl of RNase-free water was added directly to the column filter before it was centrifuged for 1 min at 9000 rpm 20-25 C˚. This was repeated once more before the concentration of RNA in the solution was measured. The purity and yield of RNA were determined by Nanodrop (Nano-Drop Technologies, Wilmington, DE, USA). The RNA samples that had OD 260/280 (optical density) values between 1.8 and 2.1 were considered to have minimal biological contamination. The RNA samples were stored at -80 C˚ until the next step in the process, cDNA synthesis.

2.4 cDNA Synthesis and Dilution

In order to perform a qPCR study, one depends on primers being able to bind to complementary nucleotide sequences on the DNA. By converting RNA to cDNA, cDNA can be amplified under qPCR. In reverse transcription, single-stranded mRNA can be used as a template to make a new complementary cDNA using the enzyme reverse transcriptase. cDNA was synthesized using the QuantiTect Reverse Transcription Kit (Quiagen) as indicated in the manufacturer protocol and illustrated in appendix II(Quiagen, Hilden, Germany). Genomic contamination was eliminated in the first step of cDNA synthesis using the kit.

Before cDNA synthesis could begin, all RNA samples were diluted with RNase-free water to a concentration of 200 ng RNA per µl. RNA equivalent to 1000 ng (5ul) from the samples was used to perform cDNA synthesis with the QuantiTect Reverse Transcription Kit as shown in appendix II. To describe briefly, 2 µl gDNA Wipeout-buffer was added to all tubes in PCR strips containing 1000 ng of RNA in a total volume of 12 µl RNase free water. Then the strip was incubated for 2 minutes at 42 ° C in PCR machine (Applied biosystems). Furthermore, 6 µl mastermix (1 µl Quantitative Reverse Transciptase, 4 µl RT buffer and 1 µl RTprimer mix) were added to all tubes in the strip and incubated for another 15 minutes at 42 C˚ and 3 minutes at 95 C˚.

Synthesized cDNA was stored at -20 ° C until further QPCR analysis.

Before the synthesized cDNA was run in the qPCR machine, it was diluted in two rounds. First to 1: 5 to and then again further diluted 1:5 times with RNA-see free water to have working solution containing 2 ng of cDNA /uL.

Negative controls of no reverse transcript (RT-) and positive controls (RT +) were added for each tissue to ensure and control the quality of the samples and the reaction mix. The positive control was made by mixing 6 lower purity RNA samples and 6 different higher purity RNA

samples based on RNA quality from NanoDrop. The positive controls were prepared in the same manner as indicated in the protocol for cDNA synthesis from Quiagen but without gDNA wipe-out to check for possible genomic contamination of the original samples. The negative control was prepared without RNA samples, only RNase free water, as in the protocol for cDNA. The RT- was used during qPCR to indicate possible differences between QPCR boards, for example differences or contaminants in the mastermix components or contaminants in the water used in dilution.

2.5 Quantitative real-time PCR (RT qPCR)

To determine the amount of expression of a particular gene in a sample, we used quantitative real-time polymerase chain reaction (RT qPCR). During this experiment, the fluorescent dye Fast SYBR Green was used. It binds to double-stranded DNA and increases in proportion to PCR product each amplification cycle. A cycle consists of denaturing, amplification, and extraction. During denaturation, high heat is used to separate double-stranded DNA into single-stranded DNA. This allows added primers to bind to specific genes in the amplification phase. The last step in a cycle is the extraction phase where a new DNA strand is synthesized using the DNA polymerase enzyme. A forward and reverse primer is required for the new DNA strand to be able to make the desired amplicon product.

Quantitative real time qPCR was performed in 96-well plates and read in 7500 Fast real-time PCR system (Applied Biosystem, CA, USA). A qPCR mastermix of total 9 µl for each sample was made of 7.8µl SYBR Green, 0.8µl forward primer (5µM) and 0.8µl reverse primer (5µM), shown in appendix III.

6 µl cDNA equivalent to 12 ng cDNA of each sample were pipetted to each well. The total volume was 15 µl in each well. Each sample was run in duplicates. Duplicates for NTC, non-template control with 6 µl Nuclease-free water instead of sample cDNA were included to check for any nonspecific binding and/or contamination. The plates were sealed with plastic film and analysed in a qPCR machine the same way as described by Kumari et al. (2015). The program parameters used during the qPCR analysis are shown in appendix IV.

2.5.1 Primer efficiency

To quantify such genetic material, the qPCR machine depends on primers that bind to the region of the cDNA that is desired to be amplified. A primer specifically initiates replication

on a region of a DNA strand. Thus, with the choice of efficient primer, one can select which gene sequences are to be transcribed and amplified during a qPCR run. A qPCR for a total of 10 genes including reference gene were run during this experiment and 10 different forward and reverse primers were used as shown in the table 1. The concentration of working forward and reverse primer used was 5 µM. The effectiveness of the primers for each gene were tested by making a standard curve with two-fold serial dilution series.

To make a-two-fold dilution for the standard curve for each gene, stock cDNA samples from six individuals showing low Ct value for that particular gene were used and mixed. A two-fold dilution series of eight was then made and qPCR were run in three duplicates. The standard curve was created by selecting the program for standard curve analysis on 7500 Fast real-time PCR system (Applied Biosystem, CA, USA). Standard curves for all genes were created to test primer efficiency. By plotting the Ct value against the dilution log-values of the standard curve the slope was calculated. Based on the results from the standard curve the efficiency-of the primers was retrieved for each gene. The primer efficiency of each gene was calculated as described in Pfaffel (2001), shown in formula 1.

Formula 1: The formula shows how E=efficiency was calculated for each gene (Pfaffl, 2001).

$$E = 10^{\frac{1}{slope}}$$

The effect of a primer shows how it binds cDNA. If a primer is 100% efficient, the primer will multiply the amount of cDNA in each amplification cycle to the double in a q-PCR run. Efficiency lower than 100% will amplify less cDNA per cycle and higher efficiency than 100% may indicate inhibition of amplification due to high concentration of cDNA in the sample (Paffel, 2001).
All target genes and their primer sequence, access number, melting point, amplicon, R2 and efficiency can be seen from table 1 given below.

Tabell 1: The primer table shows the information about the primers used in this experiment; Primer sequences, gene bank number, and primer efficiency. concentration of the primers that were used was 5 µM.

Target gene	***Synonym***	***Primer gene sequence (5-3')***	***Accession nr.***	***Amplicon size (bp)***	***R^2***	***Efficiency (E)***	***Effecti***

							vity (%)
Elongation factor 1-alpha	**ELf1α**	F: TGCCCCTCCAGGATGTCTAC R: CACGGCCCACAGGTACTG	BG933853	57	0,999	1,93849	93,8 %
Interleukin 1 beta	**IL1ß**	F: GCTGGAGAGTGCTGTGGAAGA R: TGCTTCCCTCCTGCTCGTAG	XM_014170479	73	0,987	1,96529	96,5 %
Cathelicidin antimicrobial peptide	**CATH-2**	F: GGGGCAGCAAGGCCAACCAT R: CCGGTCAGTGTTGAGGGTGTTGC	XM_014140492.1	112	0,984	1,90441	90,4 %
Heat shock protein 70	**HSP70**	F: CTGGGCTGAATGTGCTGAGA R: CTGGACATGCCTTTGTCCATG	KU885451.1	79	0,984	1,7062	70,6 %
Immunoglobulin M (secretory)	**sIgM**	F: CTACAAGAGGGAGACCGGAG R: AGGGTCACCGTATTATCACTAGTTT	BT060420	90	0,998	1,99495	99,5 %
T-cell receptor 1-alpha	**TCR1α**	F: AACAATACAGAGGCCACCAG R: ACCAGTTTGCTTCACATTTCTC	BT057540.1	106	0,998	1,93805	93,8 %
Interleukin 10	**IL10**	F: CCTTCTCCACCATCAGAGATTAC R: AACTTCAGGATGCTGTCCATAG	EF165028	127	0,988	2,02035	102,3%
Interferon-gamma	**IFN-γ**	F: AAGGGCTGTGATGTGTTTCTG	AY795563	166	0,998	1,99594	99,6 %

		R: TGTACTGAGCGGCATTACTCC					
Tumor necrosis factor alpha1	**TNF1α**	F: ACTGGCAACGATGCAGGACAA R: GCGGTAAGATTAGGATTGTATTCACCCTCT	AY929385	144	0,826	1,84014	84%
Major Histocompatibility complex I	**MHC I**	F: GAAGAGCACTCTGATGAGGACAG R: CACCATGACTCCACTGGGGT	JN561338	112	0,998	2,00164	100, 2%

2.6 Gene Expression Analysis

During qPCR, it is recommended to normalize the target genes up to an endogenous standard, and this is done by comparing expressions to a reference gene which is stably expressed. This is necessary to compensate variations during PCR. The reference genes are often so-called "housekeeping" genes that are present in all cells with nuclei, and which are essential for the survival of the cells. The synthesis of mRNA is also considered stable in all tissues for these genes (Pfaffl, 2001). Elongation factor 1 alpha (EF1-a) was used as the reference gene for qPCR analysis during this experiment. This is one of the most abundant proteins in the cellular cytosol with important cellular functions. EF1-a has a very stable gene expression in A. salmon and is therefore well suited as a reference gene (Lock et al., 2010).

All Ct values were normalized to the mean of the reference gene. All calculations of fold gene expression (fold change) were based on this. Ct data from RT-qPCR was transferred to a spreadsheet in excel. To analyse the data, the change in the target gene in relation to the reference gene was calculated (Ratio) using formula 2 described in (Pfaffel, 2001).

Formula 2: The formula shows the mathematical model for producing a relatively expressed ratio by Real Time PCR. Etarget is the real-time PCR efficiency of a target gene. Eref is the real-time PCR efficiency of a reference gene. ΔCPtarget is the deviation between the control value minus the value of the sample from the target gene. ΔCPref is the deviation between the control value and the value of the sample from the reference gene.

$$ratio = \frac{(E_{target})\Delta CP_{target}(controll - sample)}{(E_{ref})\Delta CP_{ref}(controll - sample)}$$

2.7 Radioimmunoassay (RIA)

Radioimmunoassay (RIA) is an immunological analysis method where radioactive labelled and non-radioactive molecules which compete, reversibly, for binding to an antibody specific to the molecule of interest (PerkinElmer n.d.). This makes it possible to determine small concentrations of, for example, steroid hormones (Schulz, 1985) In the present study, RIA was used to analyse cortisol blood plasma concentrations. The method was performed according to Tveiten et al. (2010). A brief outline of the method is found below.

2.7.1 Cortisol extraction

Cortisol was extracted from 200 µl of blood plasma using diethyl ether (DEE) under vigorous shaking for four minutes. This created a water phase (plasma) and an organic phase (DEE + cortisol) in the test tube. Liquid nitrogen was used to freeze the water phase so that the organic phase (DEE) with dissolved cortisol could be decanted into a new glass tube. The new glass tubes with only organic phase were placed in a water bath at 45 C˚ until the ether had evaporated to drynessTo reconstitute the extract, 600 µl of RIA buffer (1L: 1g gelatine, 10.67 g Na2HPO4 sudium phosphate dibasic dihydrate, 9 g NaCl, 3.87 g NaH2PO4 + H2O, 1ml Na-acid (5%), in 1000 ml MilliQwater) was added and the glass tube was vortexed and incubated in the 45 C˚ water bath for 5 min. The sample/extract was vortexed a second time and transferred to 2 ml eppendorf tubes. Extracts were stored at -20 C˚ until the day of analysis.

2.7.2 Cortisol RIA

For plasma cortisol quantification by RIA tritiated (^{3}H) and non-tritiated cortisol (here the antigen), and an antibody against cortisol raised in rabbit was used according to Tveiten et al. (2010) were further assay characteristics and antibody cross-reactivity is described. Briefly, the antibody was used at a 4000 x dilutionwhich gives approximately 50% binding to the radioactive labelled cortisol under non-competing conditions. The total amount of radioactivity added was equivalent to *c.* 4500 counts per minute (c.m.p).
The standard curve was made by diluting a stock solution of cortisol (500ug / ml ethanol) in RIA buffer to 40 ng / ml. The dilution of 40 ng / ml was labelled S9 and further diluted 1: 1 to S1. Thus, 1 ml from tube S9 was pipetted into tube S8 containing 1 ml of RIA buffer and mixed well before 1 ml from S8 was pipetted into S7 etc. until S1 (0.15 ng / ml). A standard curve was created for each assay to account for possible inter-assay variation.

After incubating antibody and the labelled and non-labelled (standard or unknown sample) cortisol overnight in borosilicate glass tubes at c. 4C, 300 µl Charcoal buffer (DCC÷ 100ml RIA buffer, 0.1g dextran T70, 1g activated carbon) were added in all tubes-to bind free antigen (not bound to the antibody) that was still in the solution. After adding the charcoal solution, the tubes were incubated for five minutes before centrifugation (4200 rpm for 5 minutes) to form a charcoal pellet with free antigen. Then the supernatant, where antigen was bound to the antibody, was decanted into labelled counting tubes. Tubes of Total bound (Tb; binding of the labelled cortisol under non-competing conditions) and non-specific binding (NSB; efficiency of charcoal extraction/background radioactivity) were also included, see appendix V for components. Tb tells how much antibody binds to the tracer (3H, labelled cortisol) without competition. NBS was made to check how much activity was left after charcoal buffer extraction.

All counting tubes were supplemented with 7 ml of scintillation fluid (Ultima Gold XR scintillation fluid) and mixed well before counting in scintillation counters for β-radiation (Tri-Carb 2900TR, Perkin 18 Elmer, Illinois) at NFH. The result from the count was entered in an excel spreadsheet and the concentrations of cortisol were calculated based on the standard curve.

2.8 Statistical Analysis

All data was organized and processed in Microsoft Excel from 2016.
Statistical analyses and graphs were done in GraphPad Prism version-9.3.1 (2022). Data from all groups (i.e., sterile males, sterile females, fertile males and fertile females) within each tissue and gene were entered into the GraphPad prism. First, all data sets were checked for outliers before further statistical analysis. Outliers are samples that deviates significantly from the rest of the data within the same group. Any "outliers" were identified using ROUT method (Motulsky & Brown, 2006). In this case, Q was set to 0.1%, on all data to remove statistical outliers. This means the method aims that no more than 0.1% of identified outliers are fake and that 99.9% of identified outliers are actual outliers (outliers shown in appendix VI).
Next, the outlier cleaned data was checked for normal distribution. The Shapiro-Wilk test was used for evaluating normality since it is well suited for smaller sample numbers (<50). Significance level alpha was set to 0.05. In those cases where deviations from the normal distribution were found, the data sets were log (10) transformed to achieve a normal

distribution, if possible (Feng et al., 2014), with a follow-up Shapiro-Wilk test. Log (10) transformation resulted in normal distribution in most cases and only few data sets were ultimately not normally distributed. Results from normal distribution are shown in appendix VII for the experiments. A Levene's test was used for testing homogeneity of variance (homoscedasticity). Thus, most data had a distribution that met the criteria for use of parametric statistics (Løvås. 2018). For the few data sets that deviated from the normal distribution, non-parametric statistics was used (Løvås. 2018). Log (10) transformed data were used for statistical analysis where relevant, while the original data were used for representation of the figures. The figures show the average +/- "Standard error of mean" (SEM). A two-way (group x time-point) ANOVA (Analysis of variance) (Leuvås, 2018) was run on normally distributed and log normally distributed data to determine if there was a statistically significant difference in expression of different genes at different time-points between groups (sterile and fertile females and male fish).

A Tukey-test procedure was chosen as a post-hoc test for comparison between groups at given sample point and over time within respective groups. This test assumes that data are normally distributed (Motulsky, 2022a). The test calculates the average of each dataset and compares each average with all other averages. This test allows for uneven sample size. The Tukey test considers the spread of all groups, when several averages are compared, the differences between the average and the amount of spread to each group are also compared. This is quantified using the information obtained from all the groups, which gives more power to detect differences. The results from the Tukey test are stated as statistically significant ($P < 0.05$) or not statistically significant (Motulsky, 2022a). In this study, Tukey post hoc test was used to determine where the indicated significant differences between groups, and time was placed. Significant differences between the groups were marked with different letters in the graphs.

Non-parametric tests were also performed, for data that did not meet the normality requirement even when log (10) transformed. The Kruskal-Wallis test was used in this case. In this test, a P-value was calculated to show about what the chances are that the sum of the rankings is as far apart as they do by random sampling (Motulsky, 2022b). Dunn's post-hoc test was used in these cases.

To check for correlation between immune genes a Pearson correlation test in GraphPad Prism was done. The significance was set at $P < 0.05$. The correlation analysis says something about the covariation between two or more variables (Motulsky, 2022b) but do not imply any functional relationships. If the combined data (sterile and fertile, males and females) for two

variables resulted in a significant correlation further investigation were carried out to reveal whether these relationships were influenced by fertility or sex. This was done by running correlations for different sex and fertility backgrounds separately. A priori evaluation of genes, which were assumed to have a biological/physiological relationship (known to influence their reciprocal expression and/or being a part of the same regulatory pathway) was chosen for correlation analysis. Thus, not all possible combinations of genes were investigated. Cortisol, which is known to have an important role in development of osmoregulatory capacity/smoltification and in responses to stress, is also known to influence the expression of a range of genes within the immune system, was given special emphasis.

3 Results

Gene Expression

All the isolated RNA samples were of good quality and had a 260/280 ratio between 1.9 and 2.1, see appendix VIII. The isolated RNA was without any genomic contamination as seen from positive controls from the qPCR. The reference gene expression was very stable in all the groups at different time points and the tissue studied.

3.1 Smoltification experiment T1-T3

Results from two-way ANOVA interaction analysis for all the genes studied for the smoltification experiment are shown below in table 2.

Tabell 2: ***The two-way ANOVA results for the smoltification experiment****. The table shows an overview of the two-way ANOVA interactions between time and group (sterility and sex) in head kidney and gills for all genes studied in the smoltification experiment, time point T1-T3 using GraphpadPrism. ns= no significance p>0.05, *=p≤0.05, **= p ≤ 0.01, ***=p≤0.001, ****=p≤0.0001. Interaction shows the overall group interaction.*

Two-way ANOVA T1-T3			
Genes		**Head kidney**	**Gill**
IL1ß	Interaction	ns	ns
	Time	ns	ns
	Group (sex & sterility)	ns	ns
CATH-2	Interaction	*	ns

	Time	ns	ns
	Group	ns	ns
HSP70	Interaction	ns	*
	Time	***	*
	Group	ns	ns
sIgM	Interaction	ns	ns
	Time	ns	ns
	Group	ns	ns
TCR1α	Interaction	ns	ns
	Time	ns	**
	Group	ns	ns
IL10	Interaction	ns	ns
	Time	ns	**
	Group	*	ns
IFN-γ	Interaction	ns	ns
	Time	***	*
	Group	ns	*
TNF1α	Interaction	ns	ns
	Time	ns	ns
	Group	ns	ns
MHC I	Interaction	ns	ns
	Time	ns	ns
	Group	ns	ns

Interleukin 1 beta, *IL1ß*

The relative gene expression of IL-1ß in both head kidney and gills are presented in figure 3. The gene expression profile for IL-1ß in head kidney and gills showed no significant difference between time points or within time points for any of the groups (table 2 and fig 3). The expression of IL-1ß was quite stable around 1 fold expression for both sterile and fertile males and females throughout the smoltification process in both head kidney and gills as shown in figure 3.

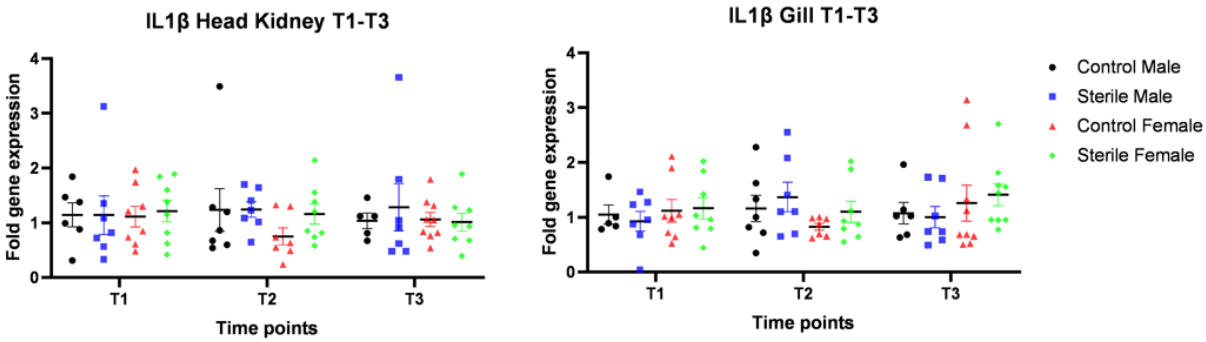


Figure 3: ***Fold gene expression of IL1ß in head kidney and gills during smoltification*** *(time points T1-T3) for Control Male (n=5-7), Sterile Male (n=5-7), Control Female (n=5-9), and Sterile female (n=5-9) A. salmon. The mean ±SEM (Standard Error of Mean) is shown for each group. The fold gene expression for individual fish in each group is shown in the interleaved scatter graph as coloured points.*

Tumor necrosis factor alpha1, *TNF1α*

The relative gene expression of TNF-1α in both head kidney and gills are presented in figure 4. The gene expression of TNF-1α, in the head kidney and gills, showed no significant difference within groups between time points or between groups within time points (there was no significant interaction between time and group) and was quite stable around 1-2 fold throughout smoltification. controls groups. (Table 2 and Fig: 4).

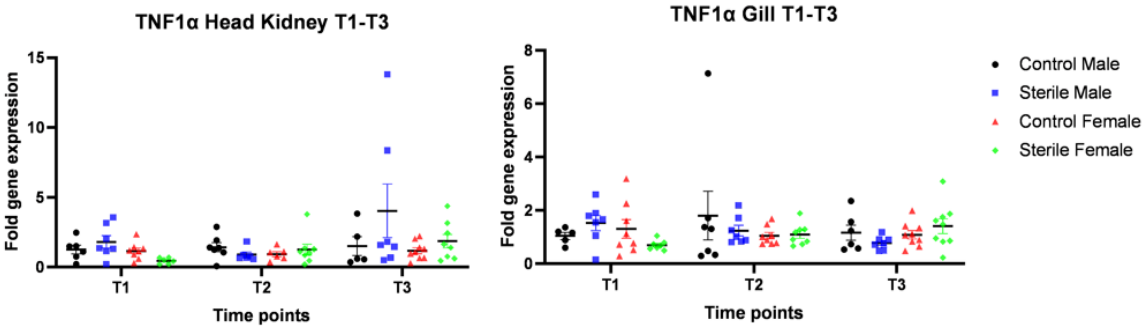


Figure 4: ***Fold gene expression of TNF1α in head kidney and gills during smoltification*** *(time points T1-T3) for Control Male (n=5-9), Sterile Male (n=5-9), Control Female (n=5-9), and Sterile female (n=5-9) A. salmon. The mean ± SEM is shown for each group. The fold gene expression for individual fish in each group is shown in the interleaved scatter graph as coloured points.*

Cathelicidin antimicrobial peptide, *CATH-2*

The relative gene expression of CATH-2 in both head kidney and gills are presented in figure 5. The two-way ANOVA (table 2) showed overall interaction effect on the expression in head kidney, indicate that expression changed differently between groups over time, but the post hoc test was not able to reveal any such difference between comparable groups. The expression of CATH-2 in the head kidney and gills showed some variation, particularly at time points T1 and T3, but with no significant difference. At time point T2, the CATH-2 expression was stable around 1 fold in all the sterile and fertile groups. Furthermore, no significant differences were observed between time points as seen in figure 5. Overall, neither time nor group had any significant influence on the CATH-2 expression during smoltification.

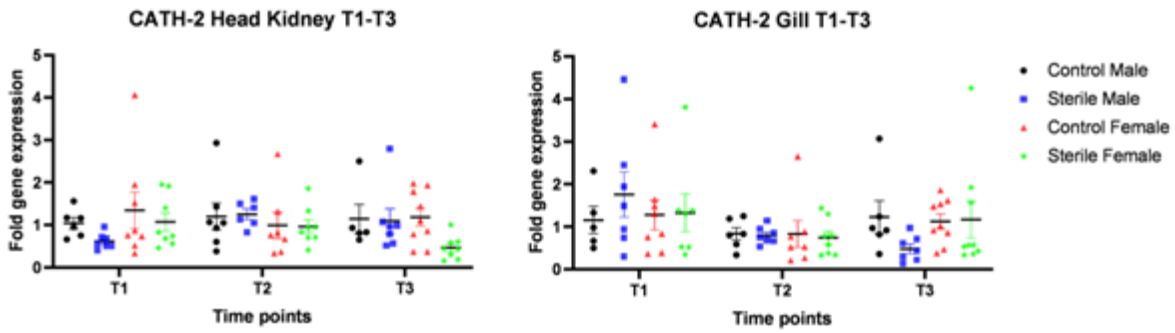


Figure 5: ***Fold gene expression of CATH-2 in Head kidney and gills during smoltification*** *(time points T1-T3) for Control Male (n=5-7), Sterile Male (n=5-7), Control Female (n=5-9), and Sterile female (n=5-9) A. salmon. The mean ± SEM is shown for each group. The fold gene expression for individual fish in each group is shown in the interleaved scatter graph as coloured points.*

Heat shock protein 70, HSP70

The relative gene expression of HSP70 in both head kidney and gills are presented in figure 6. In head kidney there was found an overall significant (decreasing) effect of time (progress of smoltification) on HSP70 expression (ANOVA Table 2), which was particularly apparent for the sterile female group (Fig.6). However, these changes did not result in any differences between fertile and sterile groups within any of the time points (Fig. 6). In gills, HSP70 expression in sterile males increased transiently (from T1 to T2) but was not significantly

elevated at T3 (Fig 6). At T1 in gills, HSP70 expression was significantly lower in sterile males than that of sterile females but not significantly differ compared to the fertile fish (Fig. xx). No other significance difference was observed for the groups over time or within time points for HSP70in the tissues (Fig 6).

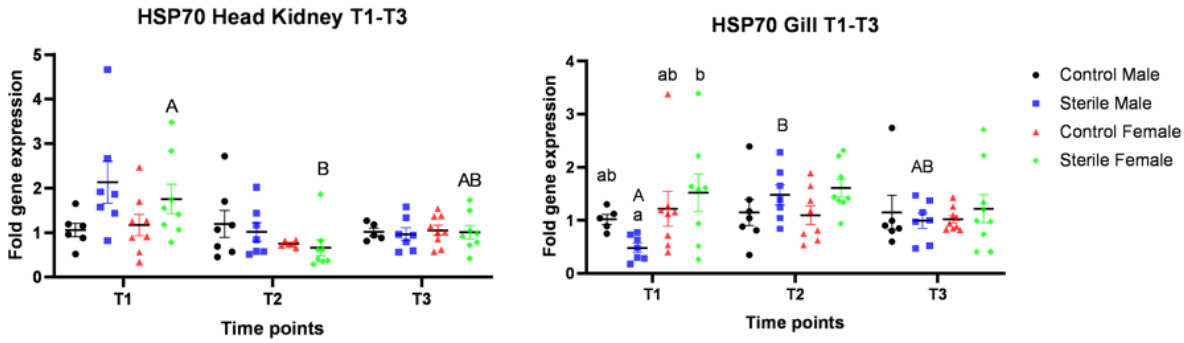


Figure 6: ***Fold gene expression of HSP70 in Head kidney and Gills during smoltification*** *(time points T1-T3) for Control Male (n=5-9), Sterile Male (n=5-9), Control Female (n=5-9), and Sterile female (n=5-9) A. salmon. The mean ± SEM is shown for each group. The fold gene expression for individual fish in each group is shown in the interleaved scatter graph as coloured points. For significance within a time point lower case letters marks the differences (p<0.05). For significance for groups between time points, uppercase letters mark the difference (p<0.05). Where it is not marked with letters there was found no significant difference.*

Secretory immunoglobulin M, sIgM

The relative gene expression of sIgM in both head kidney and gills are presented in figure 7. No significant difference in IgM expression were found between any of the control and sterile groups at any smoltification time point. The expression of sIgM in the head kidney and gills showed some variations within time points, particularly in the gills but also in T3 of head kidney, but with no significant difference. At time point T1 and T2, sIgM expression was stable (~ 1fold) in all the sterile and fertile groups, in head kidney, with the sterile groups at T3 time point expressing marginally higher than its fertile controls. The expression of sIgM followed approximately the same trend in gills as in head kidney for the female groups, while the sterile males where slightly higher expressed at T1 compared to T2 and T3 time points without it being significant. Furthermore, no significant differences were observed between time points as seen in figure 7.

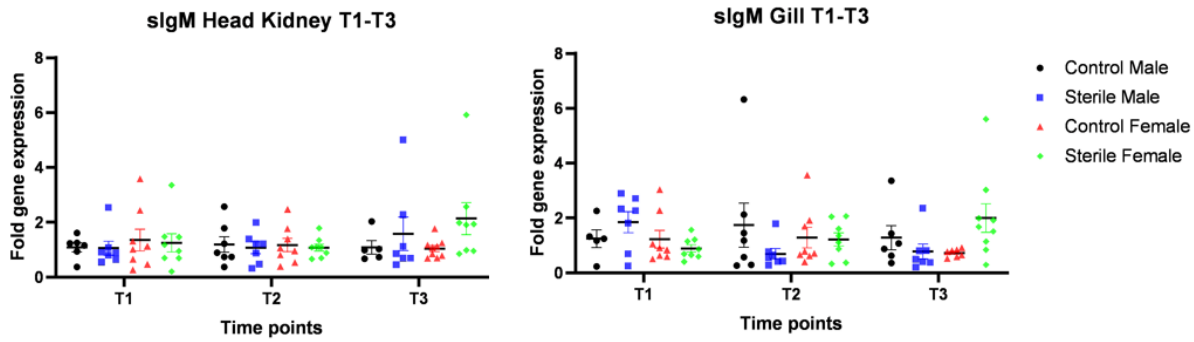


Figure 7: ***Fold gene expression of sIgM in Head kidney and Gills during smoltification*** *(time points T1-T3) for Control Male (n=5-7), Sterile Male (n=5-8), Control Female (n=5-9), and Sterile female (n=5-9) A. salmon. The mean ± SEM is shown for each group. The fold gene expression for individual fish in each group is shown in the interleaved scatter graph as coloured points.*

T-cell receptor 1-alpha, TCR1α

The relative gene expression of TCR1α in both head kidney and gills are presented in figure 8. The two-way ANOVA interaction table (table 2) showed that the time factor had significant effect on the TCR1α expression in gill tissue, but it was not discovered where this differences for comparable groups was in post hoc. The expression of TCR1α in the head kidneys and gills showed no significant difference between time points or within time points between any of the groups (sterile and fertile) in any of the tissues. The expression of TCR1α was quite stable around 1-2 fold through all the time points during the smoltification for all groups in both tissues, while the general trend of sterile expressing marginally higher at time point T2 than fertile controls, this was also the case for T1 in gills and T3 in head kidney without any significant difference (Fig: 8).

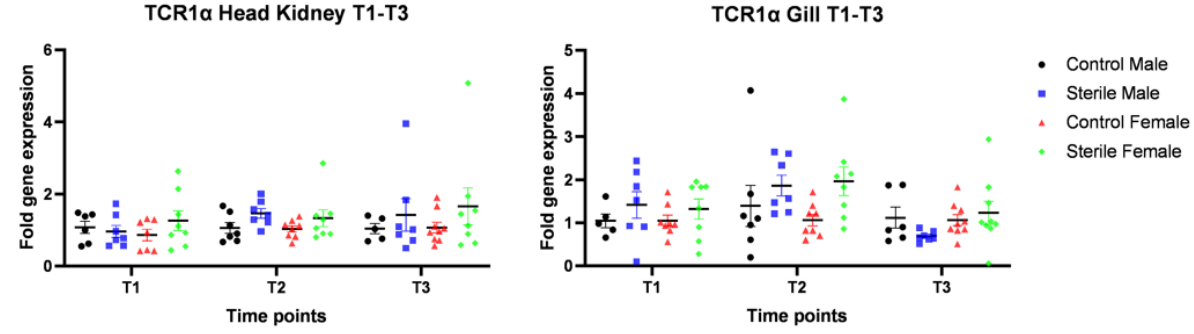


Figure 8: ***Fold gene expression of TCR1α in Head kidney and Gills during smoltification*** *(time points T1-T3) for Control Male (n=5-9), Sterile Male (n=5-9), Control Female (n=5-9), and Sterile female (n=5-9) A. salmon. The mean ± SEM is shown for each group. The fold gene expression for individual fish in each group is shown in the interleaved scatter graph as coloured points.*

Major Histocompatibility complex I, MHC I

The relative gene expression of MHC I in both head kidney and gills are presented in figure 9. The expression of MHC I in both head kidney and gills showed some variations between groups at a particular time points, but with no significant difference between sterile and fertile groups. The Anova did not find any overall interaction between time and group, but there was discovered a significant difference in post hoc. The expression of MHC I was quite stable around 1fold except for time point T2 where the males expressed 2 fold expression compared to females, without it being of significance. MHC I expression in head kidney, showed significant difference within the sterile male group over time. MHC I expression in sterile male significantly increased (P=0.0391) from T1, at the start of smoltification, to T3, at the end of smoltification, but did not significantly differ from sterile male at T2 (Fig 9.).

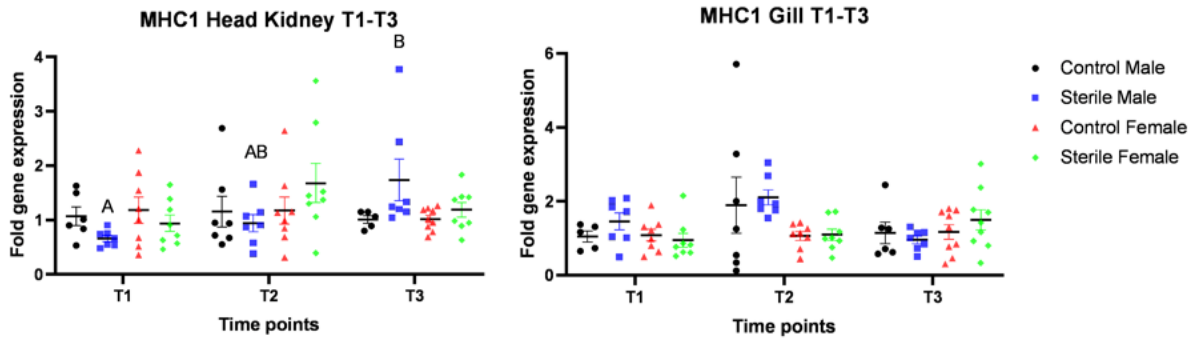


Figure 9: ***The fold gene expression of MHC I in Head kidney and Gills during smoltification*** *(time points T1-T3) for Control Male (n=5-7), Sterile Male (n=5-7), Control Female (n=5-9), and Sterile female (n=5-9) A. salmon. The mean ± SEM is shown for each group. The fold gene expression for individual fish in each group is shown in the interleaved scatter graph as coloured points. For significance within a time point lower case letters marks the differences (p<0.05). For significant between time points, uppercase letters mark the difference (p<0.05). Where it is not marked with letters there was found no significance.*

Interleukin 10, IL10

The relative gene expression IL10 in both head kidney and gills are presented in figure 10. The ANOVA showed that time had significantly affected the result in gill tissue while the group (sterility and sex) had significantly affected the result in head kidney tissue during smoltification but it was not discovered significant differences for comparable groups in post hoc. Only the time effect in gill tissue was seen in the post hoc, see figure 10. The gene expression of IL10 showed no significant difference between sterile and fertile (control) groups within a particular time points in the two tissues, even though there where some differences due to individual variations. In head kidney the expression of IL10 is quite even around 1 fold at T1, while the male groups (control and fertile) express higher at T2 as females are still stable around 1 fold expression. In contrast, at the later stage of smoltification i.e., T3 the fertile males stabilise around 1 fold again whereas the sterile female now expresses higher like sterile male, resulting in the sterile group expressing marginally more IL10 at the end of the smoltification T3 in head kidney than the fertile control group. This is not the case in the gill tissue where the expression IL10 is more scattered with higher individual differences within groups, particularly at T1 and T2 time points. Over time during the smoltification process, IL10 expression in gills showed significance difference between the sterile female group. Sterile female group in T1, at the start of smoltification, had a significantly higher expression (P=0.0338) of IL10 than sterile female in T3, at the end of smoltification, but did not significantly differ from sterile female of T2. The expression of IL10 of all the groups inn gill has a declining trend from time point T1 untilT3 with sterile female being the only one resulting in significance (Fig: 10).

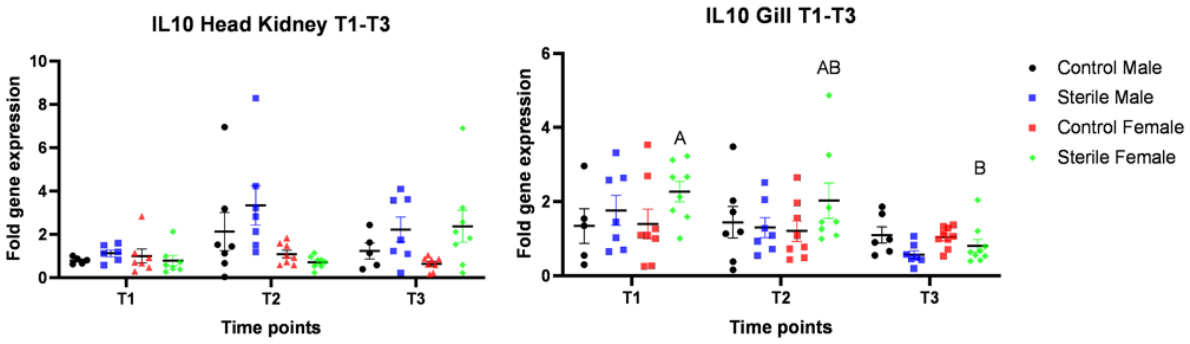


Figure 10: ***Fold gene expression of IL10 in Head kidney during smoltification*** *(time points T1-T3) for Control Male (n=5-7), Sterile Male (n=5-7), Control Female (n=5-9), and Sterile female (n=5-9)*

A. salmon. The mean ± SEM is shown for each group. The fold gene expression for individual fish in each group is shown in the interleaved scatter graph as coloured points. For significance within a time point lower case letters marks the differences (p<0.05). For significant, between time points, uppercase letters mark the difference (p<0.05). Where it is not marked with letters there was found no significance.

Interferon-gamma, *IFN-γ*

The relative gene expression of IFN-γ in both head kidney and gills are presented in figure 11. For IFN-γ there was variation within time points, but there was not found any significant difference between fertile and sterile groups in any of the tissues. In the head kidney, there was found a significant difference between the sterile female group over time. The expression of IFN-γ increased significantly (P=0.0058) from T2 to T3 for sterile female but had no significance difference from that of sterile female of T1. This increase of expression in sterile females at time T3 was most likely due to two higher expressing individuals that drew the mean up for sterile female in T3. In the gills, IFN-γ was expressed similarly to that of the head kidney at early smoltification time point, T1 with sterile, expressing higher, around 2 fold, than the fertile controls at 1 fold, although not significant. Moreover, in gills, sterile male showed a decreasing trend of IFN-γ expression over time during smoltification. Sterile male expressed significantly higher IFN-γ expression at T1 (P=0.0105) at the start of smoltification, compared to T3, at the end of smoltification, without significantly differing from T2 sterile males (Fig. 11). As shown in the interaction table (table 2), in head kidney, there was found a significant effect of only time factor, but sterility and sex factor had no effect. Time could have a possible influence on the expression of the IFN-γ gene in the head kidney during smoltification. On the other hand, gills showed significant effect at both the individual factor level i.e., time; sex and sterility, indicating the possibility that both the factors could influence IFN-γ gene expression in gills over time. but the overall interaction of both the above factors has no significant effect on IFN-γ expression in the gills.

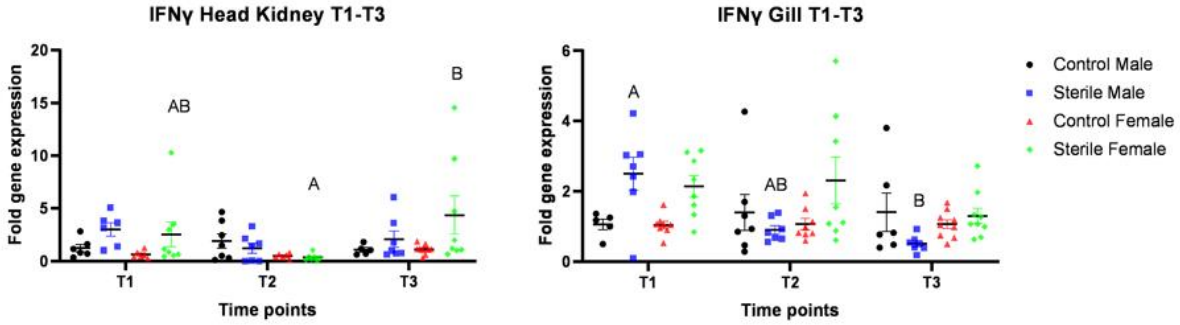
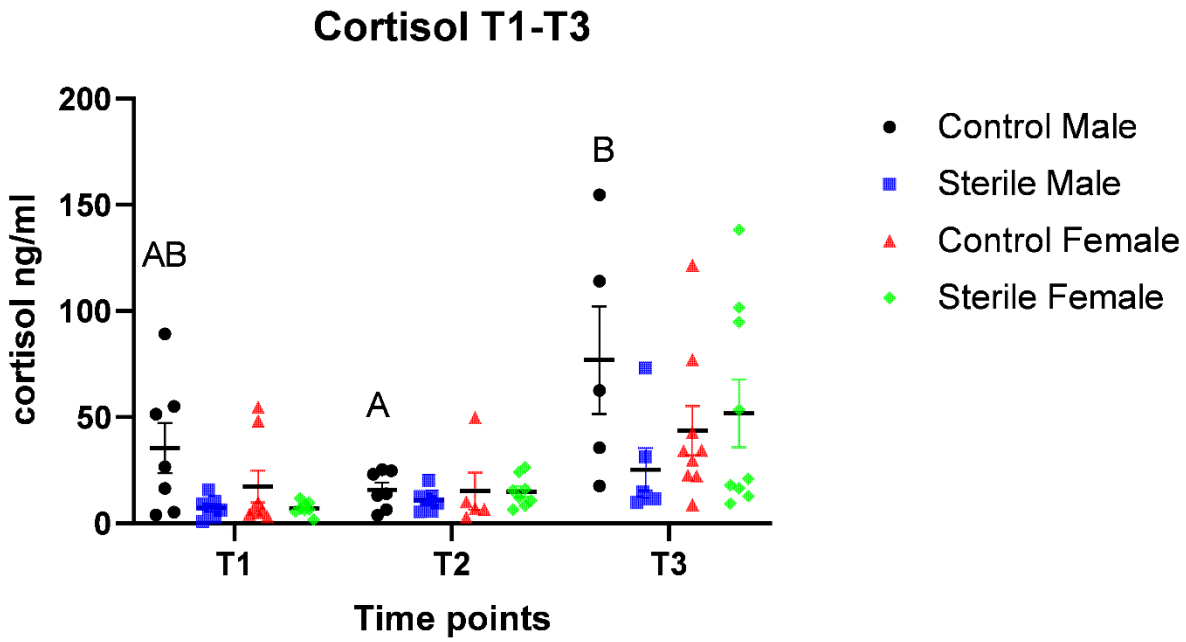


Figure 11: ***Fold gene expression of IFN-γ in Head kidney and Gills during smoltification*** *(time points T1-T3) for Control Male (n=5-7), Sterile Male (n=5-7), Control Female (n=5-9), and Sterile female (n=5-9) A. salmon. The mean and ± SEM is shown for each group. The fold gene expression for individual fish in each group is shown in the interleaved scatter graph as coloured points. For significance within a time point lower case letters marks the differences (p<0.05). For significant between time points, uppercase letters mark the difference (p<0.05). Where it is not marked with letters there was found no significance.*

Cortisol T1-T3

The cortisol concentration did not show any significant difference between sterile and fertile control salmon during smoltification. Overall, plasma cortisol changed significantly over time during smoltification (Fig. 12B), but at the group level this was apparent (statistically) only for fertile males (Fig. 12A). The general trend of cortisol level is higher at the end of smoltification i.e., at the T3 time point, for all groups. Although the ANOVA revealed an overall group effect on plasma cortisol, the post hoc test could not reveal any statistical difference between comparable groups at any of the time points (Fig. 12). The individual variation in plasma cortisol concentrations tended to increase at the end of smoltification (T3).

A



B

	Plasma Cortisol T1-T3		
	Interaction	**Time**	**Sterility and Sex**
	P-value summary	**P-value summary**	**P-value summary**
Cortisol	ns	****	*

Figure 12: ***A) Plasma cortisol concentrations (ng/ml) during smoltification (time points T1-T3)*** *for fertile Male (black dots, n=5-7), Sterile Male (blue squares, n=5-7), fertile Female (red triangles, n=5-9), and Sterile female (green diamond, n=5-9) A. salmon. Individual concentrations, mean and ± SEM is shown for each group. Individual plasma cortisol concentrations within different groups are shown in the scatter graph with coloured points. Lower case letters indicate significant differences ($p<0.05$) between groups within time points whereas uppercase letters indicate significant difference ($p<0.05$) between time points within groups. Where it is not marked with letters there was found no significance.*

B) The two way ANOVA results from the smoltification experiment. *The table show the overall interaction results between time and; sterility and sex, analysed by two-way-ANOVA using GraphpadPrism for cortisol at time points T1-T3. ns= no significance $p>0.05$, *=$p\leq0.05$, ****=$p\leq0.0001$.*

3.2 Transportation Stress Experiment T0-T24

Results from two-way ANOVA interaction analysis for all the genes studied for the transportation stress experiment are shown below in table 3.

*Tabell 3: Show the overall interaction between time and, group results from the two-way-ANOVA in head kidney and gills at time points T0-T24 using GraphpadPrism. ns= no significance p>0.05, *=p≤0.05, **= p ≤ 0.01.*

Two-way ANOVA T0-T24			
Genes		**Head kidney**	**Gill**
IL1ß	Interaction	ns	ns
	Time	ns	ns
	Group (sex and sterility)	ns	*
CATH-2	Interaction	*	ns
	Time	ns	ns
	Group	ns	ns
HSP70	Interaction	ns	ns
	Time	ns	ns
	Group	ns	ns
sIgM	Interaction	ns	**
	Time	ns	ns
	Group	ns	ns
TCR1α	Interaction	ns	ns
	Time	ns	ns
	Group	ns	ns
IL10	Interaction	ns	ns
	Time	ns	ns
	Group	ns	ns
IFN-γ	Interaction	ns	ns
	Time	ns	ns
	Group	ns	ns
TNF1α	Interaction	ns	ns
	Time	ns	ns
	Group	ns	ns

MHC I	Interaction	ns	ns
	Time	*	ns
	Group	**	ns

Interleukin 1 beta, *IL1ß*

The relative gene expression of IL1ß pre and post transport is shown in figure 13. The head kidney and gill showed no significant difference between time points or within time points for any of the groups of sterile and fertile salmon (Fig. 13). The expression of IL1ß was quite stable pre and post transport stress around 1 fold for all groups in both tissues, even though there were some individual differences within the groups especially in the head kidney. In head kidney the sterile males and females seems to be expressing higher IL1β than fertile at T24 but without it being significant.

From the interaction table (table 3) there was discovered significant interactions by the two-way ANOVA for the IL1ß gene only for group (sterility and sex) in the gill tissue, but it was not discovered significant differences for comparable groups in post hoc.

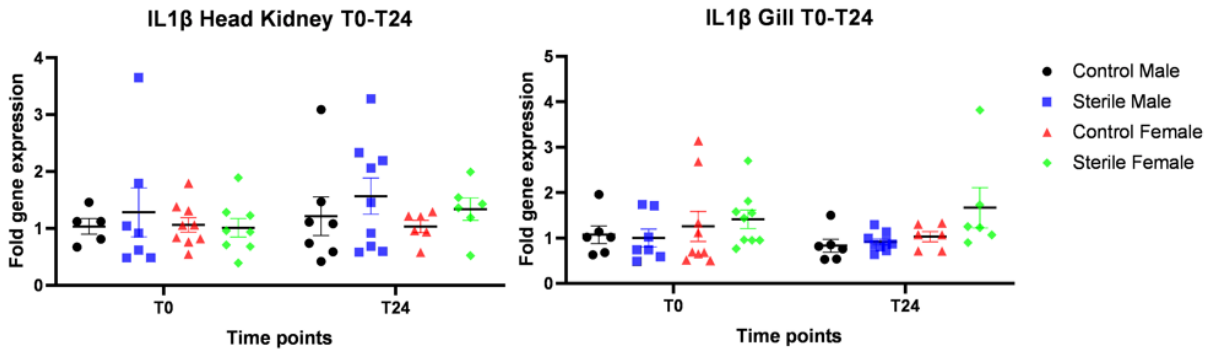


Figure 13: ***The Fold gene expression of IL1ß in Head kidney and Gills pre- and post-transport*** *(time points T0-T24) for Control Male (n=5-9), Sterile Male (n=5-9), Control Female (n=5-9), and Sterile female (n=5-9) A. salmon. The mean ± SEM is shown for each group. The fold gene expression for individual fish in each group is shown in the interleaved scatter graph as coloured points.*

Cathelicidin antimicrobial peptide, *CATH-2*

The relative gene expression of CATH-2 during the transport experiment is shown in figure 14. The expression of CATH-2 was quite even pre- and post-transport stress within the

groups, with some non-significant individual differences within groups thus driving the mean up. The result in head kidney showed a significantly higher expression of CATH-2 (p=0.0340) in sterile females post 24 h transport stress. There were no significant differences over time for any of the other groups, and there was not any significant difference between sterile and fertile groups within either pre or post transport time points. In the gills, there was not found any significant difference between the expression of CATH-2 over time or within time points for any of the groups of sterile and fertile salmon (Fig: x).

From the interaction table (table 3) there was discovered an overall significant interaction by the two-way ANOVA for the CATH-2 gene only in the head kidney tissue. Thus, there is indication of overall time and group (sterility and sex) together effecting the expression of this immune gene in head kidney pre and/or post transport but not at the individual factor level.

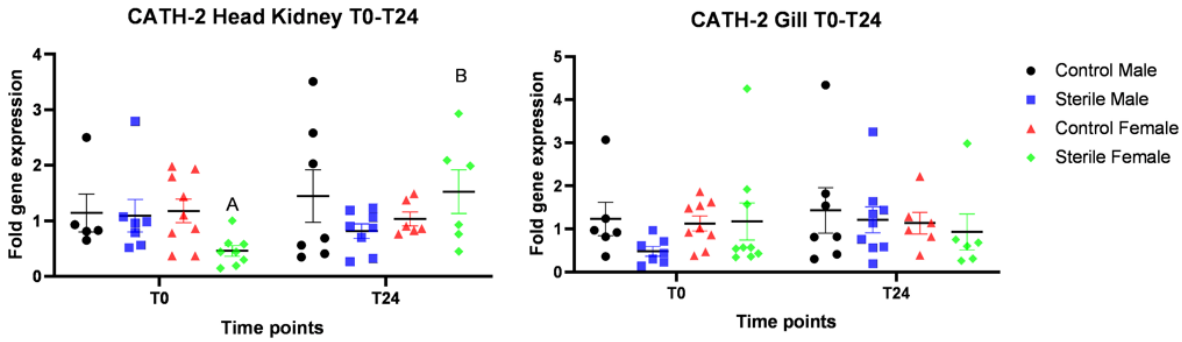


Figure 14: ***Fold gene expression of CATH-2 in Head kidney and Gills pre- and post-transport*** *(time points T0-T24) for Control Male (n=5-7), Sterile Male (n=7-9), Control Female (n=6-9), and Sterile female (n=6-9) A. salmon. The mean ± SEM is shown for each group. The fold gene expression for individual fish in each group is shown in the interleaved scatter graph as coloured points. For significance within a time point lower case letters marks the differences (p<0.05). For significant between time points, uppercase letters mark the difference (p<0.05). Where it is not marked with letters there was found no significance.*

Heat shock protein 70, HSP70

The relative gene expression of HSP70 in both tissues is shown below in figure 15. The expression of HSP70 did not reveal any significant differences between time-points or within time points for any of the groups of sterile and fertile salmon. The expression of HSP70 was stable (~1 fold) pre and post transport stress for all groups in both tissues.

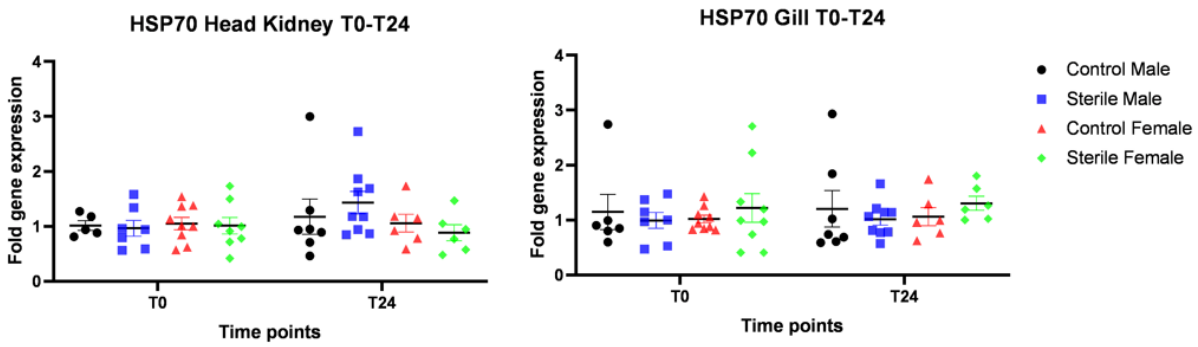


Figure 15: ***The fold gene expression of HSP70 in Head kidney and Gills pre- and post-transport*** *(time points T0-T24) for Control Male (n=5-7), Sterile Male (n=7-9), Control Female (n=6-9), and Sterile female (n=6-9) A. salmon. The mean ± SEM is shown for each group. The fold gene expression for individual fish in each group is shown in the interleaved scatter graph as coloured points.*

Secretory immunoglobulin M, *sIgM*

The relative gene expression of sIgM for both tissues are shown below in figure 16. There was not found any significant differences in sIgM expression between time points or within time points for any of the groups of sterile and fertile salmon in either tissue. The expression of sIgM showed some individual differences in expression within the groups without it being significant, particularly in gills. In the head kidney the expression levels were stable at both pre and post transport stress. The expression of sIgM in the gills for sterile female was lower post transport stress while the control female had increased but more scattered than sterile female. On the contrary, sIgM expression for sterile male is higher post transport stress than pre, while the expression for control male stays the same pre and post stress. All without being a significant difference.

From the interaction table (table 3) there was discovered an overall significant interaction by the two-way ANOVA for the sIgM gene only in the gill tissue. Thus, there is indication of overall time and sterility and sex influence on the expression of this immune gene in gills post transport.

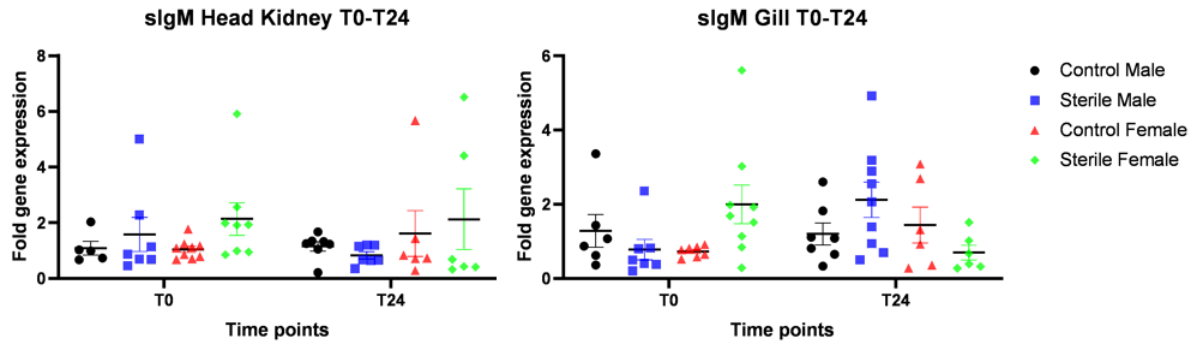


Figure 16: ***The fold gene expression of sIgM in Head kidney and Gills pre- and post-transport*** *(time points T0-T24) for Control Male (n=5-7), Sterile Male (n=7-9), Control Female (n=6-9), and Sterile female (n=6-9) A. salmon. The mean ± SEM is shown for each group. The fold gene expression for individual fish in each group is shown in the interleaved scatter graph as coloured points.*

T-cell receptor 1-alpha, *TCR1α*

The relative gene expression of TCR1α for both tissues is shown below in figure 17. There was not found any significant different expressions of TCR1α between time points or within time points for any of the groups of sterile and fertile salmon in any of the tissues. The expression of TCR1α was quite stable (~1 fold) pre and post transport stress for all groups, with some individual differences within each group without it driving the group to a significant difference. For sterile male in gills, the expression has marginally increased post transport stress but without it being significant.

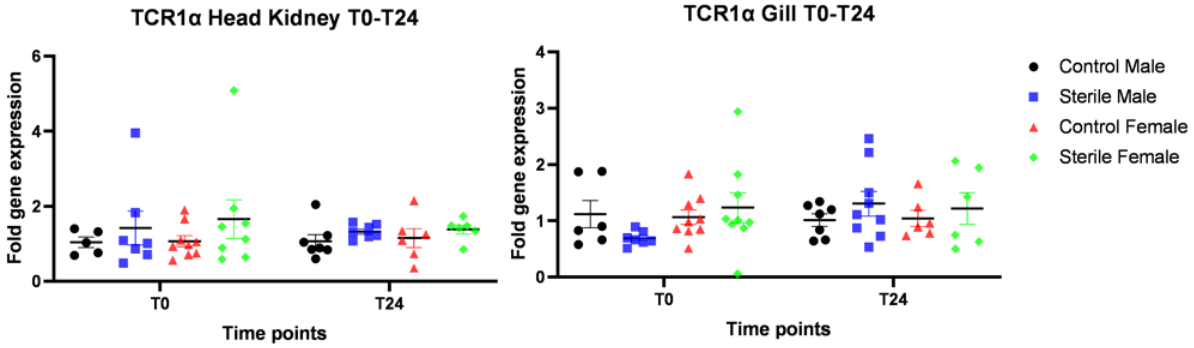


Figure 17: ***The fold gene expression of TCR1α in Head kidney and Gills pre- and post-transport*** *(time points T0-T24) for Control Male (n=5-7), Sterile Male (n=7-9), Control Female (n=6-9), and Sterile female (n=6-9) A. salmon. The mean ± SEM is shown for each group. The fold gene expression for individual fish in each group is shown in the interleaved scatter graph as coloured points.*

Interleukin 10, *IL10*

The relative gene expression of IL10 pre and post transport is shown in figure 18. The head kidney and gill showed no significant difference between time points or within time points for any of the groups of sterile and fertile salmon. The expression of IL10 in gills was quite even (~1 fold), pre and post transport stress for all groups, besides the sterile male showing lower expression (0.5) pre transport than the rest of the groups. In the head kidney, the sterile groups showed higher expression (~ 2 folds) before stress than fertile controls (~1 fold). Post transport stress, all the groups maintained the same expression levels as prestress except the sterile female where the expression had decreased to 1 fold post transport stress without being significant.

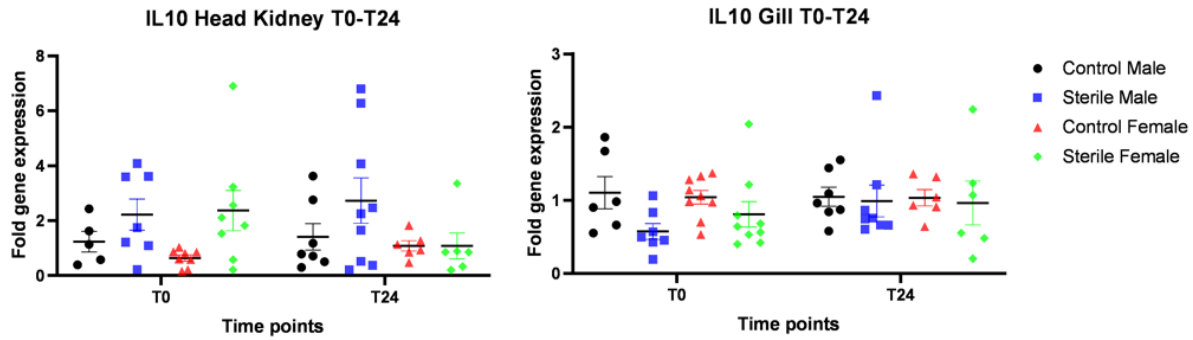


Figure 18: ***The fold gene expression of IL10 in Head kidney and gills pre- and post-transport*** *(time points T0-T24) for Control Male (n=5-7), Sterile Male (n=7-9), Control Female (n=6-9), and Sterile female (n=6-9) A. salmon. The y-axis shows the fold change of the gene expression, and the x-axis shows the time points. The mean and ± SEM is shown for each group. The fold gene expression for individual fish in each group is shown in the interleaved scatter graph as coloured points.*

Interferon-gamma, *IFN-γ*

The relative gene expression of IFN-γ pre and post transport is shown in figure 19. The head kidney and gill showed no significant difference between time points or within time points for any of the groups of sterile and fertile salmon. The expression of IFN-γ was quite similar pre and post transport stress for almost all groups in both tissues, with some fluctuations or variations due to some individual fish expressions.

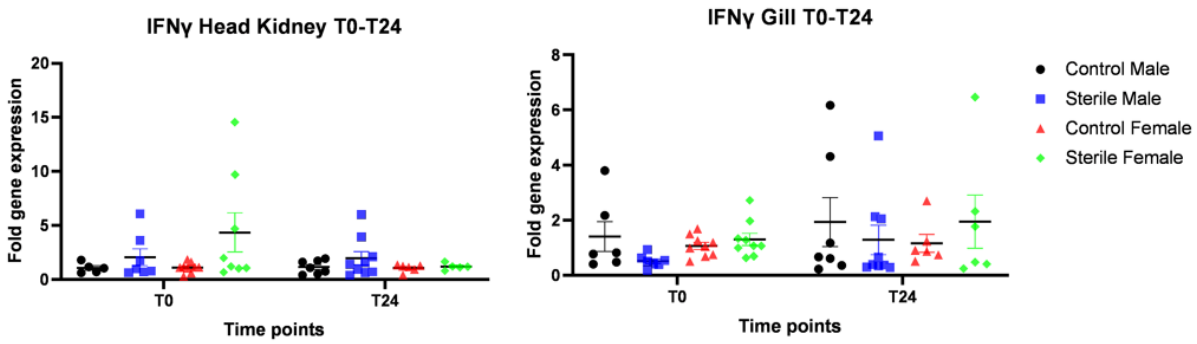


Figure 19: ***The fold gene expression of IFN-γ in Head kidney and Gills pre- and post-transport*** *(time points T0-T24) for Control Male (n=5-9), Sterile Male (n=5-9), Control Female (n=5-9), and Sterile female (n=5-9) A. salmon. The mean ± SEM is shown for each group. The fold gene expression for individual fish in each group is shown in the interleaved scatter graph as coloured points.*

Tumor necrosis factor alpha1, *TNF1α*

The relative gene expression of TNF1α pre and post transport is shown in figure 20. The head kidney and gill showed no significant difference between time points or within time points for any of the groups of sterile and fertile salmon. The expression of TNF1α was quite similar pre and post transport stress for all groups in head kidney, except for the sterile groups in gills there was a slight increase in expression of TNFα for some individuals at the post transport stress time point without it being of significant difference to the controls or pre transport corresponding groups.

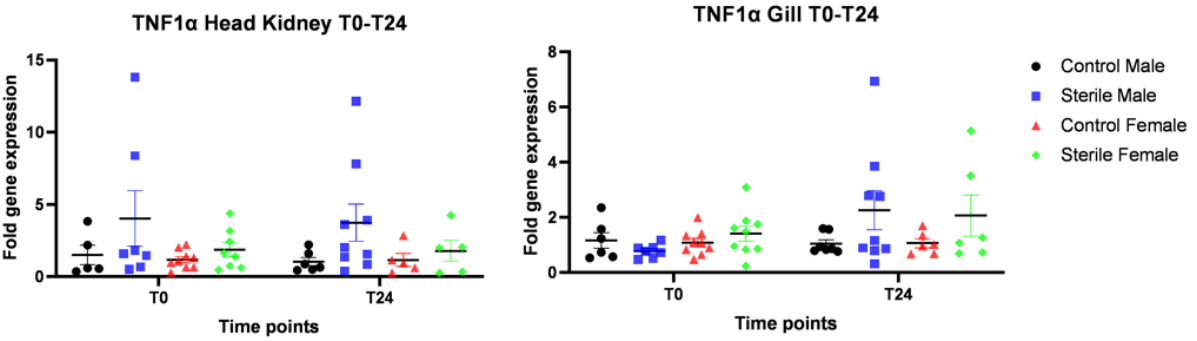


Figure 20: ***The fold gene expression of TNF1α in Head kidney and Gills pre- and post-transport*** *(time points T0-T24) for Control Male (n=5-7), Sterile Male (n=7-9), Control Female (n=6-9), and Sterile female (n=5-9) A. salmon. The mean ± SEM is shown for each group. The fold gene expression for individual fish in each group is shown in the interleaved scatter graph as coloured points.*

Major Histocompatibility complex I, *MHC I*

The relative gene expression of MHC I pre and post transport is shown in figure 21. The gill showed no significant difference between time points or within time points for any of the groups of sterile and fertile salmon. The expression of MHC I was quite similar around 1 fold pre and post transport stress for all groups except a slight decrease in expression for sterile female at T24 post-transport stress compared to pre transport stress but not significant. Similarly, this decreasing trend was also seen in the head kidney for sterile female group post stress, but here it was found to be significant (p=0,0255) compared to T0 time point in sterile female group. In addition, in head kidney, there was found significant differences between the sterile male and female groups within time point T24, where sterile male had significantly higher MHC I expression(p=0.0017) compared to sterile females while not significantly differing from any of its corresponding fertile control groups of T24... Within time point T24 post-transport stress there was significant difference between sterile male expressing higher than that of sterile female at T24. As shown in the interaction table (table 3), there was found a significant effect in head kidney at both the individual factor level i.e. time as well as Group (sex and sterility) indicating the possibility that both the factors individually might have an influence the result of MHC I gene expression in head kidney but the overall interaction of both the above factors has no significant effect on MHC I expression.

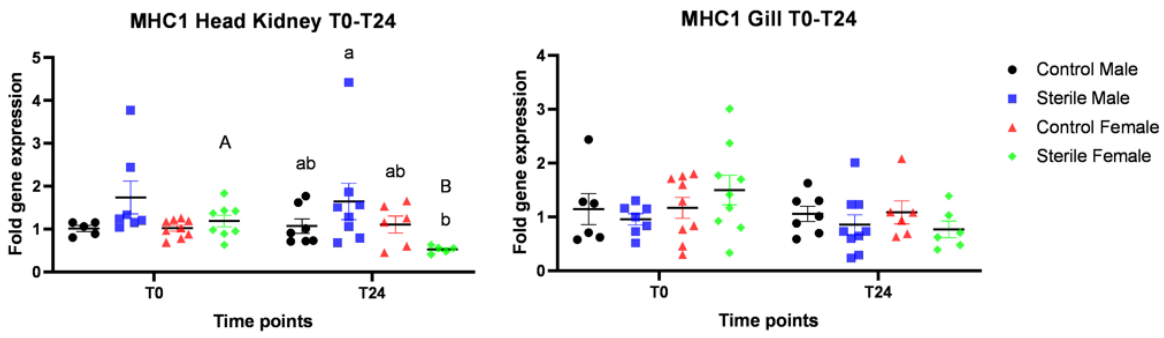
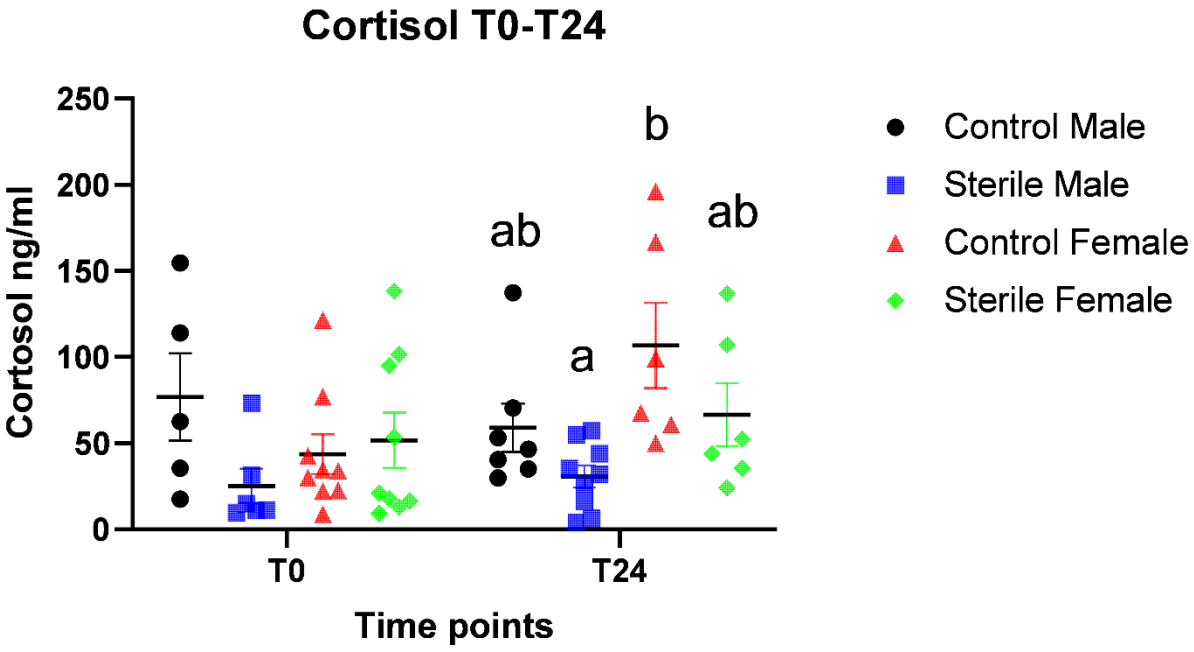


Figure 21: ***The fold gene expression of MHC I in Head kidney and Gills pre- and post-transport*** *(time points T0-T24) for Control Male (n=5-7), Sterile Male (n=7-9), Control Female (n=6-9), and Sterile female (n=5-9) A. salmon. The mean ± SEM is shown for each group. The fold gene expression for individual fish in each group is shown in the interleaved scatter graph as coloured points. For significance within a time point lower case letters marks the differences (p<0.05). For significant between time points, uppercase letters mark the difference (p<0.05). Where it is not marked with letters there was found no significance.*

Cortisol

The level of cortisol pre and post transport are shown below in Fig 22. Before transportation, plasma cortisol did not differ significantly between groups. Plasma cortisol concentrations post transport stress did not differ significantly from pre transport values for any of the groups (Fig 22). A significant effect of group was detected in the ANOVA (Fig. 22B) and could be due to the significantly higher cortisol concentrations in control females compared to that of sterile males at the 24h post-stress timepoint. Such difference(s) was not apparent compared to the other groups (Fig. 22) and cannot really be compared biologically. Post transport levels of cortisol were generally higher in the female groups than that of the corresponding male groups, and generally higher in fertile control vs. sterile fish, although not significantly according to the post hoc tests (Fig: 22).

A



B

	Plasma Cortisol T0-T24		
	Interaction	**Time**	**Group**
	P-value summary	**P-value summary**	**P-value summary**
Cortisol	ns	ns	*

Figure 22: ***A) The Amount (ng/ml) of Cortisol pre- and post-transport*** *(time points T0-T24) for Control Male (n=5-7), Sterile Male (n=6-9), Control Female (n=6-9), and Sterile female (n=6-9) A. salmon. The mean ± SEM is shown for each group. The fold gene expression for individual fish in each group is shown in the interleaved scatter graph as coloured points. For significant differences between groups, within a time point lower case letters marks the differences (p<0.05). For significant differences, between time points, uppercase letters mark the difference (p<0.05). Where it is not marked with letters there was found no significance.*

B) The two way ANOVA results from the transport stress experiment *show the overall interaction results between time and; group (sterility and sex), analysed by two-way-ANOVA using GraphpadPrism for cortisol at time points T1-T3. ns= no significance p>0.05, *=p≤0.05.*

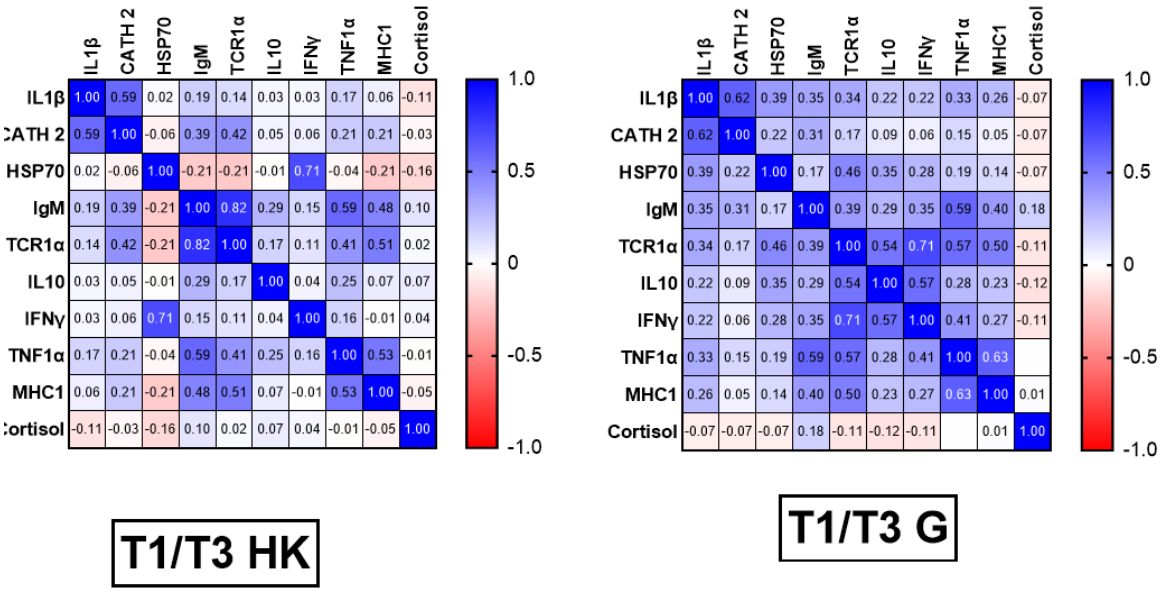
3.3 Correlation result

The correlations reveal possible relationships between gene expression at the individual level, correlational heat- maps containing gene specific correlation coefficient ("r") values were generated in Grafpad Prism (2020), as shown in figure 23 and 24. The r-values for the smoltification experiment are shown in figure 23 for head kidney and gills, while the transportation stress experiment is shown in figure 24 for the two tissues.
Overall, the heatmaps shows that both in head kidney and gills, many of the genes are positively correlated. There were few genes that expressed strong negative or positive correlations with each other where the r had a higher explanation degree. With explanation degree it is meant that the variation in one gene explains the variation of the other gene and vice versa. The percentage of explanation degree was found by squaring the r value.

The correlation results showed that there were no strong correlations found between cortisol and any of the immune genes but illustrated a weak negative correlation. r values for cortisol correlation in the smoltification experiment varied from -0,16 to 0,18 and -0,31 to 0,22 in the transport stress experiment as seen in heatmaps figure 23 and 24.

From the smoltification experiment the highest r-values in head kidney were 0,82 between TCRa1 and sIgM with an explanation degree of 67%, and r= 0,71 between HSP70 and IFNg with an explanation degree of 50% as seen in the heatmaps in figure 23. In gills, the highest r-values (> 0.6) during the smoltification were 0,71 between TCR1α and IFNg with the explanation degree of 50%, and 0,63 between TCR1α and MHC I with explanation degree of 39% as seen for heatmaps in figure 23.

From the transport stress experiment the highest r-value in head kidney was 0,83 between MHC I and sIgM with the explanation degree of 68%, and the highest r-value in gills was 0,69 between CATH-2 and ILB1 with the explanation degree of 47%, as seen in the heatmaps figure 24.



Figur 23: The heatmaps showing correlation between all the studied genes and cortisol levels from the smoltification experiment. The heatmap shows the result from a Pearsons correlations test determining the Pearson r-value between all the immune genes and cortisol in both the head kidney and gill tissues. In addition to the r-value the colour of each square determines the strength of the correlations. The stronger blue colour the closer the r-value is to 1, indicating a positive correlation. The stronger red colour the closer the r-value is to -1, indicating negative correlation.

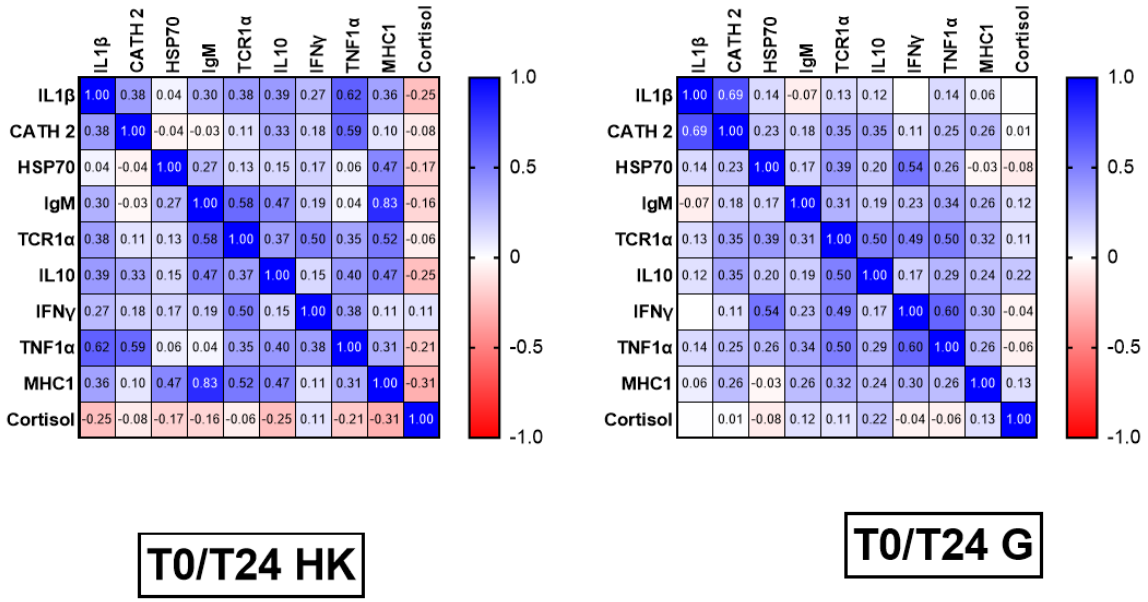


Figure 24: The heatmaps showing correlation between all the studied genes and cortisol levels from the transport experiment. The heatmap shows the result from a Pearsons correlations test determining the Pearson r-value between all the immune genes and cortisol in the head kidney and gill tissue separately. In addition to the r-value the colour of each square determines the strength of the correlations. The stronger blue colour the closer the r-value is to 1, indicating a positive correlation. The stronger red colour the closer the r-value is to -1, indicating negative correlation.

In addition to the heatmaps with r-value, p-value were also generated for all correlations between the immune genes and between immune genes and cortisol to show significant correlation with p set to 0.05. The p-values of all the immune genes and cortisol is shown in appendix IX: figure 25.

During the smoltification experiment the gill heatmap shows a much higher number and stronger positive correlations between the immune genes than that found for head kidney figure 23. The p-value revealed 27 significant correlations within the immune genes in gills and 12 in head kidney, as shown in appendix IX, table 4. Of these significant correlations, 11 of the correlated genes were common in both tissues during the smoltification experiment, as shown in the appendix IX appendix table 4,with explanation degree.

On the other hand, the heatmaps of the transportation stress experiment showed a slightly higher amount of stronger (positive) correlations in the head kidney than that in the gill tissue (Fig. 24). The p-value revealed 14 significant correlations within the immune genes in gills and 21 significant correlations in head kidney including the one between cortisol and MHC I, see appendix IX: figure 26. Among these correlations, 9 of the correlated genes were common

in both tissues during the transportation stress experiment, as shown in appendix IX: Table 5. The significant correlation of cortisol and MHC I was researched further even though it was only found in the head kidney tissue during the stress experiment, with an explanation degree of 9% for the negative correlation. Upon investigation the possible significant correlation seems to have been driven by sterile salmon data points, see appendix IX: table 8.

Only the genes that significantly correlated ($p<0.05$) in both tissues were tested further to check if these relationships were influenced by either sex or sterility. Thus, these relationships where further tested on the basis of sex (females and males separately) or fertility (sterile or fertile fish) separately. The results showed that both sex and fertility did influence relationships between expression of certain genes (appendix IX:Tab. 6 and 7) Interestingly, this was particularly apparent within the gill (T1/T3) tissue where males scored more (10 vs. 4) significant correlations than females (appendix IX: Tab. 6). On the other hand, within the same tissue, fertile fish (11 vs. 2) scored more significant correlations than sterile fish (Tab. 6). The biological background between gene known to be in the same pathway were taken into account when selecting the correlations for discussion. An overview of all correlations present in both tissue during both experiments can be seen in appendix IX as well as if significance was brought on by gender or sterility or both in.

4 Discussion

Using sterile fish in salmon production could improve the sustainability, productivity, and animal welfare. In this study we explored the immune status of the sterile salmon, produced by germ-cell ablation, in comparison to that of fertile A. salmon. Here, it was investigated whether the elimination of germ cells had any influence on the expression of some key genes related to the immune system during two challenging processes in the early part of the production cycle. To the knowledge of the current author, expression of the immune genes of germ cell free sterile A. salmon have not been reported until present. Smoltification was included in our study, since it is a critical period in the life of A. salmon where massive endocrine regulation redirects the osmotic balance and induces dramatic changes in metabolism, morphology, behaviour and immune response (Björnsson et al., 2011; Krasnov et al. 2021). Measurment's of cortisol was also included in our study because of its key roles in controlling osmoregulation during smoltification and its critical role in mounting a normal stress response. Cortisol levels is needed to understand how sterile salmon's physiology and immune function could be affected. Thus, before new methods for salmon sterilization can be

taken into commercial use, it is important that the sterile fish performs equally well to that of fertile fish. This is especially important with respect to key body functions like the immune system when the fish is under challenging physiological conditions. An immune status which is similar to that of fertile fish, may ensure that sterile salmon can handle physiological conditions and stress encountered during a production cycle in a satisfactory way.

In this study, we propose that the expression of immune genes in germ free sterile salmon is similar compared to fertile control fish during smoltification and transport stress. Moreover, no significant difference between sterile groups and fertile salmon groups has been detected within the same time point. Making it plausible that sterile salmon have the same possibility of immune genes to perform their functions as fertile salmon. There has, on the other hand, been found significant differences within groups over time/ between time points for certain groups, indicating that some immune genes change their expression during smoltification, but that these changes are dependent on both sex and fertility. The overall gene expressions for both the fish groups during this experiment are all generally low as would be expected for fish that has only been in contact with a strictly controlled environment seemingly without pathogens or infections.

4.1 The Smoltificaton Experiment

The smoltification analysis showed that almost all the genes were stable over time during smoltification for all fish groups (1-2 folds). No significant differences were found between sterile salmon and fertile control salmon at any of the given timepoints for the genes studied. Thus, it seems that the removal of germ cells has had little to no effect on the expression of these genes for sterile A. salmon during smoltification in gill and head kidney tissues. There was however found that the gene performance for HSP70, MHC I, IL10 and IFN-γ had an effect within sterile group gender during the smoltification experiment.

The general consensus surrounding immune regulation throughout smoltification seems to be associated with down regulation, but with some exceptions. Johansson et al., 2016 rapports of finding systemic repression of the expression of immune genes that manifests in smoltification and continues to decrease after sea water transfer. One notable exception was Ig's that increased (Johansson et al., 2016). Krasnov et al., 2021 also support a down regulation of many immune genes during smoltification, except some pro-inflammatory immune genes that got upregulated in gill tissue (Krasnov et al, 2021).

This was not the case for this thesis where the overall result was no significant up- or downregulation in fertile A. salmon for the immune genes studied during smoltification. This could possibly be due to difference in practises during the smoltification process such as temperatures, feed, ways of seawater transfer, sampling methods, tissues and genes included in the overall conclusion, salmon family, sources of error during this thesis and so on.

4.1.1 The expression of immune genes

4.1.1.1 Immune genes without significant differences

For the following genes, no significant difference was discovered during the smoltification experiment. The expression of these genes has not shown to be affected by sterility, gender or smoltification during this experiment.

The expression of **TCR1a** in this experiment remained stable around 1-2 fold . TCR1 alpha is important in the T-cell immunity and initiate T-cell maturation to cytotoxic T-cells (CD8+a) when interacting with antigen presented by MHC I. In studies done with A. salmon the expression of TCRa in skin and head kidney before parasite infection was also expressed low (Tadiso et al., 2011). In previous studies done with A. salmon smolts the non-virus-challenged controls expressed low TCRa, around 1 fold in both gill and head kidney tissues (Ingerslev et al., 2009). The smolt in Ingerslev's study was held in sea water at 12°C and vaccinated with Trippel forte. Although their smolt have been held in sea water concentrations, and this thesis smolt samples from freshwater, the low expression of TCR1a found in A. salmon during this thesis smoltification experiment seem to be in line with the basal fold expressions of A. salmon smolt of the above-mentioned studies. In the study Johansson (2016) of it was reported a general down regulation of TCR during smoltification, where smolts expressed around 1 fold, and stabilization first after sea water transfer for fertile salmon (Johansson et al., 2016). There was not observed any down regulation during smoltification for TCR1a in this thesis smoltification experiment. It would seem like the sterile salmon is equally equipped with TCR for T-cell activation as fertile salmon throughout smoltification.

IgM is important for the innate first line of defence in the mucosal layer of fish. Ig's are a type of glycoprotein molecules, produced by B-lymphocytes, that binds with affinity for certain specific antigens. Secretory Ig's are specialized to neutralize pathogens to maintain the homeostasis of the mucosal tissues such as gills (Abbas et al., 2020, p.). sIgM **expression** in fertile and sterile A. salmon in this thesis expressed around 1 fold, with some groups

expressing upwards to two at some timepoints. This somewhat matches the results found in rainbow trout before infection of bacteria, where the basal relative expression was around 1 fold in gills, skin and gut mucosa (Makesh et al., 2015). Durings studies on diploid and triploid chinook salmon the expression of IgM was also at a low fold prior to infection. Post smolt in the experiment of Nuñez-Ortiz et al (2018) showed a low fold of IgM (0,5-1,5 folds) prior to infection with virus, similarly to what was discovered in this thesis for smolt Ig's. Johansen et al (2016) reports of basal levels of sIgM for smolt and parr to show relative expression of upwards to 2 fold and did not significantly differ from each other. This was also the case for the sIgM expression in this thesis where it was stably expressed throughout smoltification. The antibodies perform various effector functions in the of the immune system. Ig's neutralizes antigens it finds in the body, activating the classical complement system and promote leucocyte-dependent destruction of a microbe (Abbas et al., 2020, p.261). It seems like sterile salmon is equally equipped to handle antigens by sIgM as fertile salmon throughout smoltification both in head kidney and gills.

IL1b expression around 1 fold throughout the smoltification experiment. IL1b has been shown to lay around 2 folds prior to seawater transfer in pre smolt and express around 0,5 fold at seawater transfer in intestine tissue (Wang et al., 2020). In another multigene expression assay by Krasnov et al (2020) it was not found a difference in the expression of IL1B in gills from smolt vs controls pooled from healthy salmon (Krasnov et al., 2020). For gill and head kidney tissue the gene expression lays around 1 fold for non-challenged controls in Ingerselev et al., study (2009), at the first sampling point for post smolt. The stable expression throughout smoltification in this thesis is within the expression span found for A. salmon as mentioned above but does not display any significant down regulation for smolt compared to pre-smolt AS the intestine tissue in Wang et al., (2020). Interleukins are molecules involved in the intracellular regulation of the immune defence and work as signalling proteins. IL-1ß is a pro inflammatory cytokine that has been identified in salmonids (Secombes et al., 2011). It does look like the sterile salmon is equipped for inflammation activation as fertile salmon during smoltification according to results displayed in this thesis.

Tumor necrosis factor 1 alfa (**TNF 1a**) is an immune regulatory cytokine that is identified in A. salmon. This specific type of TNF seems to be teleost specific (Zou et al., 2016). TNF1α displays overlapping functions with IL-1β and enhance microbial killing activity of macrophages and the phagocytic activity of fish leucocytes (Zou et al., 2016). The TNF

expression of sterile and fertile salmon in this thesis where about 1 with some groups expressing upwards to 2 folds. This is within what levels vas reported in Jensen et al (2019). In Jensen's study (2019) the basal TNF alpha expression in A. salmon expressed around 1-2 fold for non-infected A. salmon at throughout smoltification from gill and head kidney tissue (Jensen et al., 2019). It seems like the sterile salmon is equally equipped to perform TNFs biological effect of pro inflammatory as fertile salmon during smoltification.

CATH-2 expression was stabile around 1 fold in the smoltification experiment. There are few studies done to determine the basal levels of CATH-2 throughout smoltification in A. salmon. Krasnov 2020 discovered a slight upregulation of cathelicidin in normal smolt in fresh water, around 1 fold was observed (Krasnov et al., 2020). In another experiment the expression of CATH-2 was stable during smoltification in groups of normal oxygen levels (Krasnov et al., 2021). Basal CATH-2 expression in juvenile A. salmon has been reported by Esteves et al (2018) from 0-2 folds in skin and gills. According to Estévez CATH2 expression is highly dependent on what A. salmon family one studies, and they observed that IPNV resistant families of A. salmon showed lower basal levels of CATH-2 then non-resistant in gills (Estévez, 2018). The salmon in this thesis experiment have been vaccinated with alphaject-6 (standard 6-component-vaccine), that can protect against IPN, this could be a factor as to why the expression of CATH-2 is low in the smoltification experiment in general. Regardless, it does not look like sterile, nor fertile salmon is affected by smoltification and display the same possibility for early antimicrobial immune responses during smoltification.

4.1.1.2 Genes with significant time or gender differences

For **HSP70** there was found a time effect in sterile fish, where sterile female decreased in head kidney and males increased in gill during smoltification. There was no systematic trend for this decrease in the sterile gender groups overall, but it is possible that smoltification has affected the expression of HSP70 in sterile males and females in their corresponding tissues.

In studies done with hsp70 expression compared to cortisol levels it is apparent that HSP70 increased by heat shock is down regulated when cortisol levels have risen. As we know there is a natural rise in cortisol levels related to the evolvements during smoltification, that could possibly have affected the low expression of HSP70 and possibly down regulation of HSP70 for sterile females in head kidney during the smoltification experiment. Reports form Robertson show a fold change of 2 from liver tissues of smolt A. salmon (Robertson & McCormick, 2012). In a report form Johansson et al (2016) HSP expressed around 4 fold in

intestine tissue for smolt and showed upregulation from the pre smolts. (Krasnov et al 2020) also reported upregulation of HSP70 for normal smolt in fresh water. This was somewhat in line with the result found in this thesis experiment, where the overall mean expression was around 1-2 fold and some individuals within groups expressed upwards to 4. There was not however, a significant increase in HSP70 at the end of smoltification for any groups.

For the gill tissue data, the post hoc test showed significant difference in the expression of HSP70 between sterile males and sterile females in T1. Sterile females expressed significantly higher than that of sterile male. This could indicate a gender difference in sterile fish where females express higher HSP70 at the start of smoltification.

Few articles comment on gender differences in A-salmon when it comes to the expression of HSP70. In one study of chinook salmon there was discovered significant differences between the genders, where the control females expressed generally higher HSP70 than males from liver cells (Afonso et al., 2003). The same trend is found for sterile females during smoltification in this thesis. It could be that germ cell ablation makes the sterile female fish than sterile males. The difference could also be due to individual differences as two individuals of sterile female expressing higher and drive the mean up. There is no significant difference seen between fertile males and females, and also no significant difference between sterile and fertile groups.

4.1.1.3 **MHC I T1-13**

Although MHC I showed no interaction in the ANOVA analysis, there was discovered a significant difference over time for the sterile males during post hoc testing. Sterile males expressed MHC I significantly higher at the end of smoltification than at the start. This could mean that smoltification has affected the expression level of MHC-I in sterile males.It is possible that this time effect could be by chance due to individual differences as one individual is expressing higher (4folds) than the rest (1-2 folds). There is a lack of significance from ANOVA and no observed systematically change corresponding in both tissues that could explain or strengthen the increasing in sterile males. There was also in (Ingerslev et al (2009) study observed that MHC-I is expressed low for post smolt 7 days after seawater transfer. This was also the case in (Johansson et al. 2016) The MHC I expression in smolts (exposed to 6 weeks of constant light) was around 1 fold in gill and 2 fold in head kidney. This matches the mean folds observed for all MHC-I expressions result for this this experiment.

4.1.1.4 **IL10 T1-T3**

During the smoltification sterile females where showed a significant reduction from pre smolt to post smolt. Sterile females where significantly lower expressed and individuals with less variation in expression. This was not the trend in head kidney. IL10 is expressed by many different cell types and performs its main task as an anti-inflammatory cytokine by downregulating other cytokines. There are few studies on the effects of IL10 in fish and they have not yet concluded that IL10 has the same anti-inflammatory effects in fish as in mammals (Seppola et al., 2008).

In an experiment with Wang et al (2020) The expression of IL10 was upwards to 4 folds from samples of intestine in late freshwater stages of smoltification for salmon salar. Wang et al., has also reported a lowering of the expression of immune genes (in intestine) for fish transferred to sea water. During Wangs, experiment, the controls went from 4 folds expression in freshwater to 2,5 in sea water (Wang et al., 2020). During this thesis smoltification experiment some individuals in groups lay relatively high (upwards to 4 for) but the mean remained 2 folds. In the gill tissue in this thesis experiment there is a slight downregulation to approximately 1 fold of the IL10 expression towards the end of smoltification. This general trend is not seen in the head kidney tissue off the smoltification experiment. Although the trend is not systematic, it is possible that mucosal tissues such as gills and intestine express a lower amount of IL10 at the end of smoltification in both sterile and fertile fish alike.

In Jensen's study (2019) the smolt status of A. salmon was investigated including IL-10 gene expressions during infection with IPVN. Of the non-infected groups A. salmon expressed around 1 fold for the group exposed to week 1 on continuous light ie smoltification start. slightly rising to about 1,5 folds for the group exposed to 3 weeks of light and about 2 folds for groups exposed to 6 weeks light found in head kidney tissue. Showing a slight increase during smoltification. (Jensen et al., 2019). This somewhat matches the findings for IL10 expression in head kidney tissue of this thesis experiment. .

4.1.1.5 IFN-γ T1-T3

Throughout the smoltification experiment it was not discovered any significant differences between sterile and fertile salmon at any timepoint regarding the expression of IFN-y. Overall, the groups displayed low levels of IFN-y from 0,5-2,5 folds. It seems like the ablation of germ cells do not affect the expression of the IFN- γ and it is plausible that the

sterile fish have the same ability for cell mediated immune response during viral infection as the fertile controls.

IFN-y was expressed stably trough smoltification for the fertile salmon groups around 1-fold, and no significant up or down regulation was discovered. This aligns with discoveries found in other studies of A. salmon where the expression of IFN-y did not significantly increase or decrease for parr and smolt (Jensen et al., 2019). In Jensen's research there was not found an effect on smoltification on the expression of IFN-y. In Jensens's research (2019) there was displayed an IFN-y expression of 2,5 fold for salmon exposed to 1 and 3 weeks of continuous light 7 days post transfer to sea water. Fish exposed to 6 weeks of continuous light expressed about 3 folds at the end of smoltification for non-infected A. salmon smolts groups from head kidney tissue (Jensen et al., 2019). In vaccination studies done to indicate the presence of T-helper cells, the basal expression of IFN- γ for A. salmon was low and lay around 2 folds in head kidney pre and 30 days post vaccination (Kumari et al., 2013). These results somewhat align with the results of IFN-y expression observed in head kidney and gill tissue in this thesis results. It does not seem like smoltification has had an effect on the expression of IFN-y in fertile salmon in this thesis either.

There was however found a time effect on sterile females in head kidney tissue and sterile males in the gill tissue. Where females increased and male decreased during smoltification without significantly differing from other groups over time. This could indicate that smoltification has had an effect on the expression of IFN-y for sterile groups in their respective tissue. There is little literature supporting the significant increase of INF-γ at the end of smoltification. It is plausible that this significant difference over time for the sterile female and males are coincidental, as of low n (6-8) and some individuals within groups expressing excessively high while the rest of the individuals of the groups express from 0-2 folds. IFN is supposed to increase in expression when exposed to pathogens. This could mean that individual fish for some reason had an immune reaction, or that the immune system of sterile female is lightly upregulated at the end of smoltification in head kidney tissue. In contrast the sterile females are most upregulated pre smoltification (T1) in the gill tissue and collect itself around 2 fold at the end of smoltification (T3). Further studies with more samples of the sterile genders could help clarify this effect on sterile genders during smoltification.

4.1.2 Cortisol during the smoltification experiment T1-T3

Cortisol hormones are believed to have momentarily negative effects on the immune status of fish and expression of immune genes (Espelid et al., 1996). It is normal to see an increase in corticosteroids, including cortisol, during the smoltification of salmon (Specker & Schreck, 1982; Redding et al., 1984; Nilsen et al., 2008; McCormick, 2012), as the salmon undergoes the natural changes it needs to survive in the transition from fresh water to sea water (McCormick, 2012). During the stress experiment plasma cortisol levels displayed lower in pre smolt than in post smolt although not significantly. All groups besides control males display around the normal cortisol levels expected towards the end of smoltification, 30-50 ng/mL (Iversen et al.,2005). There was no significant difference between sterile and fertile salmon within time, so it is seems like that sterile salmon have the same ability for cortisol hormone production.

According to previous thesis by Jensen (2020) in collaboration with the "Salmosterile" project, the sweater tolerance of this type of germ cell free A. salmon was tested, under the same conditions as this thesis. It was found that there was no significant difference between sterile and fertile controls regarding the sea water tolerance and it was concluded that sterile A. salmon had the same seawater tolerance as fertile normal salmon. This align with our assumptions of no significant differences between fertile and germ cell free A. salmon at the end of smoltification of our study, and the cortisol levels are a good indication that the sterile fish has also developed normally to seawater tolerant smolt.

During the smoltification it is expected a rise in cortisol, and one would believe that the immune system would and could be repressed as is often observed with high cortisol levels during stress. In this thesis this seems to not be the case as most of the genes are stably expressed throughout the smoltification. During smoltification the levels of growth hormone (GH) and insulin-growth-factor-1 (IGF1) increase (Dickhoff et al., 1997; Prunet et al., 1989; McCormick et al., 2009) and these have shown to have a direct positive effect on the immune system of teleosts. These hormones induce reduction of inflammation, tissue repair, and maintains the production of IgM and leukocytes (Sakai et al., 1996; Yada et al., 1999; Yada, 2007). the direct positive effects of GH and IGF1 may counteract the negative effects of cortisol on the immune system during smoltification. This could be the reason that no negative effects of cortisol have been observed in this thesis smoltification experiment.

4.2 The Stress Experiment

The Aquaculture production used today involves several stress factors for the fish. If the fish is not capable of handling such stressors it can result in several unfortunate consequences for the fish welfare and financial losses for companies. Fish that cannot tolerate the physiological changes of stress often die before the production cycle is over. Although the farms try to limit stressors, they are mostly unavoidable in the production used today. The ability to handle stressors are therefore an important quality in farmed fish. Both to ensure the fish welfare, but also for a sustainable and economic production.

The stress experiment analysis showed that almost all the genes expressed were stable (1-2 fold) pre and 24 hours post transportation stress. Some individual variations were found where individual fish group expressed higher or lower before and after the stress, but the mean of all the fish did not express any significant differences pre or post stress within or between time points. No significant differences were found between sterile and fertile salmon for any of the genes in this study. Ther was also not found any significant difference over time for any of the groups for the following gens; HSP70, sIgM, TCR1α, TNF1α, IL10, IL1β, and IFN-γ . Since there was not found any significance between the sterile groups and the fertile control groups of these genes at any time point, it seems like the removal of germ cells does not affect the expression of these genes for sterile A. salmon post transportation stress.

On the other hand, there was found some significant effect on the gene expression profile of MHC I and CATH-2 of groups when compared between pre and post transportation stress especially for sterile females in head kidney.

4.2.1 Gene expressions

4.2.1.1 Genes without differences

It is well known that stress and elevated cortisol relates directly to suppression of the immune system (Tort, 2011). Elevated concentrations of corticosteroids after stress has shown to reduce the immune status by affecting the number of lymphocytes and the capacity of antibody production (Van Weerd & Komen, 1998). There has been shown reduction in lymphocytes in association with increased cortisol levels in coho salmon (Melingen et al., 2002). Also, Apoptosis of B-cells in the head kidney and blood, as well as a down regulation of the immune system is shown in correlation with increased cortisol levels due to chronical stress responses in carp (Weyts et al., 1998, Verburg-van Kemenade et al., 1999). In Trout there had been registers increased levels of cortisol simultaneously as abnormally low values

of lymphocytes (Pickering, 1984). Heightened ACTH has shown to cause acute increase in TNFa and inhibition of IL1b expression in fish. Cortisol is shown to have reduced the expression of cytokines. This downregulation of cytokines where dose dependant for TNFa and IL1b (Castillo et al., 2009). For the following genes, no significant difference was discovered during the transport stress experiment. The general fold gene expression for the genes ley around 1 fold. The expression of these genes has not shown to be affected by sterility, gender or transport stress, or sea water transfer during this experiment. All of the genes where back to "normal" prestress expressions 24 hours post the transportation stress. This is in line with other studies that show that an increase in stress only represses the immune system momentarily (Tort, 2011) and no downregulation was observed 24 h post transport stress although some individuals and groups have somewhat higher cortisol levels post transport.

In Ingerslev et al, it was reported low levels of **IL-1B**, around 1 fold, for post-smolt at 7 days post sae water transfer (Ingerslev et al., 2009), equivalent of the pre stress salmon in the transport stress experiment. In this thesis experiment it was reported low levels of IL1b post stress transport around 1 fold. Here the results are compatible with Ingerslev, which can indicate that this gene is back to their basal- pre-stress expression level 24-hours post stress, as expected with short acute stress. Thus Sterile fish expression of IL1b probably have the same ability for pro-inflammatory responses as fertile salmon post transport stress.

IFN-γ mainly promotes cell mediated immunity against viruses (Zou & Secombes, 2011.) In Ingerslev et al, it was reported low levels of IFN-y, around 1 fold, for post-smolt at 7 days post sae water transfer (Ingerslev et al., 2009). During the stress experiment of this theses there were reported low levels of IFN, around 1-2 fold, with some individual deviation. Compared to Ingerslev et al. this is within the normal spectrum of what would be expected for the pre stress groups. As sterile salmon was back to their pre transport level after stress it looks like sterile fish have the same ability for cell mediated immunity of pro-inflammatory responses as fertile salmon after transport stress.

In Ingerslev et al., (2009) it was reported low levels of IL10, around 0-1 fold, for post-smolt at 7 days post sae water transfer (Ingerslev et al., 2009). This is equivalent of the fish at the pre transport stress timepoint of this thesis transport stress experiment. In an experiment with Wang et al (2020) the expression of IL10 was upwards to 4 folds from samples of intestine in late freshwater stages of smoltification for salmon salar. Wang has also reported a lowering of

the expression of immune genes (in intestine) for fish transferred to sea water to 2,5 fold in (Wang et al., 2020). The level of IL10 in this thesis stress experiment had a mean from 1-2 for all groups but there where individuals whitin groups that express upwards to 4 folds in the head kidney tissue. It is plausible that the sterile salmon have possess the same anti inflammatory ability as fertile salmon.

As mentioned previously, the expression of **TNF1a** has shown to downregulate as an effect of Cortisol in other fish species (Castillo et al., 2009). It does not seem like this was the case For A. Salmon in this in this transport stress experiment. As low levels of cytokine were an all-round observation it could have been the heightened There was not found correlation with cortisol the post stress timepoint investigated. There was not discovered any downregulation for TNFa1 post transport stress and it is likely that the sterile salmon have the same pro inflammatory abilities in regards to the effector function of TNF as fertiles.

The expression of **TCR1a** during the transport stress experiment was stably expressed at 1 fold in both tissues and is in line with the basal fold expression level prior of infection for A. salmon smolt (0-1 fold) (Ingerslev et a., 2009; Jensen et al., 2019), that is the equivalent of the pre stress timepoint in this thesis stress experiment. The expression of TCR1a is bac to its pre stress basal level 24 hours post transportation stress. It I plausible that the sterile A.salmon is equally equipped for T-cell activation as fertile A.salmon, and that transport stress does not affect TCR expression.

The sIg's are secreted by plasma cells (activated B-cells) in the mucosal barrier tissues and have the ability to perform in an external environment and upheld the microbiota homeostasis in the mucosal surface (Salinas et al., 2021). The **IgM** concentration in mucosa is lower than of that in serum. sIgM is less abundant that sIgT in mucosal layer on the gills but they do display similar effects of surrounding intruding pathogens (Salinas et al., 2011). Increased levels of cortisol related to stress have shown to induce reduction of immunity by influencing (lowering) the production of antibodies (IgM) (Van Weerd & Komen, 1998). The closest to a basal level for sIgM for post smolt after transport stress is the number Ingerslev reported in 2009. 7 days post sea transfer of smolt the fold was around 1(Ingerslev et al., 2009). The expression of sIgM in this thesis showed no significance lowering of the sIgMs in the groups 24 hours post transport stress and expressed around 1-2 fold, not to far from levels reported for salmon by Ingerslev. It does not look like cortisol release during acute stressors, such as

transport significantly influences the expression of sIgM in sterile and fertile groups post transport.

HSP70 expressed stably around 1 fold pre and post transport stress. Sterile salmon seemingly have the same ability of cellular stress response as fertile post transport stress. According to Poltronieri et al, (2007) transport stress does not affect the expression of HSP70 in sea bass liver tissue but do increase in muscle and skin after transport stress (Poltronieri et al., 2007). It was also the case for the expression levels found in gills and liver done by Washburn et al., (2002) where no altered expressions of HSP 70 was found during handling tress. Handling and sampling have not shown signs of cellular stress with HSP response for rainbow trout (Oncorhynchus mykiss) (Vijayan et al., 1997). This matches the stable expression found in this thesis transport stress experiment. The HSP70 protein is mainly induced by exposure of salmon tissues to thermal stress(DuBeau et al., 1998; Smith et al.,1999b). According to Smith et al.(1999a) HSP70 is induced by hyperosmotic stress, and they suggest that HSP70 might have a function in regards to restoring osmotic homeostasis and protein synthesis during the early stages of osmotic chock. HSP70 is also induced by stressor such as osmotic stress, where the fish is exposed to osmotic shock (Iwama et al., 1998). In This thesis experiment there was no upregulation of HSP70 24 hours post transport stress where the fish had been put back in tanks filled with salt water. Essentially there is no sign to osmotic shock in either sterile or fertile salmon 24-hour post seawater transfer.

4.2.1.2 Genes with significant time or gender difference

1. MHC I T0-T24

There was found significant difference in the expression of MHC I in the head kidney for the stress experiment between time points for the group of sterile females. The MHC I expression level decreased significantly for sterile female post stress compared to prestress in the head kidney. Similarly, decreasing trend was also found in the gill tissue, although not significant. In the gills there was higher individual variation in the sterile females pre and post transportation stress than in head kidney. This might show that the antigen presentation capacity of the sterile females respect to MHCI expression is reduced post stress.
There were also indications of a gender difference between the sterile male and female expression of MHC I post transport stress within the head kidney. The sterile female group expressed significantly lower than the sterile males of T24. This could indicate that the expression of MHC I for sterile female might be generally lower post transport stress than that

of sterile male in the head kidney. However, of the post stress time point one individual from sterile male group had much higher expression than the rest of the group that might have contributed to the significance between the sterile genders as well as the decease of sterile female seen over time. On the other hand, the result from gills shows no significant difference that the mean of sterile male and female have the same stable level of expression, around 1fold at both time points with no significant differences. Therefore, a clear gender difference in the expression of MHC I for sterile A. salmon cannot be speculated as it is only observed in the head kidney, but we cannot overlook the possibility that female sterile fish and sterile male fish can have varying abilities to bind peptide antigens displayed by antigen presenting cells and requires further research. A project with a bigger sampling group, and more tissues could make the answer clearer and define if there is in fact a gender driven difference when removing the germ cells within the sterile groups or if this result is just by chance.

As the result presents itself now the gender difference in the expression of MHC I for sterile A. salmon as it is only observed in the head kidney, but we cannot overlook the possibility and research with more tissue samples cold clarify the result.

MHC class I like molecules are expressed on all nucleated cells and is not downregulated post tress. The expression of MHC I in sterile A. salmon does not differ from that of fertile A. salmon groups post tress during this experiment. This is a good indication that sterile salmon most likely have the peptide presenting abilities as fertile salmon in response to an infection, but this needs further research to confirm. The stable amount of TCR1α discovered in this experiment would also support this, as it does not significantly differ between sterile and fertile salmon post stress. A comparative research of sterile immune response contra fertile when exposed to infection would be a next step for further preliminary studies of germ cell free A. salmon. There are few studies that denote the MHC I expression pre and post transport stress. The normal MHC I expression in fish is also not defined by any one paper but the fold gene expression results from this thesis are similar to that of Jørgensen et al. where kidney cells from fertile A. salmon expressed circa 1-fold of MHC I pre infection, the experiments control (Jørgensen et al 2006) Stating that the MHC class I expression of both controls and sterile groups of this thesis can be presumed within normal range for uninfected salmon.

4.2.1.3 CATH-2 T0-T24

There was significant increase in CATH-2 expression of sterile females after 24 h transportation stress compared to sterile female prestress. Thus, it is possible that transport

stress has affected the expression of CATH-2 for sterile females in the gill tissue. This could mean that sterile females increase their antimicrobial activity post stress. At the T24 time point for sterile female there were 3 individuals that express higher and three that express lower. When comparing the CATH-2 expression of these individuals with their respective cortisol levels there seemed to be no trend. An experiment with more samples could clarify if the significance is real for head kidney or a coincidence due to individual reactions to a stressor as the significance is not present for the sterile female group in gills. If sterile females actually are high responders for CATH-2 expression one could speculate in the possible positive preparedness for coping with microbial challenges in the sea phase.

The effects of stress and handling on the gene expression of CATH-2 for salmonids are generally unknown and there is therefore little to compare our results with.

Results from a study of A. salmon resulted in indication of cath-2 in selected organs and upregulated in parasite exposed salmon (McGrath et al., 2022). This has also been the case in several other studies with viral infections (Guo & Dixon, 2021) As the fish in our experiment where not exposed to any pathogens the Cath-2 expression should be the same as that of control groups of other experiments. In the study done by McGrath et al (2022) in A. salmon the fold change of the control group for CATH-2 was about 1 in gills, the same as observed in our study.

4.2.2 Cortisol T0-T24

Rapid decrease of plasma cortisol levels are commonly observed after a sea water transfer for A. salmon, (Langhorne & Simpson, 1986). This was seemingly also the case in this thesis experiment, as cortisol levels are back to pre transport stress levels after 24 hours. According to Culbert et al., 2022 plasma cortisol levels for smolt are around 40 ng/ml 24 hours after sea water exposure for A. Salmon. Culbert et al., 2022 suggested that the cortisol only moderately increase during seawater transfer for smolts. This is in line with levels found post transport stress in this thesis experiment for all groups except control females where the mean was driven up by two high expressing individuals (150-200 ng/mL). The transportation stress performed in this study showed no differences in the plasma cortisol level between sterile and fertile control groups, and all groups where back to pre-stress levels 24 hours post stress. It does not seem like transportation stress followed by salt water transfer affected the cortisol level of sterile or fertile salmon 24 hours post stress. Studies have shown that the plasma cortisol levels peaked about 1h after transport (Iversen et al 1998). The recovery time of an

acute stressor of moderate intensity is said to be about 6 hours before the A. salmon is back to its original resting plasma cortisol levels (Pickering & Pottinger, 1989, Iversen et al., 1998.) Stress associated with transport or hauling has been known to result in a longer recovery for up to 24 hours or more (Pickering & Pottinger, 1989). This is also apparent in this thesis, not only for cortisol levels, but also most of the immune genes are returned to pre transport levels at 24 hours post transport.

The trend for cortisol level was slightly higher at the T24 sampling point but not so that it resulted in a significant difference from the prestress sampling point. Some even displaying lower cortisol levels than before the transport, e.g., control male at T24, but this could be due to individual differences within the sampling group at the prestress point (control males at T0). Overall, there cortisol levels of female and male sterile A. salmon seem to be lower than that of its control counterparts post transport, although not significant. In general, both the female sterile and control fertile groups have slightly higher level of plasma cortisol post transport than the sterile and fertile males. This may indicate a slight gender driven difference that could be worth researching further to ensure the females cortisol levels and possible stress handling capabilities, do in fact not differ from that of males during production. It is also worth mentioning that from previous experiments done to map out the plasma cortisol levels of salmon there was found no significant difference between concertation levels in males and females (Patiño & Schreck 1986). There have been few studies done in regards to mapping out possible gender differences in plasma cortisol. Seemingly there is not detected any gender difference in adult fertile salmon. Post stress exposure there is significance between sterile male and control female at T24. If there is a biological reasoning for this, it might be explained by gender as mentioned above as the samples of sterile male in this study holds a low concentration or that the concentration of cortisol in females in this study generally is higher than that of male's post transport. As there is no significance difference between the controls of the gender and the associated sterile group. The difference may be clarified in a study with more samples. It is important to remember that in our experiment the baseline cortisol level is equal to that of the cortisol level at the end of smoltification driving our baseline up to around 50 ng/ml, although unstressed salmon not at the end of smoltification may display a lower basal level. It is known that a natural increase in plasma cortisol is observed during smoltification of A. salmon (Iversen et al., 1998; Guo & Dixon 2021)(Forkort og klargjør hva som er våre resultat/andres) In other stress experiments imitating smolt transportation the average levels of plasma cortisol of the control A. salmon

prior to handling or transport were 54-72 ng/ml (Iversen et al.,2005), much like the levels seen in this thesis experiment pre transport stress, and was back to its resting level of plasma cortisol upon end of smolt transport (4-6 hours) (Iversen et al., 2005) and returned to pre-stress levels within 8 h (Carey & McCormick, 1998)

The result shows that the cortisol levels of sterile salmon are back to normal (baseline cortisol level of T0) and equal to the same level as the control fertile salmon, 24 hours after an acute stressor as it is not detected any significant difference between the time points. This indicates that the sterile salmon has the same ability to regulate cortisol back to its prestress baseline, 24 hours after being exposed to this studies transportation stress, as the normal fertile salmon.

There have been in vitro studies of catfish which showed that the cortisol suppression of lymphocytes has little to no significant extent beyond its momentarily impact and return to normal after a stressor (Ellsaesser & Clem, 1987). Later it was also concluded that the cortisol only has a momentarily negative effect on the immune cells of A. salmon. A momentarily increase of stress and thus cortisol, did not give the fish reduced resistance to disease or developed long lasting negative effects for the immune system after stress (Espelid et al., 1996). In studies one can see that it is mostly the excessive exposure to stressors that can lead the fish to develop chronical stress and thus can get exposed to more disease as the fish is in flight or fight mode repeatedly or constant reducing its body's energy metabolization (Tort, 2011; Barton & Iwama, 1991).

4.3 Correlations

4.3.1 Immune Gene correlations

To further strengthen the relations between immune genes studied throughout this thesis a correlation analysis was performed for the sterile and fertile fish. There was discovered many significant correlations, displayed in appendix x, and further correlation analysis was performed to establish if certain groups (sterility and/or gender) was driving the correlations. Out of the significant correlations there was correlations among the immune genes that where present in both the smoltification and stress experiment and in both tissues. I have decided to focus on some of these significant correlations as these are mostly part of the same immune pathways and it is a possibility that they express a biological functioning correlation as they are present in multiple tissues during two experiments.

During the correlation analysis it was discovered overall positive correlations between the immune genes. Meaning that the two immune genes in the significant correlations covaries, or have tendencies to increasing and decrease together.

The correlation between MHC class I and TCR1α:

MHC and TCR have a biological connection as they are both path of the T-cell mediated immune response where MCH presents antigens peptide to the TCR that activates the T-cell maturation to cytotoxic T-cells for the destruction of the infected APC (Bilal et al., 2018). During the further correlation analysis neither gender nor sterility seemed to influence the correlation alone during smoltification in head kidney tissue (HK), as seen in appendix IX, all factors displayed significantly. It must be mentioned that during smoltification in HK, the control males did have a higher significance than the rest (control females, and sterile females). In the transport stress experiment the correlation was mostly driven by control males in the gill tissue (G) and sterile males in HK. This correlation between these genes where quite strong and has an explanation degree of 48% in G of the transport stress experiment, and 34% in HK. It seems biological plausible that the MHC and TCR covaries as there must be TCR to "read" MHC antigen presenting for T-cells to further immune response (Yamaguchi & Djikstra, 2019).

The correlation between TCR1a and sIgm:

TCR and IgM have a biological connection as the both are part of the T-cell activation of T-helper cells leading to the activation of humoral immune response. MHC II binding to TCR activate Th (T-helper cells/ CD4+) that can initiate the naive antigen-specific B-cells of adaptive immunity to produce IgM antibodies (Salinas et al., 2021). During further correlation analysis all parameters (genders and sterility) influenced the correlation in HK equally during smoltification, and showed a strong correlation with 67% explanation degree. In G the correlation was driven by control males (15%). In the transport stress experiment sterile male and female drove the correlation in HK (34%), sterile males expressed higher significance than sterile females, while the correlation in G was driven by sterile males only (10%). In teleost fish the sIgM's are secreted by plasma cells (activated B-cells) locally in the mucosal barrier tissues and have the ability to perform in an external environment and upheld the microbiota homeostasis in the mucosal surface (Salinas et al., 2021). It is possible that the TCR and IgM could correlate because of their connection trough B- and T-cells during humoral immune responses in primary and secondary lymphograms.

Given that T-cell and B-cell immunity is present in teleost as presented above and the presence of these significant correlations in both tissue during both experiments it is possible that these genes do in fact functionally correlate.

4.3.2 Cortisol correlations:

For cortisol there were only one significant correlation to rapport. Cortisol only correlated with MHC I in the post-transport stress timepoint. The correlation between cortisol and MHC I only had a slight negative correlation, meaning that the increase of cortisol decreases MHC I and opposite. During further testing of the cortisol and MHC I correlation, gender was not discovered to have influenced the correlation. Only sterility showed to drive the significance of the correlation. The correlation was also only significant in the gill tissue during the stress experiment with a low explanation degree of 9 %. It is likely coincidental, as no other immune gene showed to be significantly negatively correlated to cortisol, and it was only discovered in gill tissue. As seen in the post hoc the cortisol levels post stress where generally higher for the sterile females even though the only significant difference were found between sterile males and control females, whitish is not biological comparable. Cortisol following acute stressor is often associated with a momentarily downregulation of the immune system (Tort. 2011). Contradictory, according to Guo & Dixon (2021), MHC I have been found to be upregulated during various forms of stress conditions in teleost and salmonids (Guo & Dixon, 2021). Nonetheless, as the correlation is only driven by sterile fish groups (appendix IX, Table 7) we cannot completely rule out the possibility of germ cell ablation possibly affecting the cortisol in regards to co variation with MHC I post stress in the gill tissue. If the correlation would have been found in multiple tissues that would have been a stronger indication of connection.

Possible sources of error

QPCR is a sensitive method and uncertainties must be accounted for. The amount of cDNA can be affected by many factors during synthetisation, although accounted for by using specialized-stable-kits, normalizing results to a stable reference gene and QPCR run in duplicates. Pipetting-technique and accuracy are determining factors for the qPCR method and the possibility of human error is, as always, present. Degrading/denaturation of RNA and cDNA can be a consequence of multiple defrosting, and storage on ice in room temperature for excessive amounts of time. As these tissue samples have been used for previous studies, these sample have been de frosted multiple times, this could account for some degrading of

material and must be taken into account as yield expression could have been affected. It is possible that some of the significant differences observed are found by coincidence. As this is only a preliminary study the number of samples in each group (n =5-9) is relatively low the results must be interpreted with some caution.

4.4 Further studies

As well as further studies with more samples of the sterile gender, it would be interesting to see what happens after smoltification when the fish moved onto maturation and fertile fish's expression of different sex steroids increase. Here it is possible that the differences between immune gene expression between sterile and fertile would change accordingly. Sex steroids can inflict the immune system. The relative assumption regarding HPG-axis influence on immune functions has been that androgen leads to immunosuppression and estrogen being immuno-protective. As suggested in (Segner et al., 2016) this might be overly simplified, and has suggested that the HPG-immune interaction vary depending on physiological and environmental context (Segner et al., 2016). In the study done by Tveiten et al., (2022) there was shown that sterile male salmon do express testosterone and that the ablation of germ cells do not hinder the activation of the HPG-axis to synthesise sex steroidogenesis in Dnd sterile males. The concentration for “maturing” sterile salmon was discovered to be marginally lower than that of maturing fertile counterparts (Tveiten et al., 2022). It would be interesting to see if this would influence the expression of immune genes during maturation as it does not look like germ cell ablation particularly affects the immune status for sterile smolt. Looking into the sex steroid levels of female sterile fish in regards to gene expression during maturation would also give additional information about its immune status. It would be interesting to see in the sterility effected the activation of the HPG axis steroidogenesis for sterile females to.

Farmed salmon are constantly exposed to pathogens in the water and a good immune status is essential for their survival throughout production. During the smoltification experiment it seems like the sterile salmon expresses mostly equal to that of fertile salmon. It does not look like the immune status, of sterile nor fertile, are affected by the smoltification process in regard to immune genes and cortisol explored in this thesis. This is promising for the sterile salmon's immune status and possible pathogen handling capacity. Cortisol levels of sterile fish also does not differ from that of fertile ones during smoltification, giving reason to

believe that sterile salmon follows the same development in regards to cortisol levels as fertile salmon during smoltification.

During the production of farmed salmon, the salmon has to endure multiple treatments during its lifecycle, like transport, environment changes, predator, sorting, delousing, gathering and so on, and it is essential that the fish can handle some amount of stress. Throughout the stress experiment it seems like the sterile salmon performs equal to the fertile salmon post transport stress in regards to expression of immune gene and cortisol levels. It does not look like the immune status, of sterile nor fertile salmon, is affected by the transport stress regarding the immune genes explored in this thesis. The cortisol levels of sterile fish 24 hours post transport stress and 22 hours post sea water transfer is even lower than that of fertile salmon during this thesis experiment. This is promising for the sterile salmon's stress handling capabilities if lower cortisol levels are desired. It does not look like the removal of germ cells affect the sterile salmon's capability of stress handling. On the contrary, it might actually strengthen it.

The main objective of this experiment was to investigate whether the ablation of germ cells affect the immune status and stress coping abilities during smoltification and transportation stress. From the data collected there seems to be no significant difference between the sterile groups and the fertile control groups of A. salmon during smoltification in regard to immune gene expression and cortisol levels. Thus, it does not appear like sterilization via ablation of germ cells affects the expression of the immune genes studied compared to normal salmon in the smoltification or transport stress experiments. The genes studied in this experiment are especially important for the early and innate immune response. It is plausible that the immune system of the sterile A. salmon possesses the same ability for immune system performance as fertile A. salmon in both primary lymphoid organ (head kidney) and secondary lymphoid organ (gill) during smoltification and transportation stress. There was only found significant decrease or increase within groups over time, specifically only for sterile groups, mostly sterile females, but they did not significantly differ from the fertile ones over time. This might indicate that time and sterile gender is of relevance in the expression of these genes for the sterilized groups. There was also found significant difference between sterile males and sterile females post stress for MHC I in head kidney and for HSP70 in gill at the start of smoltification (T1). That might indicate a slight gender difference at these timepoints within the sterile A. salmon as a result of sterilization by germ cell ablation. This could indicate that the loss of germ cells could have had a small impact on the expression of these genes in the tissues where it has been observed. It is possible that some of these significant differences

were found purely by coincident as many combinations were tested against each other with the significance level of 0.05, the significant differences were only found in certain genes and in single tissues, and seemingly displayed no systematic changes.

Possible correlation was investigated between the immune genes but also between the immune genes and cortisol that could have a biological connection. There were found numerous correlations that showed possible co variation between immune gene expressions. The up regulation of certain immune genes showed to either up or down regulate others, and vice versa, but only the correlations present in both tissues where tested further and connections with obvious biological connections were discussed. No significant correlations were discovered between immune genes and cortisol. If sterility were to affect the expression of an immune gene this would also impact and potentially change the expression of another gene. As of this experiment this shall seemingly not be the case for genes studied here as no significant difference was found between sterile and fertile salmon.

This experiment could benefit from a bigger scale experiment with more samples, more tissues, and ideally with more genes to further broaden our knowledge. For now, it looks like there are some minor differences in the gene expression for sterile female over time and some for sterile males as well and a slight indication of a gender difference in the sterile salmon in MHC I and HSP70. There also seems to be generally higher cortisol levels for the female contra males post transport stress and that could be interesting to research further. The levels of cortisol have not been shown to correlate significantly with any of the immune genes in this research and there has also not been found any significant difference between sterile or fertile A. salmon. As this is only a preliminary study of the immune status of germ cell free salmon during smoltification further studies are needed to confirm that the performance of sterile fish is equal to that of fertile salmon used in production today. Comparative research of sterile immune response when exposed to infection would be a natural next step in the further studies of germ cell free A. salmon to ensure it is equally as robust as fertile during production. With more research on germ cell free sterile salmon, it can become operative in the future and reduce the risk of gene pollution of escapees to the wild salmon in spawning rivers. Based on the current findings, we can hypothesize that germ-cell free sterile salmon could perform more or less similar to the normal salmon and can be a better option in compared to the previously used sterile salmon, thus contributing to better fish health.

5 Conclusion

To conclude from this preliminary study, sterility has minimum to no effect on the immune gene profile during smoltification or post transportation stress. This experiment yields a simple overview of the performance of sterile germ cell free salmon compared to its fertile counterparts during smoltification and post transport stress. Since no major difference in the relative gene expression or cortisol was discovered, it is promising for the further production of sterile fish by ‘knockdown’ of Dnd , evidently leading to possible benefits and solutions for a sustainable development of the fish farming industry.

References

Abbas, K.A., Lichtman, H.A., & Pillai, S. (2020). *Basic Immunology: Functions and Disorders of the immune system 4th ed.*, Elsevier inc.

Adamo, S. A. (2014). The Effects of Stress Hormones on Immune Function May be Vital for the Adaptive Reconfiguration of the Immune System During Fight-or-Flight Behavior. *Integrative and Comparative Biology*, 54, 419-426. https://doi.org/10.1093/icb/icu005

Afonso, L. O. B., Basu, N., Nakano, K., Devlin, R. H., & Iwama, G. K. (2003). Sex-related differences in the organismal and cellular stress response in juvenile salmon exposed to treated bleached kraft mill effluent. *Fish physiology and biochemistry*, *29*(2), 173-179. https://doi.org/10.1023/B:FISH.0000035939.81588.09

Aspen, M (2020) Stress hos kjønnscellefri atlantisk laks (Salmo Salar L.) *Effekter av transport og sjøvannseksponering på sentrale faktorer i hypothalamus-hypofyse-interrenale (HPI)-aksen (*Master) https://hdl.handle.net/10037/22599

Barton, B. A. (2002). Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. *Integrative and Comparative Biology*, 42, 517-525. https://doi.org/10.1093/icb/42.3.517

Barton, B. A. & Iwama, G. K. (1991). Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. Annual Review of fish diseases, 1, 3-26. https://doi.org/10.1016/0959-8030(91)90019-G

Bassity, E. & Clark, T. G. (2012) Functional identification of dendritic cells in the teleost model, rainbow trout (Oncorhynchus mykiss). PLoS One, 7 (3), e33196. https://doi.org/10.1371/journal.pone.0033196

Bilal, S., Lie, K. K., Sæle, Ø., & Hordvik, I. (2018). T cell receptor alpha chain genes in the teleost ballan wrasse (Labrus bergylta) are subjected to somatic

hypermutation. *Frontiers in immunology*, *9*, 1101. https://doi.org/10.3389/fimmu.2018.01101

Björnsson, B. T., Stefansson, S. O. & McCormick, S. D. (2011) Environmental endocrinology of salmon smoltification. *General and Comparative Endocrinology*, 170 (2), 290-298. https://doi.org/10.1016/j.ygcen.2010.07.003

Bonga, S. E. W. (1997). The stress response in fish. Physiological Reviews, 77, 591-625.

Brunner, S. R., Varga, J. F. A., Dixon, B. (2020) Antimicrobial Peptides of Salmonid Fish: From Form to Function. *Biology, 9(8),* 233. https://doi.org/10.3390/biology9080233

Bridle, A., Nosworthy, E., Polinski, M., & Nowak, B. (2011). Evidence of an antimicrobial-immunomodulatory role of Atlantic salmon cathelicidins during infection with Yersinia ruckeri. *PloS one*, *6*(8), e23417. https://doi.org/10.1371/journal.pone.0023417

Carey, J. B., & McCormick, S. D. (1998). Atlantic salmon smolts are more responsive to an acute handling and confinement stress than parr. *Aquaculture*, *168*(1-4), 237-253. https://doi.org/10.1016/S0044-8486(98)00352-4

Castillo, J., Teles, M., Mackenzie, S., & Tort, L. (2009). Stress-related hormones modulate cytokine expression in the head kidney of gilthead seabream (Sparus aurata). *Fish & Shellfish Immunology*, *27*(3), 493-499. https://doi.org/10.1016/j.fsi.2009.06.021

Chrousos, G. P. & Gold, P. W. (1992). The concepts of stress and system disorders – overview of physical and behavioural homeostasis. Jama-Journal of the American Medical Association, 267, 1244-1252. https://pubmed.ncbi.nlm.nih.gov/1538563/

Ciocca, D. R. & Calderwood, S. K. (2005). Heat shock proteins in cancer: diagnostic, prognostic, predictive, and treatment implications. *Cell Stress & Chaperones,* 10, 86-103. https://doi.org/10.1379/csc-99r.1

Cotter, D., O'Donovan, V., O'Maoiléidigh, N., Rogan, G., Roche, N., & Wilkins, N. P. (2000). An evaluation of the use of triploid Atlantic salmon (Salmo salar L.) in

minimising the impact of escaped farmed salmon on wild populations. *Aquaculture, 186(1), 61-75*
https://doi.org/https://doi.org/10.1016/S0044-8486(99)00367-1

Culbert, B. M., Regish, A. M., Hall, D. J., McCormick, S. D., & Bernier, N. J. (2022). Neuroendocrine Regulation of Plasma Cortisol Levels During Smoltification and Seawater Acclimation of Atlantic Salmon. *Frontiers in endocrinology*, *13*. https://doi.org/10.3389/fendo.2022.859817

Deane, E. E., Kelly, S. P., Luk, J. C., & Woo, N. (2002). Chronic salinity adaptation modulates hepatic heat shock protein and insulin-like growth factor I expression in black sea bream. *Marine Biotechnology*, *4*(2), 193-205. https://doi.org/10.1007/PL00021690

Deane, E. E. & Woo, N. Y. S. (2011). Advances and perspectives on the regulation and expression of piscine heat shock proteins. *Reviews in Fish Biology and Fisheries*, 21, 153-185. https://doi.org/10.1007/s11160-010-9164-8

Dickhoff, W. W., Beckman, B. R., Larsen, D. A., Duan, C. & Moriyama, S. (1997) The role of growth in endocrine regulation of salmon smoltification. *Fish Physiology and Biochemistry*, 17 (1-6), 231-236. https://doi.org/10.1023/A:1007710308765

DuBeau, S. F., Pan, F., Tremblay, G. C., & Bradley, T. M. (1998). Thermal shock of salmon in vivo induces the heat shock protein hsp 70 and confers protection against osmotic shock. *Aquaculture*, *168*(1-4), 311-323. https://doi.org/10.1016/S0044-8486(98)00358-5

Ellsaesser, C. F. & Clem, L. (1987) Cortisol-induced hematologic and immunologic changes in channel catfish (Ictalurus punctatus). *Comparative Biochemistry and Physiology Part A: Physiology*, 87 (2), 405-408. https://doi.org/10.1016/0300-9629(87)90143-5

Espelid, S., Løkken, G. B., Steiro, K. & Bøgwald, J. (1996) Effects of cortisol and stress on the immune system in Atlantic Salmon (Salmo salarL.). *Fish and Shellfish*

Immunology, 6 (2), 95-110. https://doi.org/10.1006/fsim.1996.0011

Feng, C., Wang, H., Lu, N., Chen, T., He, H., Lu, Y., & Tu, X. M. (2014). Logtransformation and its implications for data analysis. *Shanghai archives of psychiatry*, *26*(2), 105-109. https://doi.org/10.3969/j.issn.1002-0829.2014.02.009

Fischer, U., Koppang, E. O. & Nakanishi, T. (2013) Teleost T and NK cell immunity. *Fish and Shellfish Immunology*, 35 (2), 197-206. https://doi.org/10.1016/j.fsi.2013.04.018

Fjelldal, P. G., Hansen, T., & Huang, T.-S. (2011). Continuous light and elevated temperature can trigger maturation both during andimmediately after smoltification in male Atlantic salmon (Salmo salar). *Aquaculture*, 321, ss. 93-100. https://doi.org/10.1016/j.aquaculture.2011.08.017

Fjelldal, P. G., Schulz, R., Nilsen, T. O., Andersson, E., Norberg, B., & Hansen, T. J. (2018). Sexual maturation and smoltification in domesticated Atlantic salmon (Salmo salar L.) – is there a developmental conflict? *Physiological Reports*, 6, s. N/a. https://doi.org/10.14814/phy2.13809

Folmar, L. C. & Dickhoff, W. W. (1980) The Parr-Smolt Transformation (Smoltification) and Seawater Adaptation in Salmonids - a Review of Selected Literature. *Aquaculture*, 21 (1), 1-37. https://doi.org/10.1016/0044-8486(80)90123-4

Forseth, T., Barlaup, B. T., Finstad, B., Fiske, P., Gjøsæter, H., Falkegård, M., . . . Wennevik, V. (2017). The major threats to Atlantic salmon in Norway. *ICE Journal of Marine Science*, ss. 1496-1513. https://doi.org/10.1093/icesjms/fsx020

Fraser, T. W., Fjelldal, P. G., Hansen, T. & Mayer, I. (2012). Welfare considerations of triploid fish. *Reviews in Fisheries Science*, 20(4), 192-211. https://doi.org/10.1080/10641262.2012.704598

Gross-Thebing, T., Yigit, S., Pfeiffer, J., Reichman-Fried, M., Bandemer, J., Ruckert, C., . . . Raz, E. (2017). The Vertebrate Protein Dead End Maintains Primordial Germ Cell

Fate by Inhibiting Somatic Differentiation. *Developmental Cell*, 43, ss. 651-78. https://doi.org/10.1016/j.devcel.2017.11.019

Guo, H., & Dixon, B. (2021). Understanding acute stress-mediated immunity in teleost fish. *Fish and Shellfish Immunology Reports*, *2*, 100010. https://doi.org/10.1016/j.fsirep.2021.100010

Hansen, J. D., Landis, E. D. & Phillips, R. B. (2005) Discovery of a unique Ig heavy-chain isotype (IgT) in rainbow trout: Implications for a distinctive B cell developmental pathway in teleost fish. *Proceedings of the National Academy of Sciences of the United States of America*, 102 (19), 6919-6924. https://doi.org/10.1073/pnas.0500027102

Hansen, J. D. & Zapata, A. G. (1998) Lymphocyte development in fish and amphibians. *Immunological Reviews*, 166 (1), 199-220. https://doi.org/10.1111/j.1600-065x.1998.tb01264.x

Hansen, T. J., Olsen, R. E., Stien, L., Oppedal, F., Torgersen, T., Breck, O., . . . Fjelldal, P. G. (2015). Effect of water oxygen level on performance of diploid and triploid Atlantic salmon post-smolts reared at high temperature. *Aquaculture* 435, ss. 354-360. https://doi.org/10.1016/j.aquaculture.2014.10.017

Hansen, T., Wargelius, A., Taranger, G. L. & Fjelldal, P. G. (2012). Oppdrett av steril fisk. *Rapport fra Havforskningen. Nr. 18.* http://hdl.handle.net/11250/116725

Haugarvoll, E., Bjerkås, I., Nowak, B. F., Hordvik, I. & Koppang, E. O. (2008) Identification and characterization of a novel intraepithelial lymphoid tissue in the gills of Atlantic salmon. *Journal of Anatomy*, 213 (2), 202-209. https://doi.org/10.1111/j.1469-7580.2008.00943.x

Heggberget, T. G. Staurnes, M., Strand, R. & Husby, J. (1992). Smoltifisering hos laksefisk. - *NINA Forskningsrapport* 31: 1-42. https://www.nina.no/archive/nina/pppbasepdf/forskningsrapport/031.pdf

Hikima, J. I., Jung, T.S. & Aoki, T. (2011) Immunoglobulin genes and their transcriptional control in teleosts. *Developmental & Comparative Immunology*, 35 (9), 924-936. https://doi.org/10.1016/j.dci.2010.10.011

Hoar, W. S. (1988). The physiology of smolting salmonids. *Fish Physiology*, 11(275-343) https://doi.org/10.1016/S1546-5098(08)60216-2

Imsland, A.K., Handeland, S.O. & Stefansson, S.O. (2014). Photoperiod and temperature effects on growth and maturation of pre- and post-smolt Atlantic salmon. *Aquacult Int* 22, 1331–1345. https://doi.org/10.1007/s10499-014-9750-1

Ingerslev, H. C., Rønneseth, A., Pettersen, E. F., & Wergeland, H. I. (2009). Differential expression of immune genes in Atlantic salmon (Salmo salar L.) challenged intraperitoneally or by cohabitation with IPNV. *Scandinavian Journal of Immunology*, *69*(2), 90-98. https://doi.org/10.1111/j.1365-3083.2008.02201.x

Irwin, M. J. & Kaattari, S. L. (1986) Salmonid B lymphocytes demonstrate organ dependent functional heterogeneity. *Veterinary Immunology and Immunopathology*, 12 (1), 39-45. https://doi.org/10.1016/0165-2427(86)90108-x

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

Iversen, M., Finstad, B., McKinley, R. S., Eliassen, R. A., Carlsen, K. T., & Evjen, T. (2005). Stress responses in Atlantic salmon (Salmo salar L.) smolts during commercial well boat transports, and effects on survival after transfer to sea. *Aquaculture*, *243*(1-4), 373-382. https://doi.org/10.1016/j.aquaculture.2004.10.019

Iwama, G. K., Thomas, P. T., Forsyth, R. B., & Vijayan, M. M. (1998). Heat shock protein expression in fish. *Reviews in Fish Biology and Fisheries*, *8*(1), 35-56. https://doi.org/10.1023/A:1008812500650

Jacquiersarlin, M. R., Fuller, K., Dinhxuan, A. T., Richard, M. J. & Polla, B. S. (1994). Protective effects of hsp70 in inflammation. *Experientia*, 50, 1031-1038. https://doi.org/10.1007/bf01923458

Jensen, I., Overrein, M. C., Fredriksen, B. N., Strandskog, G., & Seternes, T. (2019). Differences in smolt status affect the resistance of Atlantic salmon (Salmo salar L.) against infectious pancreatic necrosis, while vaccine-mediated protection is unaffected. *Journal of Fish Diseases*, *42*(9), 1271-1282. https://doi.org/10.1111/jfd.13049

Jenssen, E. B. (2020). Utvikling av smoltegenskaper og sjøvannstoleranse hos kjønnscellefri atlantisk laks (Salmo salar L.) *UiT The Arctic University of Norway.* https://hdl.handle.net/10037/19360

Johansen, L. H., Dahle, M. K., Wessel, Ø., Timmerhaus, G., Løvoll, M., Røsæg, M., ... & Krasnov, A. (2016). Differences in gene expression in Atlantic salmon parr and smolt after challenge with Piscine orthoreovirus (PRV). *Molecular immunology*, *73*, 138-150. https://doi.org/10.1016/j.molimm.2016.04.007

Johansson, L. H., Timmerhaus, G., Afanasyev, S., Jørgensen, S. M., & Krasnov, A. (2016). Smoltification and seawater transfer of Atlantic salmon (Salmo salar L.) is associated with systemic repression of the immune transcriptome. *Fish & shellfish immunology*, *58*, 33-41. https://doi.org/10.1016/j.fsi.2016.09.026

Jørgensen, M. S., Lyng-Sivertsen, B., Lukacs, M., Grimholt, U., Gjøen, T. (2006). Expression of MHC class I pathway genes in response to infectious salmon anaemia virus in Atlantic almon *(Salmo salar L.)* cells. *Fish & Shellfish Immunollogy. 21(5)*, 548-560. https://doi.org/10.1016/j.fsi.2006.03.004

Kaattari, S. L. & Irwin, M. J. (1985) Salmonid spleen and anterior kidney harbor populations of lymphocytes with different B cell repertoires. *Developmental & Comparative Immunology*, 9 (3), 433-444. https://doi.org/10.1016/0145-305x(85)90006-0

Karlsen, C., Ytteborg, E., Timmerhaus, G., Høst, V., Handeland, S., Jørgensen, S. M., & Krasnov, A. (2018). Atlantic salmon skin barrier functions gradually enhance after seawater transfer. *Scientific reports*, *8*(1), 1-12. https://doi.org/10.1038/s41598-018-27818-y

Karlsen, K. (2020) Bortfall av kjønnsceller hos atlantisk laks (Salmo salar L.) – effekter på vekst og uttrykk av gener som er sentrale i regulering av hypothalamus- hypofyse-gonade aksen. *Masteroppgave i Fiskeri- og havbruksvitenskap.* https://hdl.handle.net/10037/22650

Katzenback, B. A. (2015). Antimicrobial peptides as mediators of innate immunity in teleosts. *Biology*, *4*(4), 607-639. https://doi.org/10.3390/biology4040607

Kleppe, L., Andersson, E., Skaftnesmo, K. O., Edvardsen, R. B., Fjelldal, P. G., Norberg, B., Bogerd, J., Schulz, R. W., & Wargelius, A. (2017). Sex steroid production associated with puberty is absent in germ cell-free salmon. Sci Rep, 7(1), 12584. https://doi.org/10.1038/s41598-017-12936-w

Koppang, E. O., Fischer, U., Moore, L., Tranulis, M. A., Dijkstra, J. M., Köllner, B., Aune, L., Jirillo, E. & Hordvik, I. (2010) Salmonid T cells assemble in the thymus, spleen and in novel interbranchial lymphoid tissue. *Journal of Anatomy*, 217 (6), 728-739 https://doi.org/10.1111/j.1469-7580.2010.01305.x

Krasnov, A., Afanasyev, S., Nylund, S., & Rebl, A. (2020). Multigene Expression Assay for Assessment of the Immune Status of Atlantic Salmon. *Genes (Basel)*, 11(11). https://doi.org/10.3390/genes11111236

Krasnov, A., Burgerhout, E., Johnsen, H., Tveit, H., Bakke, A. F., Lund, H., Afanasyev, S., Rebl, A., Johansen, L. H. (2021) Development of Atlantic Salmon (*Salmo salar* L.) Under Hypoxic Conditions Induced Sustained Changes in Expression of Immune Genes and Reduced Resistance to *Moritella viscosa*, *Frontiers in Ecology and Evolution.* https://doi.org/10.3389/fevo.2021.722218

Kumari, J., Bøgwald, J., & Dalmo, R. A. (2013) Vaccination of Atlantic salmon, Salmo salar L., with Aeromonas salmonicida and infectious pancreatic necrosis virus (IPNV)

showed a mixed Th1/Th2/Treg response. *Journal of fish diseases*, 36(10). https://doi.org/10.1111/jfd.12100

Kumari, J., Zhang, Z., Swain, T., et al. (2015) Transcription Factor T-Bet in Atlantic Salmon: Characterization and Gene Expression in Mucosal Tissues during Aeromonas Salmonicida Infection. *Front Immunol* 6, 345. https://doi.org/10.3389/fimmu.2015.00345

Langhorne, P. & Simpson T. H. (1986) The interrelationship of cortisol, gill (Na + K) ATPase, and homeostasis during the Parr-Smolt transformation of Atlantic salmon (Salmo salar L.). *General and Comparative Endocrinology*, 61(2), 203-213. https://pubmed.ncbi.nlm.nih.gov/3007267/

Li, J., Barreda, D. R., Zhang, Y. A., Boshra, H., Gelman, A. E., LaPatra, S., Tort, L. & Sunyer, J. O. (2006) B lymphocytes from early vertebrates have potent phagocytic and microbicidal abilities. *Nature Immunology*, 7 (10), 1116-1124. https://www.nature.com/articles/ni1389#citeas

Lock, E. F., Ziemiecki, R., Marron, J. & Dittmer, D. P. (2010). Efficiency clustering for low-density microarrays and its application to QPCR. *BMC bioinformatics, 11(1), 386.* https://link.springer.com/article/10.1186/1471-2105-11-386#citeas

Løvås, G.G. (2018). *Statistikk for universiteter og høgskoler* (4. utg.). Universitetsforlaget.

Magnadóttir, B. (2010) Immunological control of fish diseases. *Marine Biotechnology*, 12 (4), 361-379. https://doi.org/10.1007/s10126-010-9279-x

Makesh, M., Sudheesh, P. S., & Cain, K. D. (2015). Systemic and mucosal immune response of rainbow trout to immunization with an attenuated Flavobacterium psychrophilum vaccine strain by different routes. *Fish & Shellfish Immunology, 44*(1), 156-163. https://doi.org/https://doi.org/10.1016/j.fsi.2015.02.003

Maule, A. G., Schreck, C. B. & Kaattari, S. L. (1987) Changes in the immune system of coho salmon (Oncorhynchus kisutch) during the parr-to-smolt transformation and after implantation of cortisol. *Canadian Journal of Fisheries and Aquatic Sciences*, 44

(1), 161-166.
https://doi.org/10.1139/f87-021

Maxime, V. (2008). The physiology of triploid fish: current knowledge and comparisons with diploid fish. *Fish and Fisheries*, 9(1), 67-78.
https://doi.org/10.1111/j.1467-2979.2007.00269.x

McCormick, S. D. & Saunders, R. L. (1987) Preparatory physiological adaptations for marine life of salmonids: osmoregulation, growth, and metabolism. *American Fisheries Society Symposium*, 1, 211-229.
https://webpages.uidaho.edu/fish511/readings/readings%202010/mccormick%20and%20saunders.pdf

McCormick, S. D., Hansen, L. P., Quinn, T. P. & Saunders, R. L. (1998) Movement, migration, and smolting of Atlantic salmon (Salmo salar). *Canadian Journal of Fisheries and Aquatic Sciences*, 55 (S1), 77-92.
https://doi.org/10.1139/d98-011

McCormick, S. D., Lerner, D. T., Monette, M. Y., Nieves-Puigdoller, K., Kelly, J. T. & Björnsson, B. T. (2009) Taking it with you when you go: how perturbations to the freshwater environment, including temperature, dams, and contaminants, affect marine survival of salmon. *American Fisheries Society Symposium*, 69, 195-214.
http://www.bio.umass.edu/biology/mccormick/pdf/AFS%20symposium%202009%20mccormick%20et%20al.pdf

McCormick, S. D. (2012) Smolt Physiology and Endocrinology. *Euryhaline Fishes. Amsterdam, Academic Press*, 199-251.
https://doi.org/10.1016/B978-0-12-396951-4.00005-0

McEwen, B. S. (1998). Protective and damaging effects of stress mediators. *New England Journal of Medicine*, 338, 171-179.
https://doi.org/10.31887%2FDCNS.2006.8.4%2Fbmcewen

McGrath, L., O'Keeffe, J., & Slattery, O. (2022). Antimicrobial peptide gene expression in Atlantic salmon (Salmo salar) seven days post-challenge with Neoparamoeba perurans. *Developmental & Comparative Immunology*, *127*, 104287.
https://doi.org/10.1016/j.dci.2021.104287

Melingen, G. O., Pettersen, E. F. & Wergeland, H. I. (2002) Leucocyte populations and responses to immunization and photoperiod manipulation in Atlantic salmon (Salmo salar L.) 0+ smolt. *Aquaculture*, 214 (1-4), 381-396. https://doi.org/10.1016/S0044-8486(01)00866-3

Melingen, G., Stefansson, S., Berg, A. & Wergeland, H. (1995) Changes in serum protein and IgM concentration duringsmolting and early post-smolt period in vaccinated and unvaccinated Atlantic salmon (Salmo salar L.). *Fish and Shellfish Immunology*, 5 (3), 211-221. https://doi.org/10.1016/S1050-4648(05)80015-4

Melo, M. C., Andersson, E., Fjelldal, P. G., Bogerd, J., Franca, L., Taranger, G. L., & Schulz, R. W. (2014). Salinity and photoperiod modulate pubertal development in Atlantic salmon (Salmo salar). *Journal of Endocrinology*, 229, ss. 319-332. 220, 1-15. https://doi.org/10.1530/JOE-13-0240

Michelsen, F. A., Klebert, P., Broch, O. J. & Alver, M. O. (2019). Impacts of fish farm structures with biomass on water currents: A case study from Frøya. *Journal of Sea Research*, 154, 101806. https://doi.org/10.1016/j.seares.2019.101806

Mommsen, T. P., Vijayan, M. M. & Moon, T. W. (1999). Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries*, 9(3), 211-268. https://link.springer.com/article/10.1023/A:1008924418720#citeas

Morrison, R. N. & Nowak, B. F. (2002) The antibody response of teleost fish. *Seminars in Avian and Exotic Pet Medicine*, 11 (1), 46-54. https://doi.org/10.1053/saep.2002.28241

Motulsky, H. J., & Brown, R. E. (2006). Detecting outliers when fitting data with nonlinear regression - a new method based on robust nonlinear regression and the false discovery rate. *BMC Bioinformatics*, *7*, 123. https://doi.org/10.1186/1471-2105-7-123

Motulsky, H.J. (2022a). Tukey and Dunnett methods. *GraphPad Statistics guide*.

https://www.graphpad.com/guides/prism/latest/statistics/stat_the_methods_of_tukey_and_dunne.htm

Motulsky, H.J. (2022b). Interpreting results: Kruskal-Wallis test. *GraphPad Statistics Guide.* Retrieved from https://www.graphpad.com/guides/prism/latest/statistics/how_the_kruskal-wallis_test_works.htm

Nilsen, T. O., Ebbesson, L. O., Kiilerich, P., Björnsson, B. T., Madsen, S. S., McCormick, S. D. & Stefansson, S. O. (2008) Endocrine systems in juvenile anadromous and landlocked Atlantic salmon (Salmo salar): seasonal development and seawater acclimation. *General and Comparative Endocrinology*, 155 (3), 762-772. https://doi.org/10.1016/j.ygcen.2007.08.006

Nuñez-Ortiz, N., Moore, L. J., Jarungsriapisit, J., Nilsen, T. O., Stefansson, S., Morton, H. C., ... & Patel, S. (2018). Atlantic salmon post-smolts adapted for a longer time to seawater develop an effective humoral and cellular immune response against Salmonid alphavirus. *Fish & shellfish immunology*, *82*, 579-590. https://doi.org/10.1016/j.fsi.2018.08.059

O'Flynn, F. M., McGeachy, S. A., Friars, G. W., Benfey, T. J., & Bailey, J. K. (1997). Comparisons of cultured triploid and diploid Atlantic salmon. *ICES Journal of Marine Science*, 54, ss. 1160- 1165. https://doi.org/10.1016/S1054-3139(97)80022-7

Quiagen. (2009). Quantitect Reverse Transpriction Handbook. https://www.quiagen.com/us/resources/resourcedetail?id=f0de5533-3dd1-4835-8820-1f5c088dd800&land=en

Quiagen. (2019). RNeasy Mini Handbook. https://www.qiagen.com/us/resources/resourcedetail?id=14e7cf6e-521a-4cf7-8cbc-bf9f6fa33e24&lang=en

Patiño, R., & Schreck, C. B. (1986). Sexual dimorphism of plasma sex steroid levels in juvenile coho salmon, Oncorhynchus kisutch, during smoltification. *General and comparative endocrinology*, *61*(1), 127-133. https://doi.org/10.1016/0016-6480(86)90257-1

Pauli, A., Montague, T. G., Lennox, K. A., Behlke, M. A. & Schier, A. F. (2015). Antisense oligonucleotide-mediated transcript knockdown in zebrafish. PLoS One, 10(10). Pfaffl, M.
https://doi.org/10.1371/journal.pone.0139504

PerkinElmer (u.å) *Radioimmnoassay*.
https://www.perkinelmer.com/no/lab-products-and-services/application-support-knowledgebase/radiometric/radioimmunoassays.html

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, 29, 6.
https://doi.org/10.1093/nar/29.9.e45

Pickering, A. (1984) Cortisol-induced lymphocytopenia in brown trout, Salmo trutta L. *General and Comparativ Endocrinology*, 53 (2), 252-259
https://doi.org/10.1016/0016-6480(84)90250-8

Pickering, A. D., & Pottinger, T. G. (1989). Stress responses and disease resistance in salmonid fish: effects of chronic elevation of plasma cortisol. *Fish physiology and biochemistry*, *7*(1), 253-258.
https://doi.org/10.1007/BF00004714

Pike, K. (1993). Multinational Companies and genetic resources. WORLD BANK TECHNICAL PAPER, 51-51. *Agricultural Technologies for Market-led Development Opportunities in the 1900s*
Ebook link

Poltronieri, C., Maccatrozzo, L., Simontacchi, C., Bertotto, D., Funkenstein, B., Patruno, M., & Radaelli, G. (2007). Quantitative RT-PCR analysis and immunohistochemical localization of HSP70 in sea bass Dicentrarchus labraxexposed to transport stress. *European Journal of Histochemistry*, *51*(2), 125-136
https://doi.org/10.1007/s00300-004-0697-y

Press, C. M. & Evensen, Ø. (1999) The morphology of the immune system in teleost fishes. *Fish and Shellfish Immunology*, 9 (4), 309-318.
https://doi.org/10.1006/fsim.1998.0181

Prunet, P., Boeuf, G., Bolton, J. P. & Young, G. (1989) Smoltification and Seawater Adaptation in Atlantic Salmon (Salmo-Salar) - Plasma Prolactin, Growth-Hormone, and ThyroidHormones. *General and Comparatve Endocrinology*, 74 (3), 355-364. https://doi.org/10.1016/s0016-6480(89)80031-0

Rauta, P. R., Nayak, B., & Das, S. (2012). Immune system and immune responses in fish and their role in comparative immunity study: a model for higher organisms. *Immunol Lett*, 148(1), 23-33. https://doi.org/10.1016/j.imlet.2012.08.003

Raz, E. (2003). Primordial germ-cell development: the zebrafish perspective. *Nature Reviews Genetics*, 4(9), 690-700. https://doi.org/10.1038/nrg1154

Redding, J. M., Schreck, C. B., Birks, E. K. & Ewing, R. D. (1984) Cortisol and its effects on plasma thyroid hormone and electrolyte concentrations in fresh water and during seawater acclimation in yearling coho salmon, Oncorhynchus kisutch. *General and Comparative Endocrinology*, 56 (1), 146-155. https://doi.org/10.1016/0016-6480(84)90071-6

Reid, S. G., Bernier, N. J. & Perry, S. F. (1998). The adrenergic stress response in fish: control of catecholamine storage and release. Comparative Biochemistry and Physiology C-Toxicology & Pharmacology, 120, 1-27. https://doi.org/10.1016/S0742-8413(98)00037-1

Regjeringen (2021). Norsk Havbruksnæring. https://www.regjeringen.no/no/tema/mat-fiske-og-landbruk/fiskeri-og-havbruk/Norskhavbruksnaring/id754210/

Regjeringen (2015). Bærekraftig og forutsigbar vekst for laks. *Nærings-og fiskedepartementet.* https://www.regjeringen.no/no/aktuelt/barekraftig-og-forutsigbar-vekst-for-laks/id2401801 (accessed 24.04.2022)

Roberts, R. J., Agius, C., Saliba, C., Bossier, P. & Sung, Y. Y. (2010). Heat shock proteins (chaperones) in fish and shellfish and their potential role in relation to fish health: a

review. *Journal of Fish Diseases*, 33, 789-801.
https://doi.org/10.1111/j.1365-2761.2010.01183.x

Robertson, L. S., & McCormick, S. D. (2012). Transcriptional profiling of the parr–smolt transformation in Atlantic salmon. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, *7*(4), 351-360.

https://doi.org/10.1016/j.cbd.2012.07.003

Sakai, M., Kobayashi, M. & Kawauchi, H. (1996) Mitogenic effect of growth hormone and prolactin on chum salmon Oncorhynchus keta leukocytes in vitro. *Veterinary Immunology and Immunopathology*, 53 (1), 185-189
https://doi.org/10.1111/j.1365-2761.2010.01183.x

Sakai, M., Hikima, J. I., & Kono, T. (2020). Fish cytokines: current research and applications. *Fisheries Science*, *87*(1), 1-9.
https://doi.org/10.1007/s12562-020-01476-4

Salinas, I., Fernández-Montero, Á., Ding, Y., & Sunyer, J. O. (2021). Mucosal immunoglobulins of teleost fish: A decade og advances. *Dev Comp Immunol*, 121, 104079.
https://doi.org/10.1016/j.dci.2021.104079

Salinas, I. (2015). The Mucosal Immune System of Teleost Fish. *Biology (Basel)*, 4(3), 525-539.
https://doi.org/10.3390/biology4030525

Salinas, I., Zhang, Y.-A. & Sunyer, J. O. (2011) Mucosal immunoglobulins and B cells of teleost fish. *Developmental & Comparative Immunology*, 35 (12), 1346-1365.
https://doi.org/10.1016/j.dci.2011.11.009

Sambraus, F., Olsen, R. E., Remen, M., Hansen, T. J., Torgersen, T., & Fjelldal, P. G. (2017). Water temperature and oxygen: The effect of triploidy on performanceand metabolism in farmed Atlantic salmon (Salmo salarL.) post-smolts. *Aquaculture*, 473, ss. 1-12.
https://doi.org/10.1016/j.aquaculture.2017.01.024

Schulz, R. (1985). Measurement of five androgens in the blood of immature and maturing male rainbow trout, Salmo gairdneri (Richardson). *Steroids*, 46(2-3), 717-726. https://doi.org/10.1016/0039-128x(85)90051-0

Secombes, C. J., Wang, T. & Bird, S. (2011) The interleukins of fish. Developmental & Comparative Immunology 35(12) 1336-1345 https://doi.org/10.1016/j.dci.2011.05.001

Selye, H. (1946). The general adaptation syndrome and the diseases of adaptation. *The journal of clinical endocrinology*, 6(2), 117-230. https://doi.org/10.1210/jcem-6-2-117

Segner, H., Verburg-van Kemenade, B. L., & Chadzinska, M. (2016). The immunomodulatory role of the hypothalamus-pituitary-gonad axis: Proximate mechanism for reproduction-immune trade offs?. *Developmental & Comparative Immunology*, *66*, 43-60.

https://doi.org/10.1016/j.dci.2016.07.004

Seppola, M., Larsen, A. N., Steiro, K., Robertsen, B., & Jensen, I. (2008). Characterisation and expression analysis of the interleukin genes, IL-1β, IL-8 and IL-10, in Atlantic cod (Gadus morhua L.). *Molecular Immunology*, 45(4), 887-897. https://doi.org/10.1016/j.molimm.2007.08.003

Slanchev, K., Stebler, J., De La Cueva-Méndez, G., Raz, E., & Hopkins, N. (2005). Development without germ cells: The role of the germ line in zebrafish sex differentiation. *Proceedings of the National Academy of Sciences of the United States of America*, 102(11), ss. 4074-4079. https://doi.org/10.1073/pnas.0407475102

Smith, T. R., Tremblay, G. C., & Bradley, T. M. (1999a). Hsp70 and a 54 kDa protein (Osp54) are induced in salmon (Salmo salar) in response to hyperosmotic stress. *Journal of Experimental Zoology*, *284*(3), 286-298. https://doi.org/10.1002/(SICI)1097-010X(19990801)284:3%3C286::AID-JEZ6%3E3.0.CO;2-J

Smith TR, Tremblay G.C, Bradley T.M. (1999b). Characteriza-tion of the heat shock protein response of Atlantic salmon (Salmo salar). *Fish Physiol Biochem (in press) 20, 279-292.* https://link.springer.com/article/10.1023/A:1007743329892#citeas

Specker, J. L. & Schreck, C. B. (1982) Changes in plasma corticosteroids during smoltification of coho salmon, Oncorhynchus kisutch. *General and Comparative Endocrinology*, 46 (1), 53-58 https://doi.org/10.1016/0016-6480(82)90162-9

Strand, J. E., Hazlerigg, D., & Jørgensen, E. H. (2018). Photoperiod revisited: is there a critical day length for triggering a complete parr–smolt transformation in Atlantic salmon Salmo salar?. *Journal of Fish Biology*, *93*(3), 440-448. https://doi.org/10.1111/jfb.13760

Summerfelt, S., Waldrop, T., Davidson, J., Good, C., Kenney, P. B., Terjesen, B. F. & Wolters, W. (2012). Atlantic salmon growout trials in freshwater closed-containment systems at The Conservation Fund Freshwater Institute. https://0201.nccdn.net/4_2/000/000/07d/95b/06-summerfelt_update-on-growout-trials.pdf

Tadiso, T. M., Krasnov, A., Skugor, S., Afanasyev, S., Hordvik, I., & Nilsen, F. (2011). Gene expression analyses of immune responses in Atlantic salmon during early stages of infection by salmon louse (Lepeophtheirus salmonis) revealed bi-phasic responses coinciding with the copepod-chalimus transition. *BMC Genomics*, 12(1), 141. https://doi.org/10.1186/1471-2164-12-141

Tanriverdi, F., Silveira, L. F., MacColl, G. S., & Bouloux, P. M. (2003). The hypothalamicpituitary-gonadal axis: immune function and autoimmunity. J Endocrinol, 176(3), 293-304. https://doi.org/10.1677/joe.0.1760293

Taranger, G. L., Carrillo, M., Schulz, R. W., Fontaine, P., Zanuy, S., Felip, A., . . . Norberg, B. (2010). Control of puberty in farmed fish. *General and comparative endocrinology,* 165(3), 483-515. https://doi.org/10.1016/j.ygcen.2009.05.004

Tort, L. (2011). Stress and immune modulation in fish. Developmental and Comparative Immunology, 35, 1366-1375. https://doi.org/10.1016/j.dci.2011.07.002

Tveiten, H., Bjørn, P., Johnsen, H., Finstad, B. & McKinley, R. (2010). Effects of the sea louse Lepeophtheirus salmonis on temporal changes in cortisol, sex steroids, growth and reproductive investment in Arctic charr Salvelinus alpinus. *Journal of Fish Biology*, 76(10), 2318-2341. https://doi.org/10.1111/j.1095-8649.2010.02636.x

Tveiten, H., Skugor, A., Andersen, Ø., Slanchev, K., Mommens, M., & Santi, N. (2015). Steril laks – kartlegging av faktorer sentrale i dannelsen av kjønnsceller hos Atlantisk laks (Rapport K-36/2015). Nofima https://www.fhf.no/prosjekter/prosjektbasen/900791/

Tveiten, H., Karlsen, K., ThessLund, T., Johanson, G, S., Thiyagarajan, D, B., Andersen, Ø. (2022) Impact of germ cell ablation on the activation of the brain–pituitary–gonadal axis in precocious Atlantic salmon (*Salmo salar* L.) male 89(10), 471-484. https://doi.org/10.1002/mrd.23635

Uribe, C., Folch, H., Enríquez, R., & Moran, G. (2011). Innate and adaptive immunity in teleost fish: A review. *Veterinarni Medicina*, 56, 486-503. https://doi.org/10.17221/3294-VETMED

Van Weerd, J. & Komen, J. (1998). The effects of chronic stress on growth in fish: a critical appraisal. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 120(1), 107-112. https://doi.org/10.1016/S1095-6433(98)10017-X

Veterinærinstituttet. (2018). Triploid laks – mottakelighet for smittsomme sykdommer. (*Rapport 27 – 2018*). file:///C:/Users/mgtei/Downloads/2018-27%20Triploid%20laks.pdf

Verburg-van Kemenade, B., Nowak, B., Engelsma, M. & Weyts, F. (1999) Differential effects of cortisol on apoptosis and proliferation of carp B-lymphocytes from head

kidney, spleen and blood. *Fish and Shellfish Immunology*, 9 (5), 405-415. https://doi.org/10.1006/fsim.1998.0197

Vijayan, M. M., Pereira, C., Forsyth, R. B., Kennedy, C. J. & Iwama, G. K. (1997). Handling stress does not affect the expression of hepatic heat shock protein 70 and conjugation enzymes in rainbow trout treated with beta-naphthoflavone. *Life Sciences*, 61(2), 117- 127. https://doi.org/10.1016/s0024-3205(97)00366-4

Wang, T., & Secombes, C. J. (2013). The cytokine networks of adaptive immunity in fish. *Fish Shellfish Immunol*, 35(6), 1703-1718. https://doi.org/10.1016/j.fsi.2013.08.030

Wang, J., Kortner, T. M., Chikwati, E. M., Li, Y., Jaramillo-Torres, A., Jakobsen, J. V., ... & Krogdahl, Å. (2020). Gut immune functions and health in Atlantic salmon (Salmo salar) from late freshwater stage until one year in seawater and effects of functional ingredients: A case study from a commercial sized research site in the Arctic region. *Fish & Shellfish Immunology*, *106*, 1106-1119. https://doi.org/10.1016/j.fsi.2020.09.019

Wargelius, A., Leininger, S., Skaftnesmo, K. O., Kleppe, L., Andersson, E., Taranger, G. L., … Edvardsen, R. B. (2016). Dnd knockout ablates germ cells and demonstrates germ cell independent sex differentiation in Atlantic salmon. *National Library of Medicing*, 6(1), 1-8. https://doi.org/10.1038/srep21284

Washburn, B. S., Moreland, J. J., Slaughter, A. M., Werner, I., Hinton, D. E., & Sanders, B. M. (2002). Effects of handling on heat shock protein expression in rainbow trout (Oncorhynchus mykiss). *Environmental Toxicology and Chemistry: An International Journal*, *21*(3), 557-560.

https://doi.org/10.1002/etc.5620210313

Wei, Y. Q., Zhao, X., Kariya, Y., Teshigawara, K. & Uchida, A. (1995). Inhibition of proliferation and induction of adaptosis by abrogation of heat-shock protein (HSP)-70 expression in tumor-cells. *Cancer Immunology Immunotherapy*, 40, 73-78. https://doi.org/10.1007/bf01520287

Weidinger, G., Stebler, J., Slanchev, K., Dumstrei, K., Wise, C., Lovell-Badge, R., . . . Raz, E. (2003). Dead end, a Novel Vertebrate Germ Plasm Component, Is Required for Zebrafish Primordial Germ Cell Migration and Survival. *Current Biology*, 13(16), ss. 1429-1434. https://doi.org/10.1016/s0960-9822(03)00537-2

Welch, W. J. (1993). How cells respond to stress. *Scientific American*, 268, 56–64. https://doi.org/10.1038/scientificamerican0593-56

Weyts, F., Flik, G., Rombout, J. & Verburg-van Kemenade, B. (1998) Cortisol induces apoptosis in activated B cells, not in other lymphoid cells of the common carp, Cyprinus carpio L. *Developmental & Comparative Immunology*, 22 (5), 551-562. https://doi.org/10.1016/s0145-305x(98)00033-0

Wieten, L., Broere, F., Van Der Zee, R., Koerkamp, E. K., Wagenaar, J. & Van Eden, W. (2007). Cell stress induced HSP are targets of regulatory T cells: A role for HSP inducing compounds as anti-inflammatory immuno-modulators? *FEBS Letters*, 581, 3716-3722. https://doi.org/10.1016/j.febslet.2007.04.082

Wong, T.-T. & Zohar, Y. (2015). Production of reproductively sterile fish by a non-transgenic gene silencing technology. *The Proceedings of the National Academy of Sciences* 5, 15822. https://doi.org/10.1073/pnas.0407475102

Workenhe, S. T., Rise, M. L., Kibenge, M. J. & Kibenge, F. S. (2010) The fight between the teleost fish immune response and aquatic viruses. Molecular Immunology, 47 (16), 2525-2536. https://doi.org/10.1016/j.molimm.2010.06.009

Yada, T. (2007) Growth hormone and fish immune system. *General and Comparative Endocrinology*, 152 (2), 353-358. https://doi.org/10.1016/j.ygcen.2007.01.045

Yada, T., Nagae, M., Moriyama, S. & Azuma, T. (1999) Effects of prolactin and growth hormone on plasma immunoglobulin M levels of hypophysectomized rainbow

trout, Oncorhynchus mykiss. *General and Comparative Endocrinology*, 115 (1), 46-52. https://doi.org/10.1006/gcen.1999.7282

Yamaguchi, T., & Dijkstra, J. M. (2019). Major Histocompatibility Complex (MHC) Genes and Disease Resistance in Fish. *Cells*, 8(4). https://doi.org/10.3390/cells8040378

Yaron, Z. & Levavi-Sivan, B. (2011). Endocrine regulation of fish reproduction. Encyclopedia of fish physiology: from genome to environment, 2, 1500-1508 https://www.tarjomefa.com/wp-content/uploads/2018/01/8460-EnglishTarjomeFa.pdf

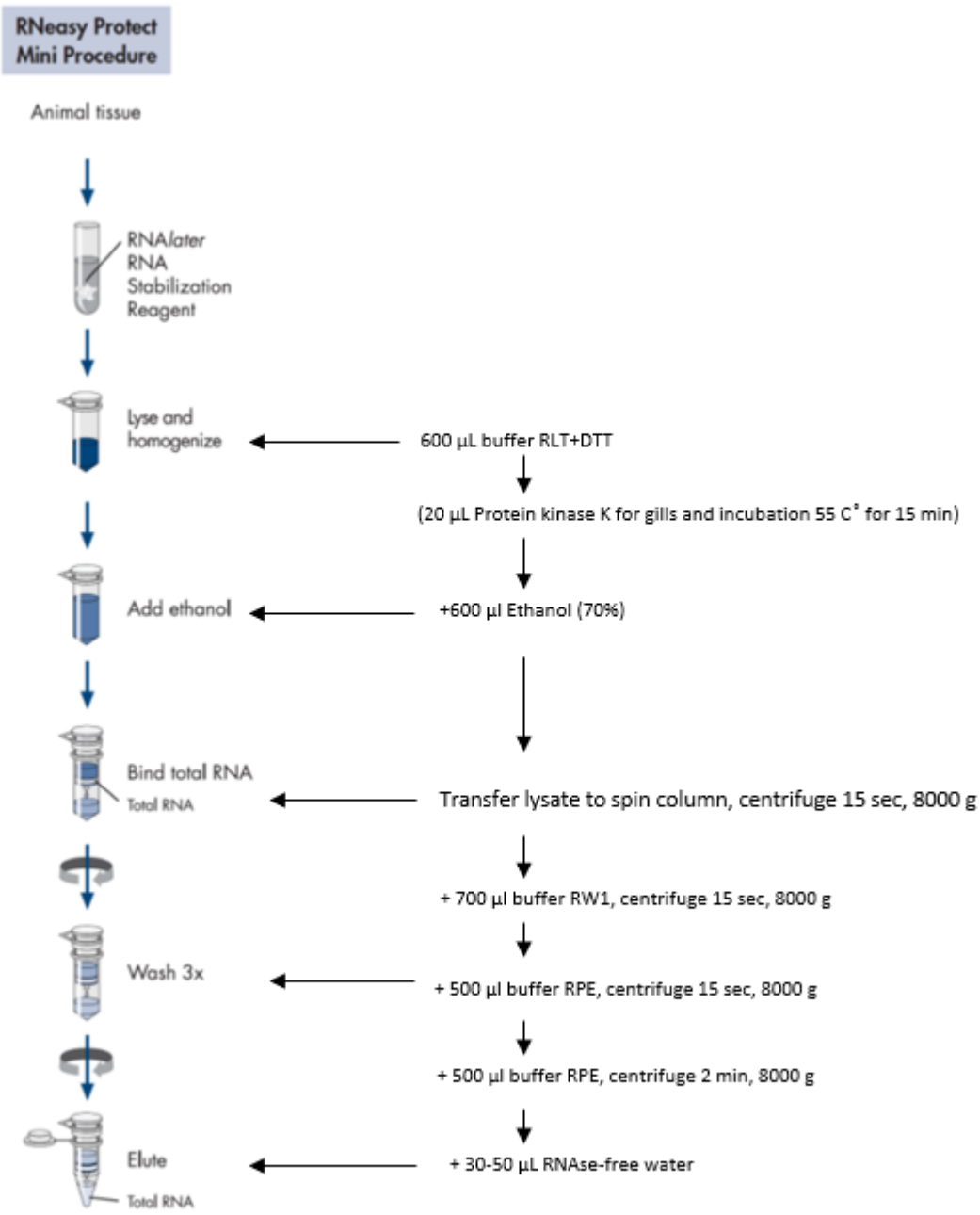
Zapata, A., Diez, B., Cejalvo, T., Gutierrez-de Frias, C. & Cortes, A. (2006) Ontogeny of the immune system of fish. *Fish and Shellfish Immunology*, 20 (2), 126-136. https://doi.org/10.1016/j.fsi.2004.09.005

Zou, J., & Secombes, C. J. (2011). Teleost fish interferons and their role in immunity. *Developmental & Comparative Immunology*, *35*(12), 1376-1387. https://doi.org/10.1016/j.dci.2011.07.001

Zou, J., & Secombes, C. J. (2016). The function of fish cytokines. *Biology*, *5*(2), 23 https://doi.org/10.3390/biology5020023

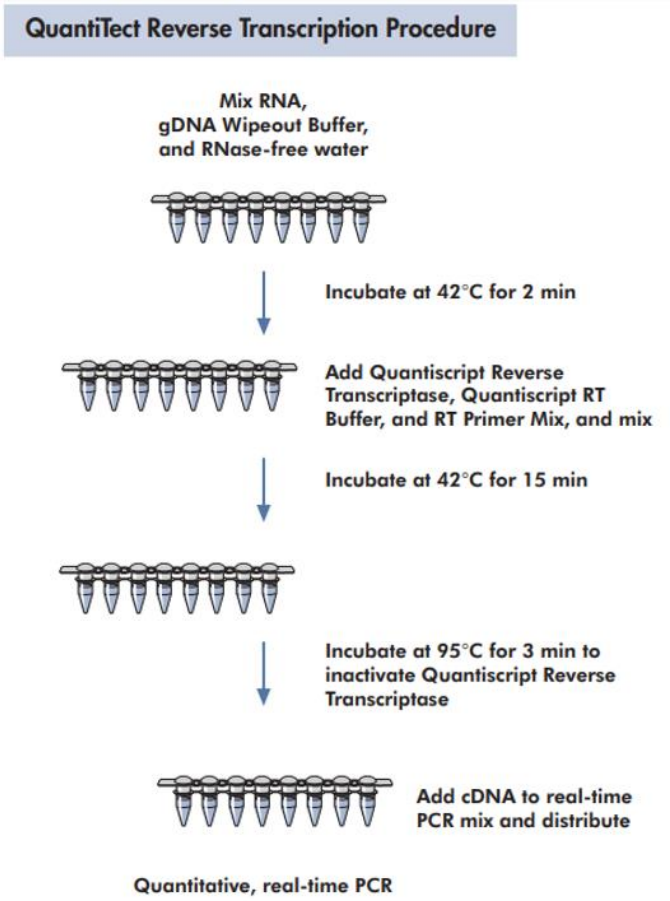
Zwollo, P., Cole, S., Bromage, E. & Kaattari, S. (2005) B cell heterogeneity in the teleost kidney: evidence for a maturation gradient from anterior to posterior kidney. *The Journal of Immunology*, 174 (11), 6608-6616. https://doi.org/10.4049/jimmunol.174.11.6608

Appendix I



The figure shows a simple outline of the main procedures during RNA isolation using the RNeasy Mini Kit (Quiagen, Hilden, Germany)

Appendix II



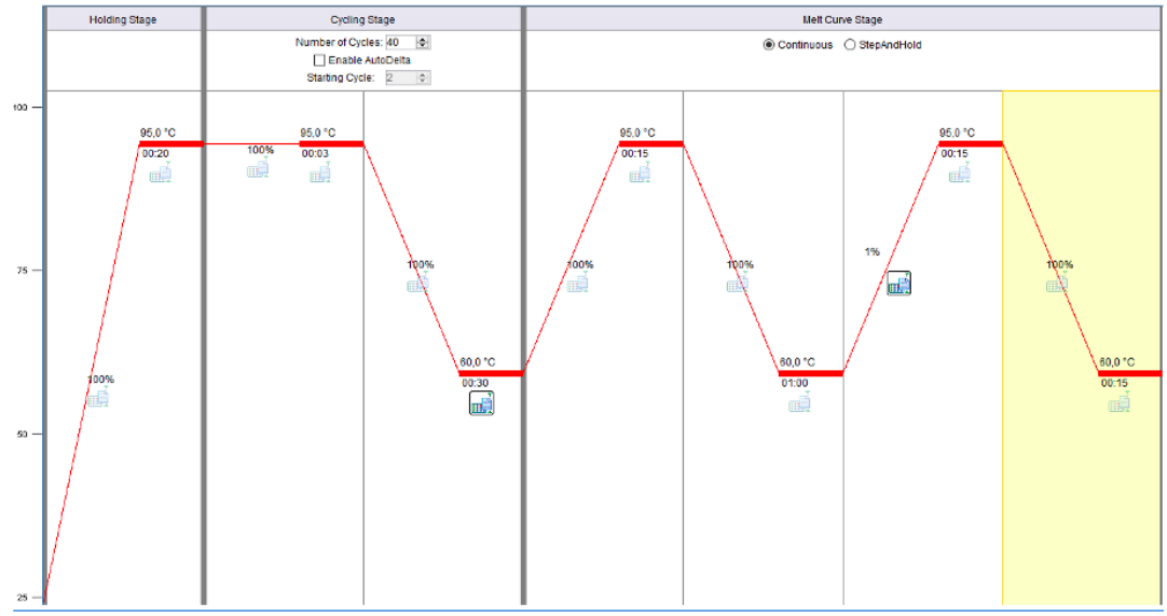
The figure shows a simple outline of the main procedures during cDNA synthesis using the QuantiTect Reverse Transcription Kit (Quiagen, Hilden, Germany).

Appendix III

The table shows the amount of components added to the master mix per well and per qPCR 96-well tray. The amount is calculated for four extra wells per tray to adjust for mast mixes that adhere to surfaces of pipettes and master mix tubes, as well as changing pipettes or pipette tips along the way.

Coponents in the mastermix	**Per well (µl)**	**Mastermix (µl) (96-wells +4 extra wells)**
Fast SYBR® Green I	7,8	780
Forward primer (5µM)	0,8	80
Reverse primer (5µM)	0,8	80

Appendix IV



Program used for SYBR® Green reagents during qPCR. Stages are shown in the header as well as number of cycles, temperature at each stage are given over the red line and duration in minutes and seconds under the line, also shown in the table under.

The table shows the cycle parameters the samples have undergone during a 7500 Fast-Realtime PCR system program.

Program step	**Temperature (C°)**	**Duration**	**Cycles**
Pre-PCR reading	50	2 min	Hold
Denaturation	95	20 sec	Hold
Denaturation	95	3 sec	40
Amplification and extraction	60	30 sec	40
Melt Curve	95	15 sec	Continuous
	60	1 min	
	95	15 sec	
	60	15 sec	

Appendix V

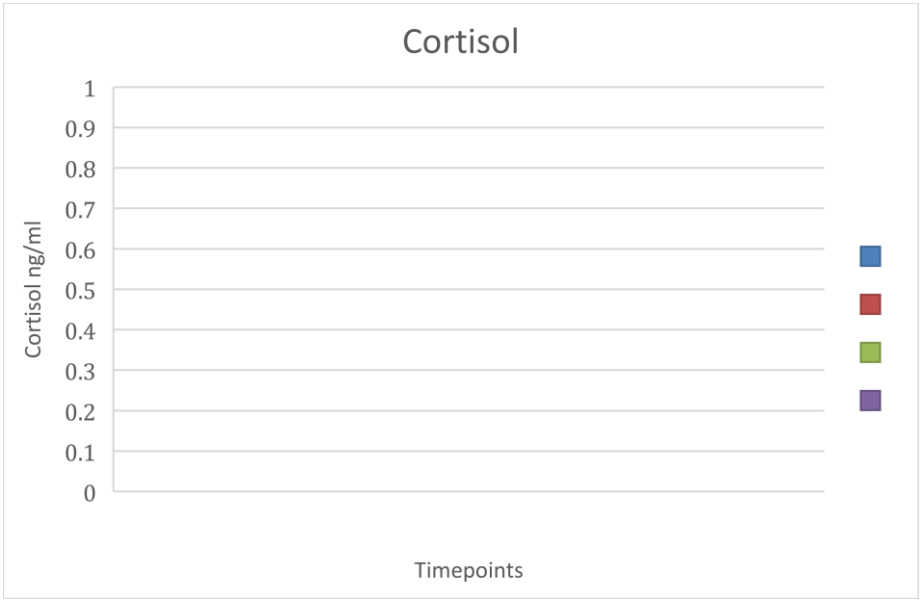
Tabell Shows what is added to the different samples in the RIA assay in ul. TC = total count. TB = total bound. NSB = non-specific bound. STD = standard curve solution.

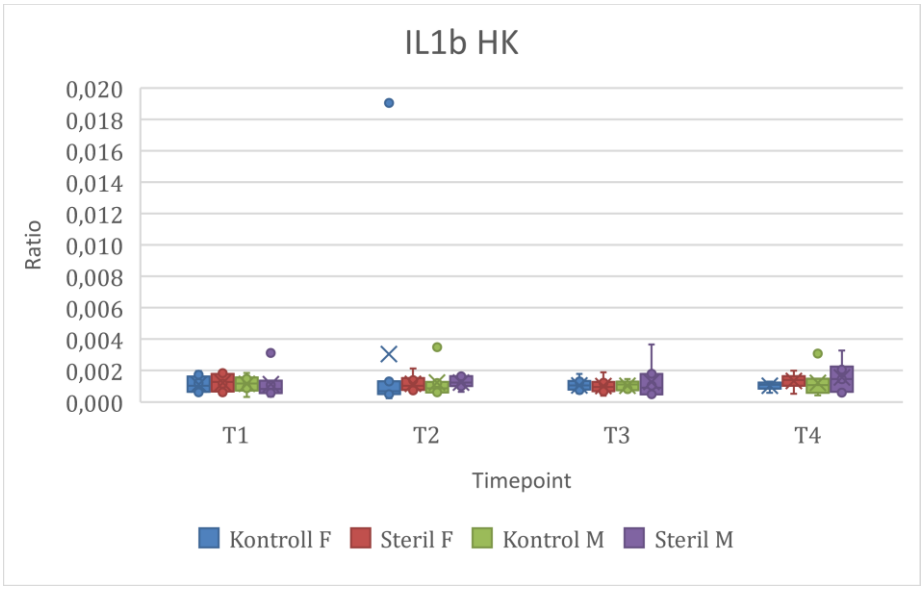
	Buffer	**Standard**	**Sample**	**3H**	**Antibody**
TC	600	-	-	50	-
TB	100	-	-	50	200
NSB	300	-	-	50	-
Std. S1-S9	50	50	-	50	200
Sample 1-30	-	-	100	50	200

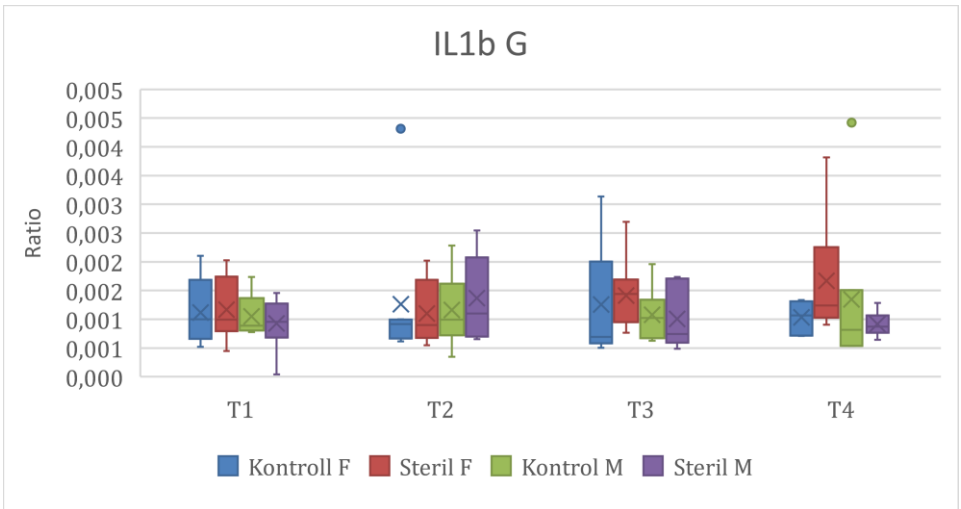
Appendix VI

Graphs With Outliers

The original data as a boxplot with mean and standard deviation whit outliers. Produced in excel (2017). SF=sterile female, SM=sterile male, KF=control female, KM=control male. T3 is corresponds to the same sampling as T0, and T4 corresponds to T24 of the transport stress experiment.


Cortisol
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Cortisol ng/ml
Timepoints


IL1b HK
0,020
0,018
0,016
0,014
0,012
0,010
0,008
0,006
0,004
0,002
0,000
Ratio
T1
T2
T3
T4
Timepoint
Kontroll F
Steril F
Kontrol M
Steril M


IL1b G
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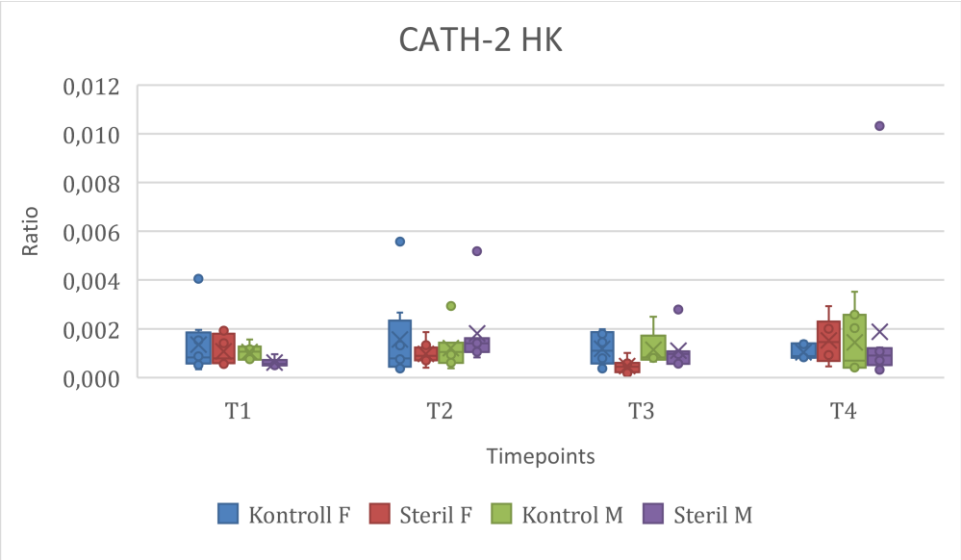
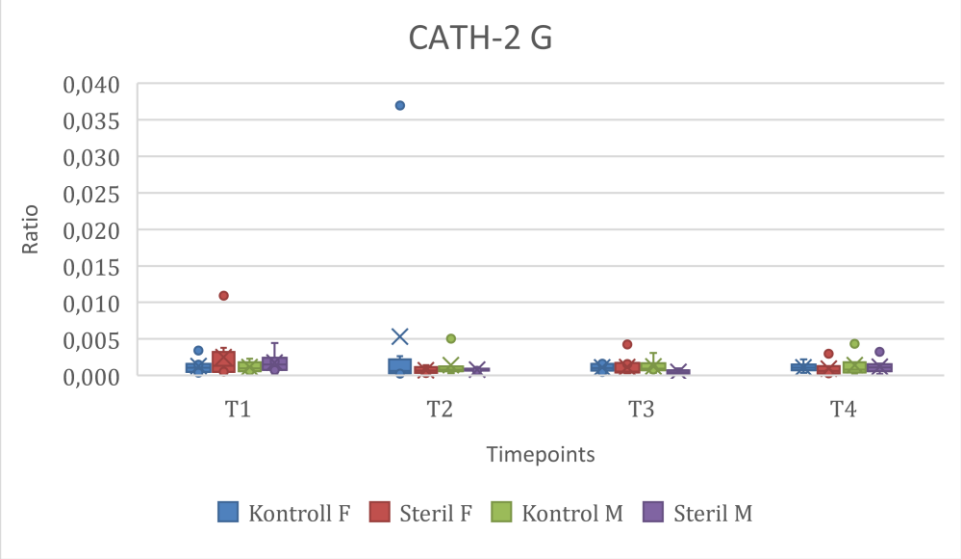
0,005
0,004
0,004
0,003
0,003
0,002
0,002
0,001
0,001
0,000
Ratio
T1
T2
T3
T4
Kontroll F
Steril F
Kontrol M
Steril M


CATH-2 HK
0,012
0,010
0,008
0,006
0,004
0,002
0,000
Ratio
T1
T2
T3
T4
Timepoints
Kontroll F
Steril F
Kontrol M
Steril M


CATH-2 G
0,040
0,035
0,030
0,025
0,020
0,015
0,010
0,005
0,000
Ratio
T1
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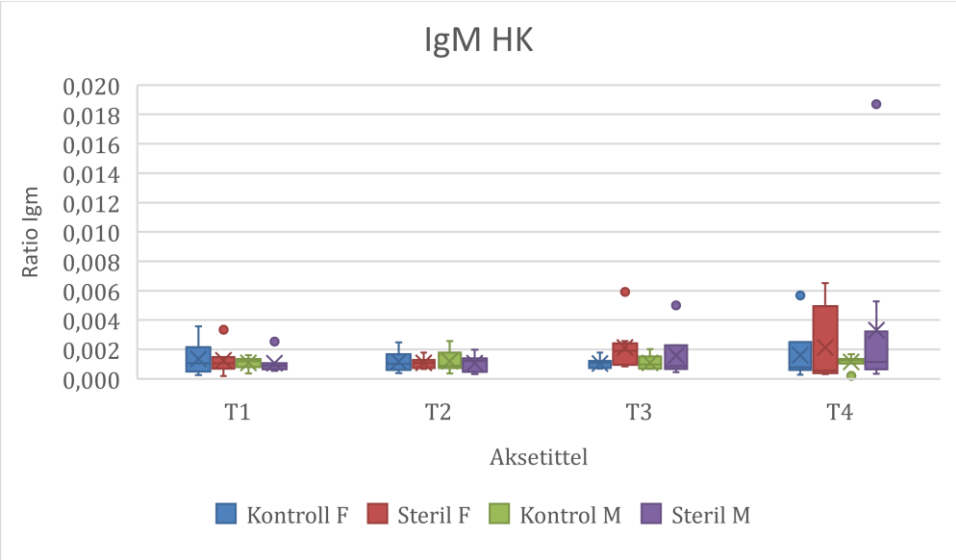
T4
Timepoints
Kontroll F
Steril F
Kontrol M
Steril M


IgM HK
Ratio Igm
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0,018
0,016
0,014
0,012
0,010
0,008
0,006
0,004
0,002
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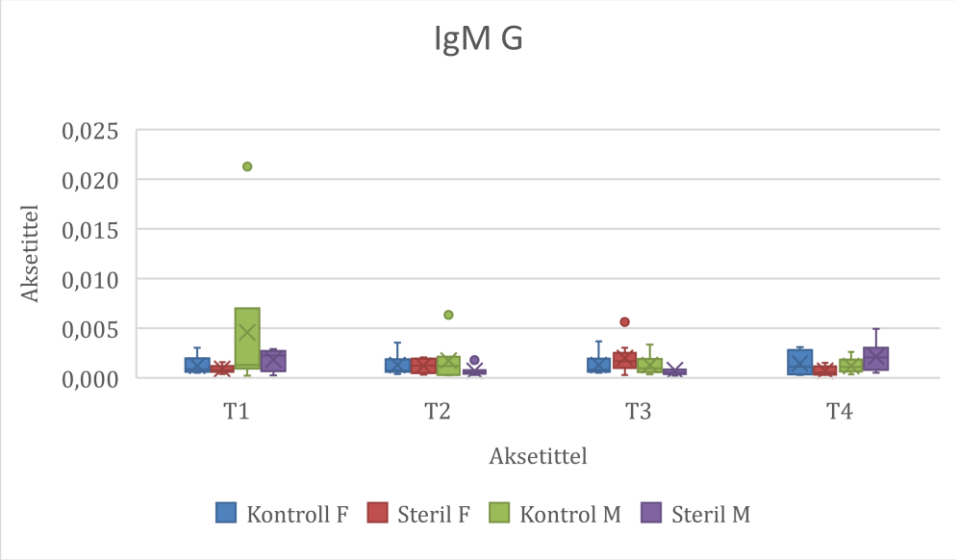
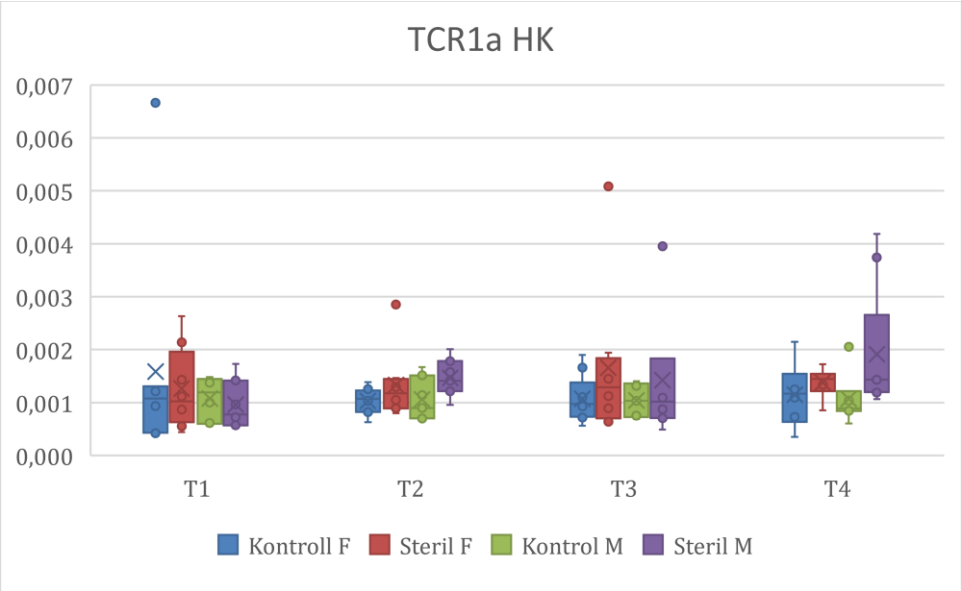
Aksetittel
Kontroll F
Steril F
Kontrol M
Steril M


IgM G
Aksetittel
0,025
0,020
0,015
0,010
0,005
0,000
T1
T2
T3
T4
Aksetittel
Kontroll F
Steril F
Kontrol M
Steril M


TCR1a HK
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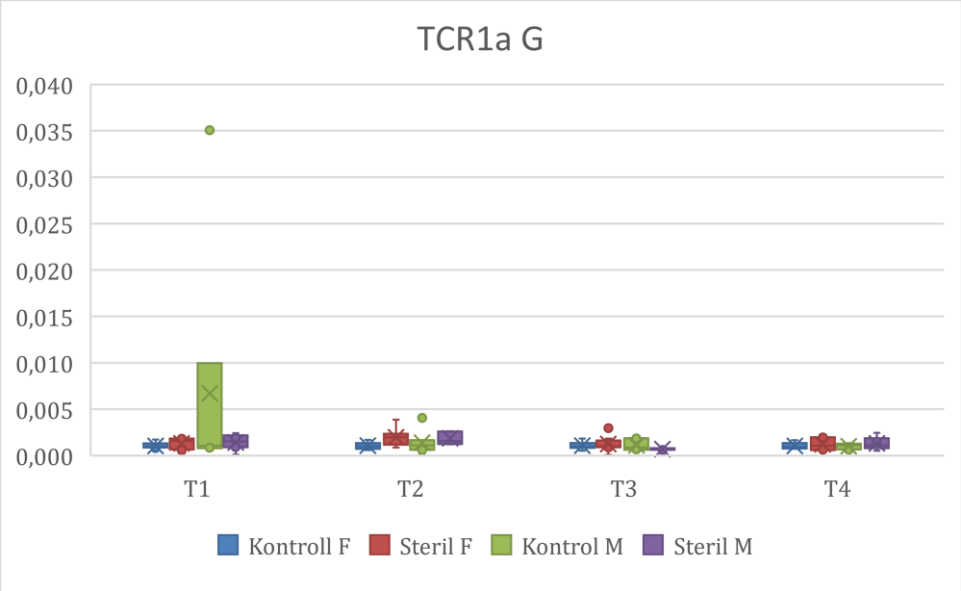
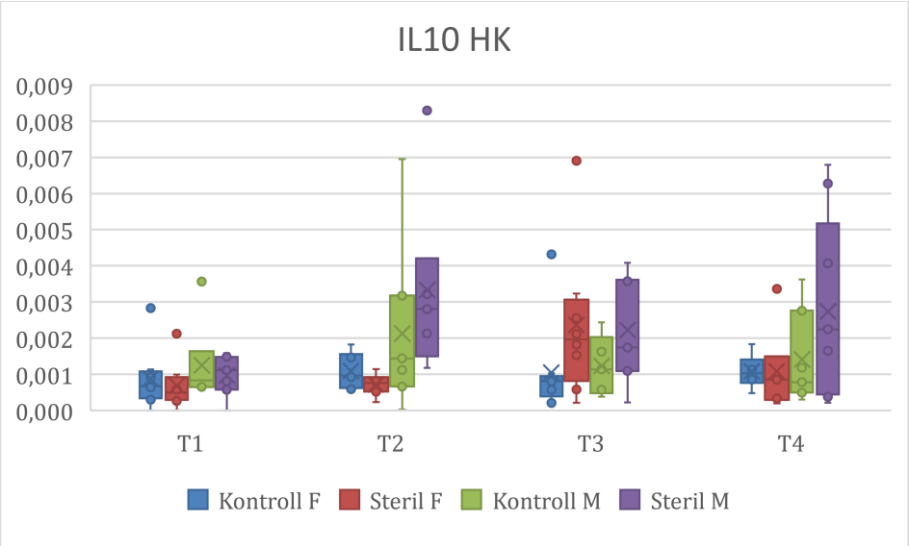
0,006
0,005
0,004
0,003
0,002
0,001
0,000
T1
T2
T3
T4
Kontroll F
Steril F
Kontrol M
Steril M


TCR1a G
0,040
0,035
0,030
0,025
0,020
0,015
0,010
0,005
0,000
T1
T2
T3
T4
Kontroll F
Steril F
Kontrol M
Steril M


IL10 HK
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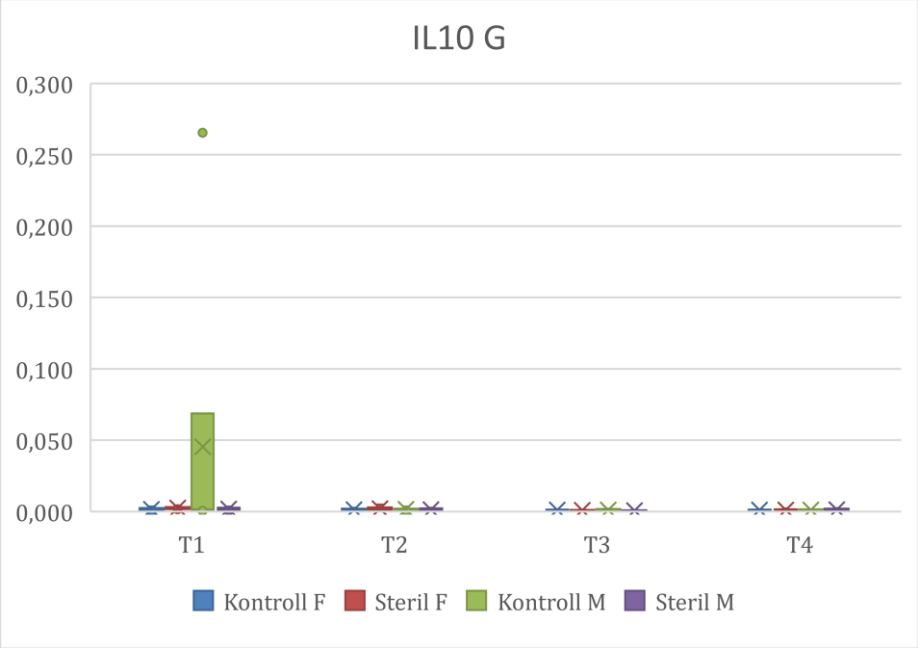
0,007
0,006
0,005
0,004
0,003
0,002
0,001
0,000
T1
T2
T3
T4
Kontroll F
Steril F
Kontroll M
Steril M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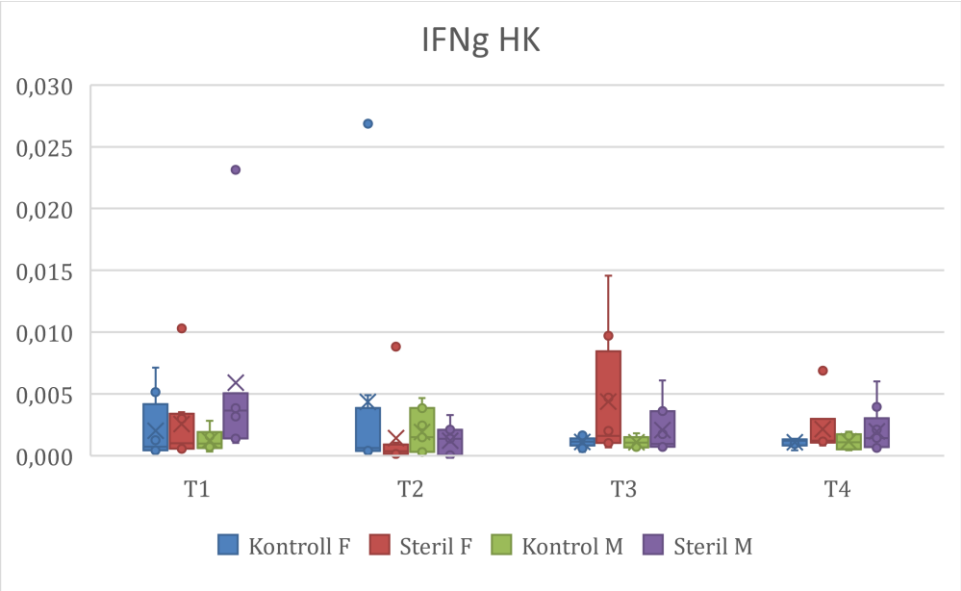
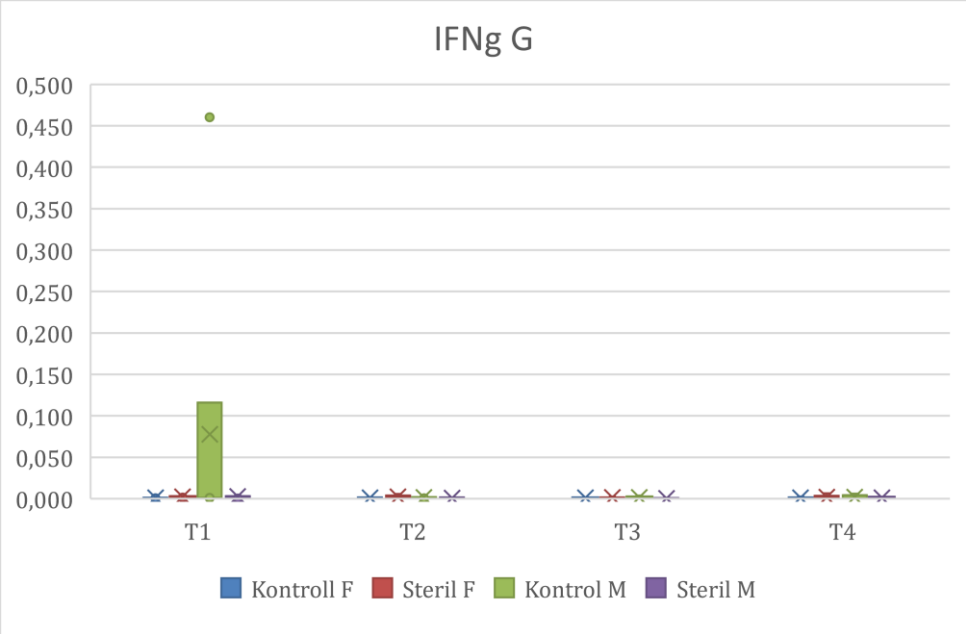
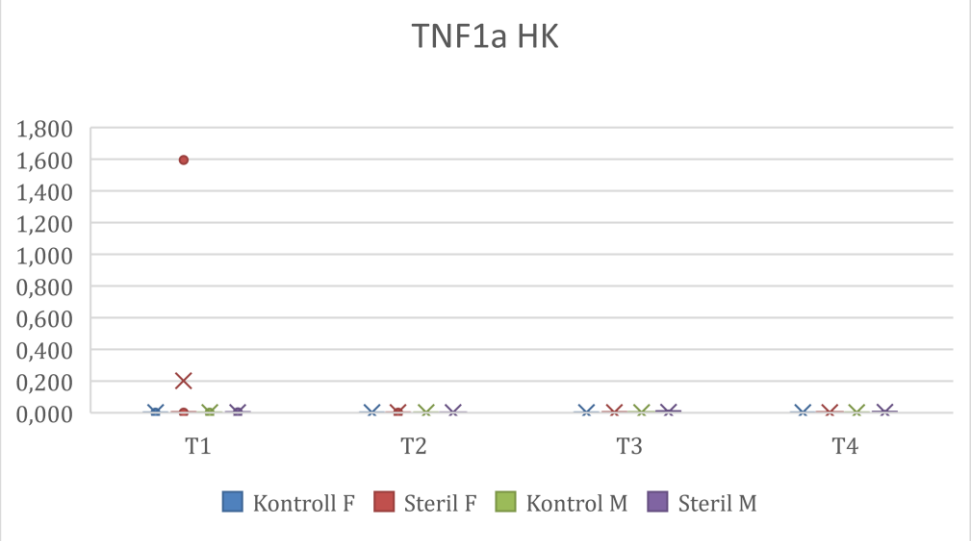
0,250
0,200
0,150
0,100
0,050
0,000
T1
T2
T3
T4
Kontroll F
Steril F
Kontroll M
Steril M


IFNg HK
0,030
0,025
0,020
0,015
0,010
0,005
0,000
T1
T2
T3
T4
Kontroll F
Steril F
Kontrol M
Steril M


IFNg G
0,500
0,450
0,400
0,350
0,300
0,250
0,200
0,150
0,100
0,050
0,000
T1
T2
T3
T4
Kontroll F
Steril F
Kontrol M
Steril M


TNF1a HK
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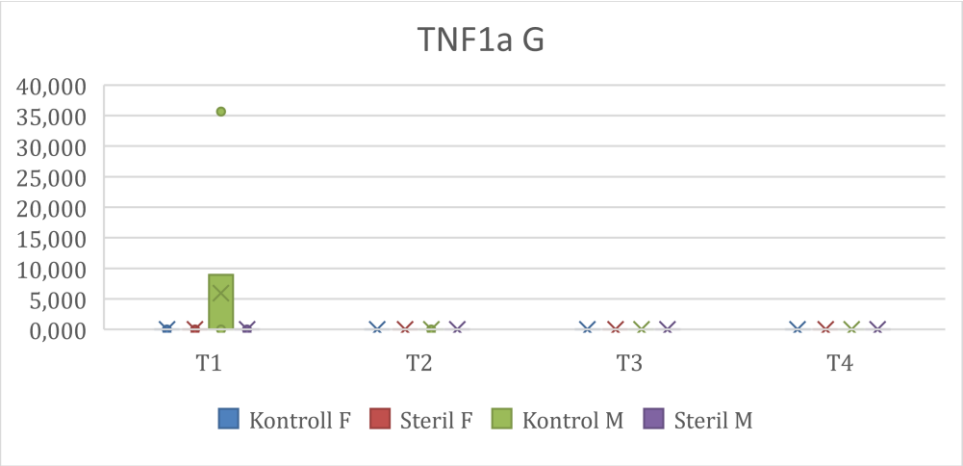
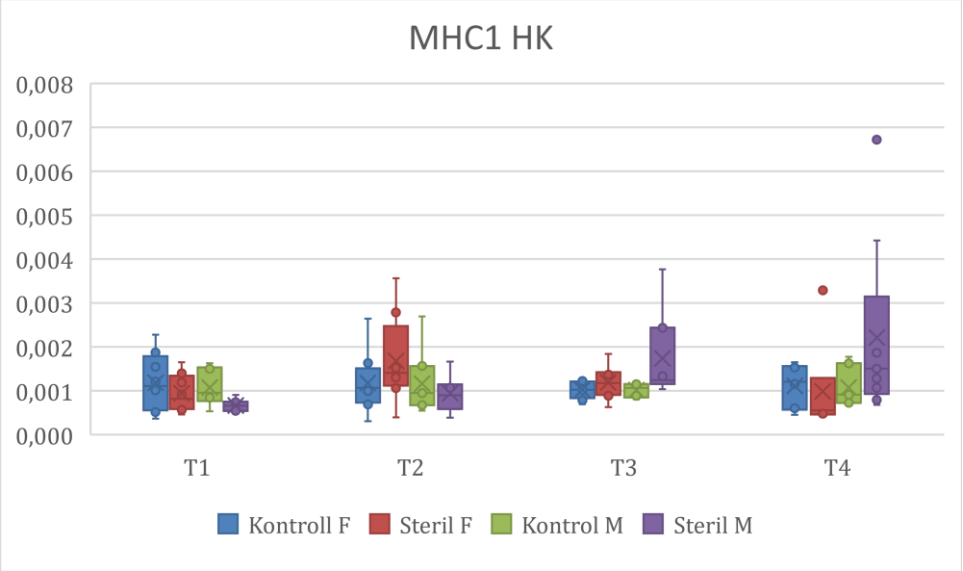
1,600
1,400
1,200
1,000
0,800
0,600
0,400
0,200
0,000
T1
T2
T3
T4
Kontroll F
Steril F
Kontrol M
Steril M


TNF1a G
40,000
35,000
30,000
25,000
20,000
15,000
10,000
5,000
0,000
T1
T2
T3
T4
Kontroll F
Steril F
Kontrol M
Steril M


MHC1 HK
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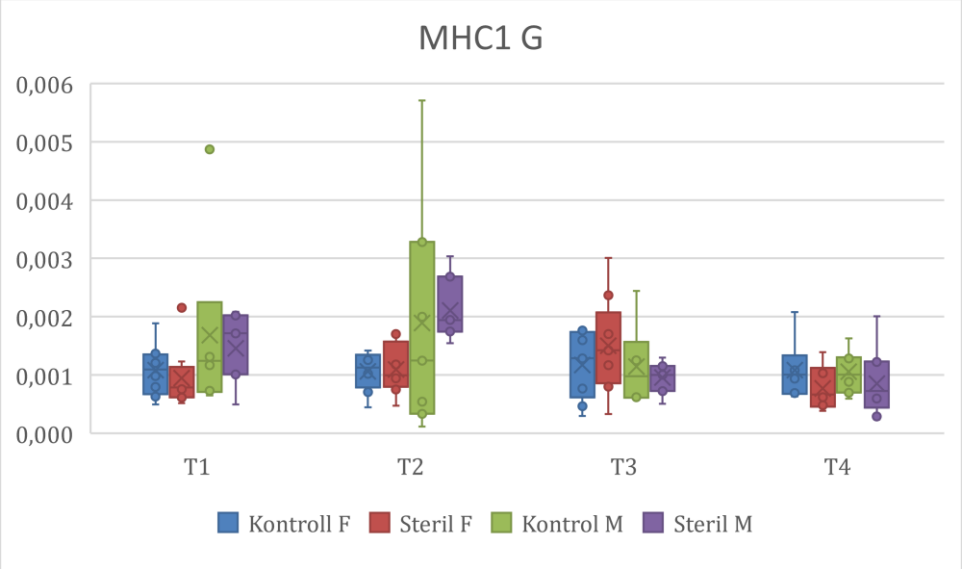
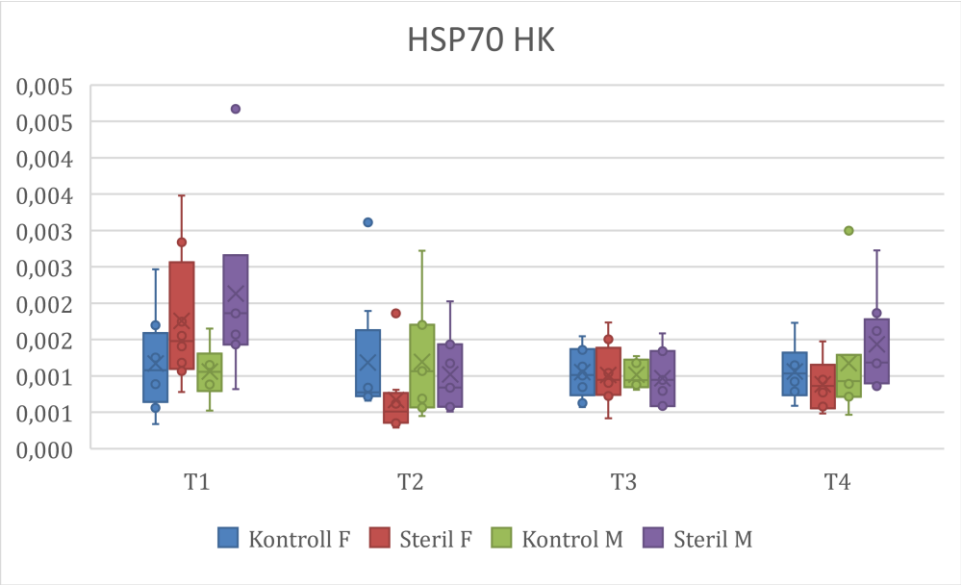
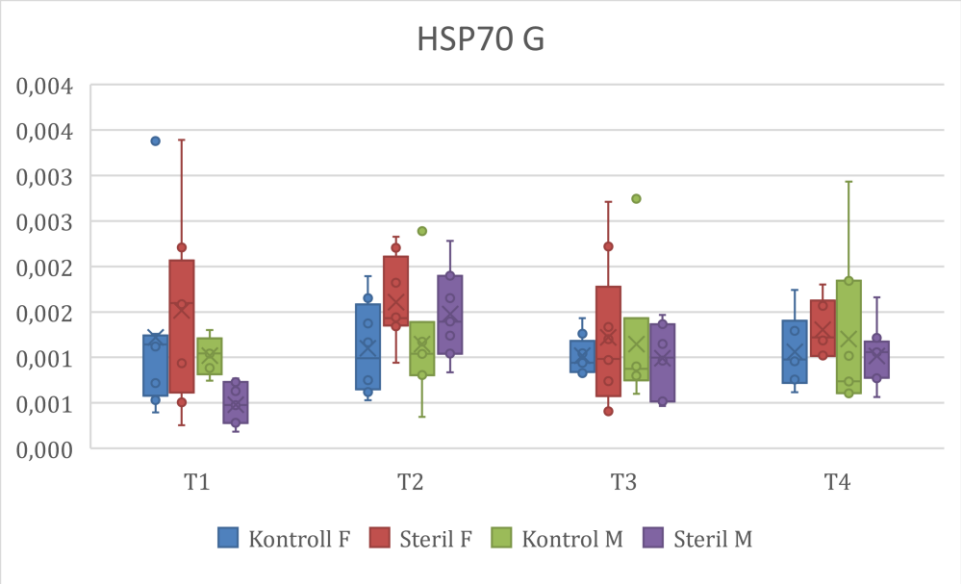
0,007
0,006
0,005
0,004
0,003
0,002
0,001
0,000
T1
T2
T3
T4
Kontroll F
Steril F
Kontrol M
Steril M

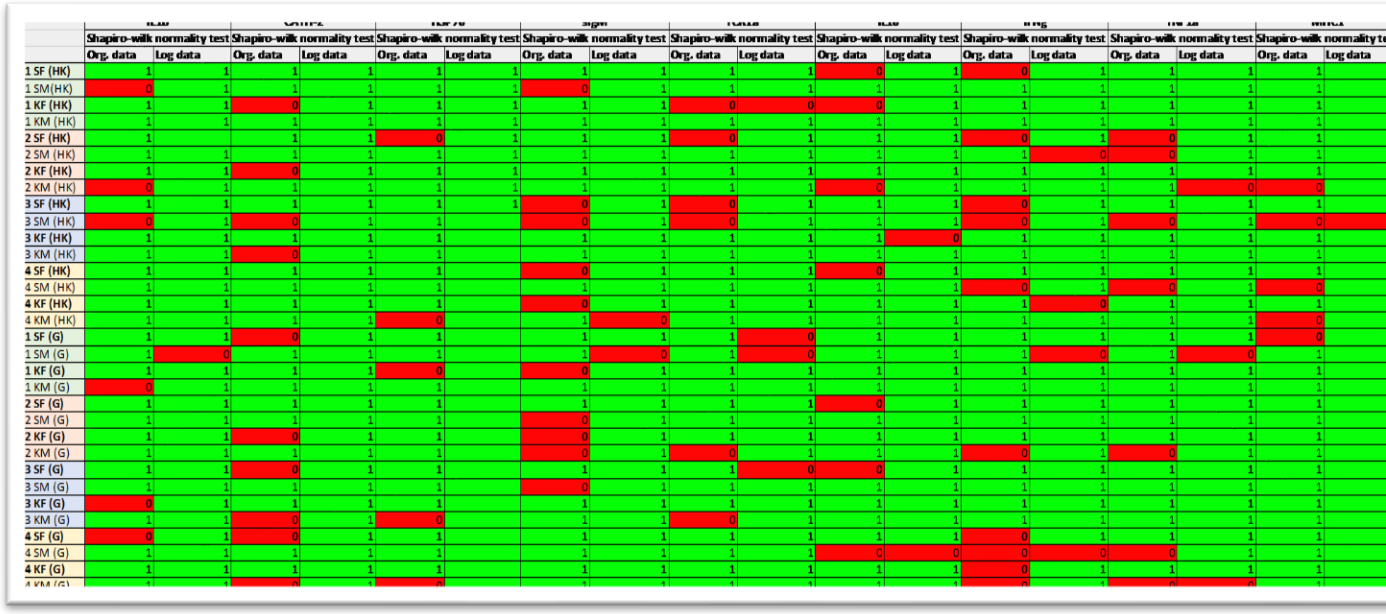

MHC1 G
0,006
0,005
0,004
0,003
0,002
0,001
0,000
T1
T2
T3
T4
Kontroll F
Steril F
Kontrol M
Steril M





Appendix VII

Normality tests



	Shapiro-wilk normality test																			
	Org. data	Log data	Org. data	Log data	Org. data	Log data	Org. data	Log data	Org. data	Log data	Org. data	Log data	Org. data	Log data	Org. data	Log data	Org. data	Log data	Org. data	Log data
1 SF (HK)	1	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1			
1 SM(HK)	0	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1			
1 KF (HK)	1	1	0	1	1	1	1	1	0	0	0	1	1	1	1	1	1			
1 KM (HK)	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
2 SF (HK)	1		1	1	0	1	1	1	0	1	1	1	0	1	0	1	1			
2 SM (HK)	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1			
2 KF (HK)	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
2 KM (HK)	0	1	1	1	1	1	1	1	1	1	0	1	1	1	1	0	0			
3 SF (HK)	1	1	1	1	1	1	0	1	0	1	1	1	0	1	1	1	1			
3 SM (HK)	0	1	0	1	1		0	1	0	1	1	1	0	1	0	1	0			
3 KF (HK)	1	1	1	1	1		1	1	1	1	1	0	1	1	1	1	1			
3 KM (HK)	1	1	0	1	1		1	1	1	1	1	1	1	1	1	1	1			
4 SF (HK)	1	1	1	1	1		0	1	1	1	0	1	1	1	1	1	1			
4 SM (HK)	1	1	1	1	1		1	1	1	1	1	1	0	1	0	1	0			
4 KF (HK)	1	1	1	1	1		0	1	1	1	1	1	1	0	1	1	1			
4 KM (HK)	1	1	1	1	0		1	0	1	1	1	1	1	1	1	1	0			
1 SF (G)	1	1	0	1	1		1	1	1	0	1	1	1	1	1	1	0			
1 SM (G)	1	0	1	1	1		1	0	1	0	1	1	1	0	1	0	1			
1 KF (G)	1	1	1	1	0		0	1	1	1	1	1	1	1	1	1	1			
1 KM (G)	0	1	1	1	1		1	1	1	1	1	1	1	1	1	1	1			
2 SF (G)	1	1	1	1	1		1	1	1	1	0	1	1	1	1	1	1			
2 SM (G)	1	1	1	1	1		0	1	1	1	1	1	1	1	1	1	1			
2 KF (G)	1	1	0	1	1		0	1	1	1	1	1	1	1	1	1	1			
2 KM (G)	1	1	1	1	1		0	1	0	1	1	1	0	1	0	1	1			
3 SF (G)	1	1	0	1	1		1	1	1	0	0	1	1	1	1	1	1			
3 SM (G)	1	1	1	1	1		0	1	1	1	1	1	1	1	1	1	1			
3 KF (G)	0	1	1	1	1		1	1	1	1	1	1	1	1	1	1	1			
3 KM (G)	1	1	0	1	0		1	1	0	1	1	1	1	1	1	1	1			
4 SF (G)	0	1	0	1	1		1	1	1	1	1	1	0	1	1	1	1			
4 SM (G)	1	1	1	1	1		1	1	1	1	0	0	0	0	0	1	1			
4 KF (G)	1	1	1	1	1		1	1	1	1	1	1	0	1	1	1	1			

The results of the Shapiro-wilk normality test for all the genes. Both for the original and lotransformed data where tested. 1 (green) indicates that the data has passed the normality test and 0 (red) indicates that the data sample did not pass the normality test. SF=sterile female, SM= sterile male, KF= control female, KM= control male. All normality tests were done in Grafpad Prism (2022).

	Cortisol	
	Shapiro-wilk normality test	
	Org. data	Log data
Γ1 SF	1	0
Γ1 SM	1	1
Γ1 KF	1	1
Γ1 KM	1	1
Γ2 SF	1	1
Γ2 SM	1	1
Γ2 KF	0	1
Γ2 KM	1	1
Γ3 SF	0	1
Γ3 SM	0	1
Γ3 KF	0	1
Γ3 KM	1	1
Γ4 SF	1	1
Γ4 SM	1	1
Γ4 KF	1	1
Γ4 KM	0	1

The results of the Shapiro-wilk normality test for Cortisol samples. Both for the original and lotransformed data where tested. 1 (green) indicates that the data has passed the normality test and 0 (red) indicates that the data sample did not pass the normality test.SF=sterile female, SM= sterile male, KF= control female, KM= control male. All All normality tests were done in Grafpad Prism (2022).

Appendix VIII

Results from nanodrop and dilution calculations, as well as an overview of number of fish, fish-group, tank and tissue

Tid	Kar	Group	Sex	Fiskenr	Katarina's Løpenr.	Status	RNA conc. ng/uL	260/280	260/230	Vol RNA µl	totRNA kons. (NanoDrop) µg/uL	Ønsket kons. µg	Fortynnes til vol. (totalvolumet i en tube)	Tilsatt H20	Til cDNA syntese				1000 ng tot RNA Direktefortynning	vann	tot
T1	E104-1	S	F	4	HK1	RNA isolert	690,82	1,92	1,97	3	0,69082	0,2	10,4	7	5 uL RNA	H20 7 uL	tot 12 uL				
T1	E104-1	S	F	5	HK2	RNA isolert	2743,93	1,93	1,81	3	2,74393	0,2	41,2	38	5	7	12				
T1	E104-1	S	M	7	HK3	RNA isolert	1275,94	1,97	1,64	3	1,27594	0,2	19,1	16	5	7	12				
T1	E104-1	S	F	9	HK4	RNA isolert	1402,92	1,99	2,04	3	1,40292	0,2	21,0	18	5	7	12				
T1	E104-1	S	F	10	HK5	RNA isolert	379,06	1,9	1,23										2,6	9,4	12
T1	E104-2	K	M	3	HK6	RNA isolert	1508,43	1,97	1,75	3	1,50843	0,2	22,6	20	5	7	12				
T1	E104-2	K	F	4	HK7	RNA isolert	1837,31	1,98	2,19	3	1,83731	0,2	27,6	25	5	7	12				
T1	E104-2	K	M	7	HK8	RNA isolert	3930,22	1,59	1,68	3	3,93022	0,2	59,0	56	5	7	12				
T1	E104-2	K	M	8	HK9	ikke nok RNA	6,3	1,2	0,08												
T1	E104-2	K	F	9	HK10	RNA isolert	1022,71	1,92	1,08	3	1,02271	0,2	15,3	12	5	7	12				
T1	E104-3	K	M	4	HK11	RNA isolert	2193,06	2	2,02	3	2,19306	0,2	32,9	30	5	7	12				
T1	E104-3	K	M	5	HK12	RNA isolert	4443,85	1,65	1,64	3	4,44385	0,2	66,7	64	5	7	12				
T1	E104-3	K	F	7	HK13	RNA isolert	3681,84	1,92	1,9	3	3,68184	0,2	55,2	52	5	7	12				
T1	E104-3	K	F	8	HK14	RNA isolert	2879,44	1,97	2,06	3	2,87944	0,2	43,2	40	5	7	12				
T1	E104-3	K	M	9	HK15	RNA isolert	1673,59	1,98	1,95	3	1,67359	0,2	25,1	22	5	7	12				
T1	E104-4	K	F	3	HK16	Tissue	534,5	2	2,26	5	0,5345	0,1	26,7	22	10	2	12				
T1	E104-4	K	M	6	HK17	Tissue	378,43	1,97	1,65	5	0,37843	0,1	18,9	14	10	2	12				
T1	E104-4	K	F	7	HK18	Tissue	687,37	2,06	2,28	5	0,68737	0,1	34,4	29	10	2	12				
T1	E104-4	K	F	9	HK19	Tissue	646,22	1,99	2,01	5	0,64622	0,1	32,3	27	10	2	12				
T1	E104-4	K	F	10	HK20	Tissue	292,41	2,01	2,29	5	0,29241	0,1	14,6	10	10	2	12				
T1	E104-5	S	M	2	HK21	Tissue	341,14	1,97	1,05	5	0,34114	0,1	17,1	12	10	2	12				
T1	E104-5	S	F	3	HK22	Tissue	719,81	2,05	2,3	5	0,71981	0,1	36,0	31	10	2	12				
T1	E104-5	S	M	6	HK23	Tissue	341,24	2,01	2,27	5	0,34124	0,1	17,1	12	10	2	12				
T1	E104-5	S	M	7	HK24	Tissue	236,8	2,01	1,6	10	0,2368	0,1	23,7	14	10	2	12		4,2	7,8	12
T1	E104-5	S	F	8	HK25	Tissue	440,99	2	1,88	10	0,44099	0,1	44,1	34	10	2	12				
T1	E104-6	S	M	5	HK26	Tissue	511,12	2	2,12	10	0,51112	0,1	51,1	41	10	2	12				
T1	E104-6	S	F	6	HK27	Tissue	497,04	1,98	2,25	10	0,49704	0,1	49,7	40	10	2	12				
T1	E104-6	S	M	7	HK28	Tissue	288,54	2,05	1,75	10	0,28854	0,1	28,9	19	10	2	12				
T1	E104-6	S	M	8	HK29	Tissue	208,86	2,01	2,12	10	0,20886	0,1	20,9	11	10	2	12				
T1	E104-6	S	F	9	HK30	Tissue	260,54	2,05	1,13	10	0,26054	0,1	26,1	16	10	2	12				
T2	E104-1	S	M	2	HK31	Tissue	353,46	2,01	2,29	4	0,35346	0,1	14,1	10	10	2	12				
T2	E104-1	S	F	3	HK32	Tissue	2327	1,97	2,06	4	2,327	0,1	93,1	89	10	2	12				
T2	E104-1	S	M	4	HK33	Tissue	793,5	2,07	2,38	4	0,7935	0,1	31,7	28	10	2	12				
T2	E104-1	S	F	5	HK34	Tissue	1100,47	2,04	2,37	4	1,10047	0,1	44,0	40	10	2	12				
T2	E104-1	S	M	7	HK35	Tissue	627,36	2,06	2,36	4	0,62736	0,1	25,1	21	10	2	12				
T2	E104-2	K	F	2	HK36	Tissue	1018,08	2,02	2,3	4	1,01808	0,1	40,7	37	10	2	12				
T2	E104-2	K	F	4	HK37	Tissue	474,79	2,02	2,06	4	0,47479	0,1	19,0	15	10	2	12				
T2	E104-2	K	F	5	HK38	Tissue	838,9	2,08	2,25	4	0,8389	0,1	33,6	30	10	2	12				
T2	E104-2	K	M	6	HK39	Tissue	435,06	2	2,3	4	0,43506	0,1	17,4	13	10	2	12				
T2	E104-2	K	F	9	HK40	Tissue	550,55	1,97	2,3	4	0,55055	0,1	22,0	18	10	2	12				
T2	E104-3	K	M	3	HK41	Tissue	645,06	2,03	2,13	4	0,64506	0,1	25,8	22	10	2	12				
T2	E104-3	K	M	4	HK42	Tissue	974,39	2,06	2,33	4	0,97439	0,1	39,0	35	10	2	12				
T2	E104-3	K	M	5	HK43	Tissue	372,02	1,98	1,38	4	0,37202	0,1	14,9	11	10	2	12				
T2	E104-3	K	M	7	HK44	Tissue	1235,24	2,08	2,25	4	1,23524	0,1	49,4	45	10	2	12				
T2	E104-3	K	M	8	HK45	Tissue	328,44	2,04	2,03	4	0,32844	0,1	13,1	9	10	2	12				
T2	E104-4	K	F	2	HK46	Tissue	1057,9	2,01	2,23	4	1,0579	0,1	42,3	38	10	2	12				
T2	E104-4	K	M	3	HK47	Tissue	1540,42	2,03	2,22	4	1,54042	0,1	61,6	58	10	2	12				
T2	E104-4	K	F	4	HK48	Tissue	1557,08	2,05	2,38	4	1,55708	0,1	62,3	58	10	2	12				
T2	E104-4	K	F	5	HK49	Tissue	531,91	2,03	2,15	4	0,53191	0,1	21,3	17	10	2	12				
T2	E104-4	K	F	8	HK50	Tissue	392,67	2	1,77	4	0,39267	0,1	15,7	12	10	2	12				
T2	E104-5	S	F	2	HK51	Tissue	1474,7	2,05	2,34	4	1,4747	0,2	29,5	25	5	7	12				
T2	E104-5	S	F	4	HK52	Tissue	1886,2	2,05	2,4	4	1,8862	0,2	37,7	34	5	7	12				
T2	E104-5	S	F	5	HK53	Tissue	1440,21	2,13	2,36	4	1,44021	0,2	28,8	25	5	7	12				
T2	E104-5	S	M	6	HK54	Tissue	713,07	2,08	2,29	4	0,71307	0,2	14,3	10	5	7	12				
T2	E104-5	S	F	7	HK55	Tissue	843,9	2,13	2,25	4	0,8439	0,2	16,9	13	5	7	12				
T2	E104-6	S	M	1	HK56	Tissue	1526,77	2,06	2,29	4	1,52677	0,2	30,5	27	5	7	12				
T2	E104-6	S	M	2	HK57	Tissue	1278,7	2,1	2,32	4	1,2787	0,2	25,6	22	5	7	12				
T2	E104-6	S	F	4	HK58	Tissue	2239,59	2,07	2,35	4	2,23959	0,2	44,8	41	5	7	12				
T2	E104-6	S	M	5	HK59	Tissue	2209,12	2,1	2,37	4	2,20912	0,2	44,2	40	5	7	12				
T2	E104-6	S	F	7	HK60	Tissue	2596,95	2,1	2,38	4	2,59695	0,2	51,9	48	5	7	12				
T3	E104-1	S	M	4	HK61	RNA isolert	1204,53	2	1,84	3	1,20453	0,2	18,1	15	5	7	12				
T3	E104-1	S	M	6	HK62	RNA isolert	1678,06	2	1,39	3	1,67806	0,2	25,2	22	5	7	12				
T3	E104-1	S	F	7	HK63	RNA isolert	3876,86	1,87	1,86	3	3,87686	0,2	58,2	55	5	7	12				
T3	E104-1	S	M	8	HK64	RNA isolert	3519,27	1,92	1,91	3	3,51927	0,2	52,8	50	5	7	12				
T3	E104-1	S	F	9	HK65	RNA isolert	2744,06	1,99	2,02	3	2,74406	0,2	41,2	38	5	7	12				
T3	E104-1	S	M	10	HK66	RNA isolert	3848,83	1,9	2	3	3,84883	0,2	57,7	55	5	7	12				
T3	E104-2	K	F	4	HK67	RNA isolert	4255,03	1,78	1,76	3	4,25503	0,2	63,8	61	5	7	12				
T3	E104-2	K	M	5	HK68	RNA isolert	1311,48	1,97	1,41	3	1,31148	0,2	19,7	17	5	7	12				
T3	E104-3	K	F	1	HK69	RNA isolert	2515,5	1,95	1,61	3	2,5155	0,2	37,7	35	5	7	12				
T3	E104-3	K	M	2	HK70	RNA isolert	4436,65	1,67	1,36	3	4,43665	0,2	66,5	64	5	7	12				
T3	E104-3	K	F	3	HK71	RNA isolert	4543,69	1,56	1,43	3	4,54369	0,2	68,2	65	5	7	12				
T3	E104-3	K	F	6	HK72	RNA isolert	140,91	2,01	2,09										7,1	4,9	12
T3	E104-3	K	F	8	HK73	RNA isolert	4564,62	1,59	1,73	3	4,56462	0,2	68,5	65	5	7	12				
T3	E104-3	K	F	9	HK74	RNA isolert	4021,03	1,86	1,92	3	4,02103	0,2	60,3	57	5	7	12				
T3	E104-4	K	F	1	HK75	RNA isolert	2382,6	1,97	2,05	3	2,3826	0,2	35,7	33	5	7	12				
T3	E104-4	K	M	2	HK76	RNA isolert	3696,74	1,91	1,74	3	3,69674	0,2	55,5	52	5	7	12				
T3	E104-4	K	M	3	HK77	RNA isolert	2445,52	1,97	1,34	3	2,44552	0,2	36,7	34	5	7	12				
T3	E104-4	K	M	4	HK78	ikke nok RNA															
T3	E104-4	K	F	6	HK79	RNA isolert	3264,3	1,95	1,91	3	3,2643	0,2	49,0	46	5	7	12				
T3	E104-4	K	F	7	HK80	RNA isolert	2175,91	1,98	1,51	3	2,17591	0,2	32,6	30	5	7	12				
T3	E104-4	K	M	8	HK81	RNA isolert	4692,3	1,31	1,46	3	4,6923	0,2	70,4	67	5	7	12				
T3	E104-5	S	M	2	HK82	RNA isolert	2077,16	1,98	2,04	3	2,07716	0,2	31,2	28	5	7	12				
T3	E104-5	S	F	5	HK83	RNA isolert	4296,99	1,75	1,89	3	4,29699	0,2	64,5	61	5	7	12				
T3	E104-5	S	F	6	HK84	RNA isolert	3980,48	1,78	1,71	3	3,98048	0,2	59,7	57	5	7	12				
T3	E104-5	S	F	7	HK85	RNA isolert	2628,17	1,96	1,64	3	2,62817	0,2	39,4	36	5	7	12				
T3	E104-5	S	M	8	HK86	RNA isolert	1785,2	1,98	2,1	3	1,7852	0,2	26,8	24	5	7	12				
T3	E104-6	S	F	2	HK87	RNA isolert	4283,37	1,78	1,92	3	4,28337	0,2	64,3	61	5	7	12				
T3	E104-6	S	M	4	HK88	RNA isolert	892,86	1,98	1,27	3	0,89286	0,2	13,4	10	5	7	12				
T3	E104-6	S	F	5	HK89	RNA isolert	685,98	1,83	1,37	3	0,68598	0,2	10,3	7	5	7	12				
T3	E104-6	S	F	7	HK90	RNA isolert	3629,96	1,89	1,85	3	3,62996	0,2	54,4	51	5	7	12				
T3	E104-6	S	F	9	HK91	RNA isolert	4097,67	1,83	1,76	3	4,09767	0,2	61,5	58	5	7	12				
T4	E104-1	S	F	1	HK92	RNA isolert	3475,81	1,92	1,9	3	3,47581	0,2	52,1	49	5	7	12				
T4	E104-1	S	M	5	HK93	RNA isolert	4171,86	1,78	1,87	3	4,17186	0,2	62,6	60	5	7	12				
T4	E104-1	S	M	6	HK94	RNA isolert	3703,75	1,88	1,85	3	3,70375	0,2	55,6	53	5	7	12				
T4	E104-1	S	F	8	HK95	RNA isolert	2775,6	1,96	2,08	3	2,7756	0,2	41,6	39	5	7	12				
T4	E104-2	K	M	3	HK96	RNA isolert	3252,89	1,94	2,02	3	3,25289	0,2	48,8	46	5	7	12				
T4	E104-2	K	F	5	HK97	RNA isolert	3340,11	1,92	1,77	3	3,34011	0,2	50,1	47	5	7	12				
T4	E104-2	K	F	6	HK98	RNA isolert	3496,43	1,9	1,82	3	3,49643	0,2	52,4	49	5	7	12				
T4	E104-2	K	F	7	HK99	RNA isolert	2615,95	1,97	1,81	3	2,61595	0,2	39,2	36	5	7	12				
T4	E104-2	K	F	8	HK100	RNA isolert	3921,69	1,85	1,96	3	3,92169	0,2	58,8	56	5	7	12				
T4	E104-3	K	F	3	HK101	RNA isolert	4538,15	1,64	1,74	3	4,53815	0,2	68,1	65	5	7	12				
T4	E104-3	K	F	4	HK102	RNA isolert	4297,82	1,75	1,86	3	4,29782	0,2	64,5	61	5	7	12				
T4	E104-3	K	M	7	HK103	RNA isolert	105,8	1,85	1,02										9,5	2,5	12
T4	E104-3	K	M	8	HK104	RNA isolert	31,6	1,87	0,58									250 ug RN	7,9	4,1	12
T4	E104-4	K	F	4	HK105	RNA isolert	3261,74	1,93	1,92	3	3,26174	0,2	48,9	46	5	7	12				
T4	E104-4	K	F	5	HK106	RNA isolert	4227,12	1,78	1,91	3	4,22712	0,2	63,4	60	5	7	12				
T4	E104-4	K	M	7	HK107	RNA isolert	2402,77	1,97	1,73	3	2,40277	0,2	36,0	33	5	7	12				
T4	E104-4	K	M	8	HK108	RNA isolert	2390,56	1,98	1,94	3	2,39056	0,2	35,9	33	5	7	12				
T4	E104-5	S	M	1	HK109	RNA isolert	3325,26	1,96	1,98	3	3,32526	0,2	49,9	47	5	7	12				
T4	E104-5	S	F	2	HK110	RNA isolert	1911,33	1,99	2,06	3	1,91133	0,2	28,7	26	5	7	12				
T4	E104-5	S	F	3	HK111	RNA isolert	802,5	1,99	1,81	3	0,8025	0,2	12,0	9	5	7	12				
T4	E104-5	S	F	4	HK112	RNA isolert	1131,37	1,98	1,87	3	1,13137	0,2	17,0	14	5	7	12				
T4	E104-5	S	M	8	HK113	RNA isolert	1591,85	1,93	1,59	3	1,59185	0,2	23,9	21	5	7	12				
T4	E104-6	S	M	1	HK114	RNA isolert	1446,73	1,97	2,03	3	1,44673	0,2	21,7	19	5	7	12				
T4	E104-6	S	M	2	HK115	RNA isolert	1247,68	1,96	2,05	3	1,24768	0,2	18,7	16	5	7	12				
T4	E104-6	S	F	3	HK116	RNA isolert	1858,4	1,96	2,1	3	1,8584	0,2	27,9	25	5	7	12				
T4	E104-6	S	F	4	HK117	RNA isolert	2377,73	1,96	2,06	3	2,37773	0,2	35,7	33	5	7	12				
T4	E104-6	S	F	5	HK118	RNA isolert	1525,29	1,96	2,08	3	1,52529	0,2	22,9	20	5	7	12				
T4	E104-6	S	M	8	HK119	RNA isolert	2414,69	1,94	1,88	3	2,41469	0,2	36,2	33	5	7	12				

Tid	Kar	Group	Sex	Fiskenr	Katarina's Løpenr.	Status	RNA conc. ng/uL	260/280	260/230	Vol RNA µl	totRNA kons. (NanoDrop) µg	Ønsket kons. µg	Fortynnes til vol.	Tilsatt H20	Til cDNA syntese ul fortynnet RNA	ul H2O	TOT ul				
T1	E104-1	S	F	4	G1	RNA isolert	870,61	1,95	1,85										Direkte fo	VANN	TOT
T1	E104-1	S	F	5	G2	RNA isolert	331,03	1,85	0,79										1,1	10,9	12
T1	E104-1	S	M	7	G3	RNA isolert	395,09	1,87	0,91										3,0	9,0	12
T1	E104-1	S	F	9	G4	RNA isolert	524,59	1,81	1,15										2,5	9,5	12
T1	E104-1	S	F	10	G5	RNA isolert	1771,33	1,91	1,88	5	1,77133	0,2	44,3	39	5	7	12		1,9	10,1	12
T1	E104-2	K	M	3	G6	RNA isolert	571,89	1,86	1,53												
T1	E104-2	K	F	4	G7	RNA isolert	684,37	1,93	1,85	5	0,68437	0,2	17,1	12	5	7	12		1,7	10,3	12
T1	E104-2	K	M	7	G8	Ikke nok RNA	144											250 ng	1,7	10,3	12
T1	E104-2	K	M	8	G9	RNA isolert	250	1,89	1,01									400 ng	1,6	10,4	12
T1	E104-2	K	F	9	G10	RNA isolert	1473,74	1,89	1,01	5	1,47374	0,2	36,8	32	5	7	12				
T1	E104-3	K	M	4	G11	RNA isolert	324,05	1,9	1,1									500ng	1,5	10,5	12
T1	E104-3	K	M	5	G12	RNA isolert	145,36	1,79	0,68									800ng	5,5	6,5	12
T1	E104-3	K	F	7	G13	RNA isolert	672,91	1,96	1,49	5	0,67291	0,2	16,8	12	5	7	12				
T1	E104-3	K	F	8	G14	RNA isolert	427,74	1,89	1,21	5	0,42774	0,2	10,7	6	5	7	12		2,3	9,7	12
T1	E104-3	K	M	9	G15	RNA isolert	2227,62	1,97	2,13	5	2,22762	0,2	55,7	51	5	7	12				
T1	E104-4	K	F	3	G16	RNA isolert	840,51	1,93	1,76	5	0,84051	0,2	21,0	16	5	7	12				
T1	E104-4	K	M	6	G17	RNA isolert	788,26	2	1,65	5	0,78826	0,2	19,7	15	5	7	12				
T1	E104-4	K	F	7	G18	RNA isolert	443,63	1,89	1,88										2,3	9,7	12
T1	E104-4	K	F	9	G19	RNA isolert	418,71	1,93	2,03										2,4	9,6	12
T1	E104-4	K	F	10	G20	RNA isolert	1385,59	1,9	2,19	3	1,38559	0,1	41,6	39	10	2	12				
T1	E104-5	S	M	2	G21	RNA isolert	465,5	1,89	1,39										2,1	9,9	12
T1	E104-5	S	F	3	G22	RNA isolert	513,76	1,92	1,86										1,9	10,1	12
T1	E104-5	S	M	6	G23	RNA isolert	450	1,96	1,56										2,2	9,8	12
T1	E104-5	S	M	7	G24	RNA isolert	481,27	1,92	1,95										2,1	9,9	12
T1	E104-5	S	F	8	G25	RNA isolert	1314,41	1,98	2,07	3	1,31441	0,1	39,4	36	10	2	12				
T1	E104-6	S	M	5	G26	RNA isolert	516,3	1,76	1,28										1,9	10,1	12
T1	E104-6	S	F	6	G27	RNA isolert	918,02	1,92	1,21	3	0,91802	0,1	27,5	25	10	2	12				
T1	E104-6	S	M	7	G28	RNA isolert	1483,12	1,95	1,87	3	1,48312	0,1	44,5	41	10	2	12				
T1	E104-6	S	M	8	G29	RNA isolert	550,92	1,88	1,07										1,8	10,2	12
T1	E104-6	S	F	9	G30	RNA isolert	652,7	1,92	1,73										1,5	10,5	12
T2	E104-1	S	M	2	G31	RNA isolert	1093,81	1,95	2,2	5	1,09381	0,2	27,3	22	5	7	12				
T2	E104-1	S	F	3	G32	RNA isolert	884,04	1,97	1,49	5	0,88404	0,2	22,1	17	5	7	12				
T2	E104-1	S	M	4	G33	RNA isolert	1447,36	1,94	2	5	1,44736	0,2	36,2	31	5	7	12				
T2	E104-1	S	F	5	G34	RNA isolert	741,73	1,96	1,95	5	0,74173	0,2	18,5	14	5	7	12				
T2	E104-1	S	M	7	G35	RNA isolert	1381,18	1,95	1,62	5	1,38118	0,2	34,5	30	5	7	12				
T2	E104-2	K	F	2	G36	RNA isolert	1999,17	1,95	2,17	5	1,99917	0,2	50,0	45	5	7	12				
T2	E104-2	K	F	4	G37	RNA isolert	1165,62	1,95	1,63	5	1,16562	0,2	29,1	24	5	7	12				
T2	E104-2	K	F	5	G38	RNA isolert	1211,13	1,94	2,1	5	1,21113	0,2	30,3	25	5	7	12				
T2	E104-2	K	M	6	G39	RNA isolert	1706,62	1,93	2,15	5	1,70662	0,2	42,7	38	5	7	12				
T2	E104-2	K	F	9	G40	RNA isolert	516,71	1,89	1,32	5	0,51671	0,2	12,9	8	5	7	12				
T2	E104-3	K	M	3	G41	RNA isolert	1284,69	1,99	2,15	5	1,28469	0,2	32,1	27	5	7	12				
T2	E104-3	K	M	4	G42	RNA isolert	411,3	1,89	1,22	5	0,4113	0,2	10,3	5	5	7	12				
T2	E104-3	K	M	5	G43	RNA isolert	666,24	1,92	1,63	5	0,66624	0,2	16,7	12	5	7	12				
T2	E104-3	K	M	7	G44	RNA isolert	2127,42	1,94	1,59	5	2,12742	0,2	53,2	48	5	7	12				
T2	E104-3	K	M	8	G45	RNA isolert	1038,49	1,98	1,35	5	1,03849	0,2	26,0	21	5	7	12				
T2	E104-4	K	F	2	G46	RNA isolert	2918,03	1,9	2,05	5	2,91803	0,2	73,0	68	5	7	12				
T2	E104-4	K	M	3	G47	RNA isolert	1945,43	1,98	1,65	4	1,94543	0,2	38,9	35	5	7	12				
T2	E104-4	K	F	4	G48	RNA isolert	2505,17	1,91	1,96	4	2,50517	0,2	50,1	46	5	7	12				
T2	E104-4	K	F	5	G49	RNA isolert	911,48	1,92	1,85	4	0,91148	0,2	18,2	14	5	7	12		1,1	10,9	12
T2	E104-4	K	F	8	G50	RNA isolert	919,15	1,88	1,44	4	0,91915	0,2	18,4	14	5	7	12				
T2	E104-5	S	F	2	G51	RNA isolert	814,49	1,94	1,26	4	0,81449	0,2	16,3	12	5	7	12				
T2	E104-5	S	F	4	G52	RNA isolert	1699,6	1,93	1,62	4	1,6996	0,2	34,0	30	5	7	12				
T2	E104-5	S	F	5	G53	RNA isolert	2102,33	1,96	1,68	4	2,10233	0,2	42,0	38	5	7	12				
T2	E104-5	S	M	6	G54	RNA isolert	1455,6	1,95	1,64	4	1,4556	0,2	29,1	25	5	7	12				
T2	E104-5	S	F	7	G55	RNA isolert	1085,53	1,96	1,64	4	1,08553	0,2	21,7	18	5	7	12				
T2	E104-6	S	M	1	G56	RNA isolert	1444,85	1,96	1,78	4	1,44485	0,2	28,9	25	5	7	12				
T2	E104-6	S	M	2	G57	RNA isolert	1080,69	1,97	1,8	4	1,08069	0,2	21,6	18	5	7	12				
T2	E104-6	S	F	4	G58	RNA isolert	756,18	1,93	1,42	4	0,75618	0,2	15,1	11	5	7	12				
T2	E104-6	S	M	5	G59	RNA isolert	1571,81	1,98	1,66	4	1,57181	0,2	31,4	27	5	7	12				
T2	E104-6	S	F	7	G60	RNA isolert	700,38	1,95	1,55	4	0,70038	0,2	14,0	10	5	7	12				
T3	E104-1	S	M	4	G61	RNA isolert	503,09	1,93	1,83	5	0,50309	0,2	12,6	8	5	7	12				
T3	E104-1	S	M	6	G62	RNA isolert	1295,78	2,01	1,31	5	1,29578	0,2	32,4	27	5	7	12		Direkte		
T3	E104-1	S	F	7	G63	RNA isolert	1740,74	1,99	1,97	5	1,74074	0,2	43,5	39	5	7	12		RNA	VANN	TOT
T3	E104-1	S	M	8	G64	RNA isolert	1427,31	1,99	1,43	5	1,42731	0,2	35,7	31	5	7	12				
T3	E104-1	S	F	9	G65	RNA isolert	211,5	1,89	0,86										4,7	7,3	12
T3	E104-1	S	M	10	G66	RNA isolert	773,32	1,89	1,69	5	0,77332	0,2	19,3	14	5	7	12				
T3	E104-2	K	F	4	G67	RNA isolert	448,44	1,8	1,4	5	0,44844	0,2	11,2	6	5	7	12				
T3	E104-2	K	M	5	G68	RNA isolert	1665,8	2,02	1,84	5	1,6658	0,2	41,6	37	5	7	12				
T3	E104-3	K	F	1	G69	RNA isolert	1319,76	1,98	1,89	5	1,31976	0,2	33,0	28	5	7	12				
T3	E104-3	K	M	2	G70	RNA isolert	2053,82	1,93	1,42	5	2,05382	0,2	51,3	46	5	7	12				
T3	E104-3	K	F	3	G71	RNA isolert	1684,57	1,97	1,69	5	1,68457	0,2	42,1	37	5	7	12				
T3	E104-3	K	F	6	G72	RNA isolert	1544,47	2,00	1,45	5	1,54447	0,2	38,6	34	5	7	12				
T3	E104-3	K	F	8	G73	RNA isolert	1670,03	1,99	1,64	5	1,67003	0,2	41,8	37	5	7	12				
T3	E104-3	K	F	9	G74	RNA isolert	1144,31	1,98	1,96	5	1,14431	0,2	28,6	24	5	7	12				
T3	E104-4	K	F	1	G75	RNA isolert	2015,91	1,97	1,95	5	2,01591	0,2	50,4	45	5	7	12				
T3	E104-4	K	M	2	G76	RNA isolert	457,59	1,86	0,91	5	0,45759	0,2	11,4	6	5	7	12				
T3	E104-4	K	M	3	G77	RNA isolert	1495,48	1,98	1,32	5	1,49548	0,2	37,4	32	5	7	12				
T3	E104-4	K	M	4	G78	RNA isolert	957,85	1,96	0,86	5	0,95785	0,2	23,9	19	5	7	12		No Sample in HK for T3 fish 4		
T3	E104-4	K	F	6	G79	RNA isolert	1386,04	1,98	1,66	5	1,38604	0,2	34,7	30	5	7	12				
T3	E104-4	K	F	7	G80	RNA isolert	873,58	1,95	1,19	5	0,87358	0,2	21,8	17	5	7	12				
T3	E104-4	K	M	8	G81	RNA isolert	996,84	1,88	1,4	5	0,99684	0,2	24,9	20	5	7	12				
T3	E104-5	S	M	2	G82	RNA isolert	984,37	1,97	1,77	5	0,98437	0,2	24,6	20	5	7	12				
T3	E104-5	S	F	5	G83	RNA isolert	1529,08	1,97	1,95	5	1,52908	0,2	38,2	33	5	7	12				
T3	E104-5	S	F	6	G84	RNA isolert	3258,67	1,91	1,9	5	3,25867	0,2	81,5	76	5	7	12		0,3	11,7	12
T3	E104-5	S	F	7	G85	RNA isolert	2589,03	1,94	1,96	5	2,58903	0,2	64,7	60	5	7	12				
T3	E104-5	S	M	8	G86	RNA isolert	1186,12	1,97	2,07	5	1,18612	0,2	29,7	25	5	7	12				
T3	E104-6	S	F	2	G87	RNA isolert	1468,53	1,99	1,88	5	1,46853	0,2	36,7	32	5	7	12				
T3	E104-6	S	M	4	G88	RNA isolert	2073,49	1,95	2,11	5	2,07349	0,2	51,8	47	5	7	12		0,5	11,5	12
T3	E104-6	S	F	5	G89	RNA isolert	502,39	1,93	1,19	5	0,50239	0,2	12,6	8	5	7	12				
T3	E104-6	S	F	7	G90	RNA isolert	753,97	1,91	1,21	5	0,75397	0,2	18,8	14	5	7	12				
T3	E104-6	S	F	9	G91	RNA isolert	711,62	1,92	1,19	5	0,71162	0,2	17,8	13	5	7	12				
T4	E104-1	S	F	1	G92	Tissue	761,17	1,98	2,26	4	0,76117	0,2	15,2	11	5	7	12				
T4	E104-1	S	M	5	G93	Tissue	1403,18	1,99	2,28	4	1,40318	0,2	28,1	24	5	7	12				
T4	E104-1	S	M	6	G94	Tissue	868,14	1,97	2,24	4	0,86814	0,2	17,4	13	5	7	12				
T4	E104-1	S	F	8	G95	Tissue	1040,38	1,98	2,22	4	1,04038	0,2	20,8	17	5	7	12				
T4	E104-2	K	M	3	G96	Tissue	1459,93	2	2,32	4	1,45993	0,2	29,2	25	5	7	12				
T4	E104-2	K	F	5	G97	Tissue	888,84	1,99	2,25	4	0,88884	0,2	17,8	14	5	7	12				
T4	E104-2	K	F	6	G98	Tissue	1256,93	1,98	2,1	4	1,25693	0,2	25,1	21	5	7	12				
T4	E104-2	K	F	7	G99	Tissue	1036,15	1,96	2,26	4	1,03615	0,2	20,7	17	5	7	12				
T4	E104-2	K	F	8	G100	Tissue	820,66	1,99	2,29	4	0,82066	0,2	16,4	12	5	7	12				
T4	E104-3	K	F	3	G101	Tissue	816,2	1,98	2,2	4	0,8162	0,2	16,3	12	5	7	12				
T4	E104-3	K	F	4	G102	Tissue	1395,61	2	2,26	4	1,39561	0,2	27,9	24	5	7	12				
T4	E104-3	K	M	7	G103	Tissue	1268,54	1,98	2,21	4	1,26854	0,2	25,4	21	5	7	12				
T4	E104-3	K	M	8	G104	Tissue	819,07	1,93	2,22	4	0,81907	0,2	16,4	12	5	7	12				
T4	E104-4	K	F	4	G105	Tissue	1149,36	1,98	2,26	4	1,14936	0,2	23,0	19	5	7	12				
T4	E104-4	K	F	5	G106	Tissue	672,87	1,95	2,16	4	0,67287	0,2	13,5	9	5	7	12				
T4	E104-4	K	M	7	G107	Tissue	770,54	1,95	2,11	4	0,77054	0,2	15,4	11	5	7	12				
T4	E104-4	K	M	8	G108	Tissue	1152,9	1,99	2,24	4	1,1529	0,2	23,1	19	5	7	12				
T4	E104-5	S	M	1	G109	Tissue	946,36	1,98	2,11	4	0,94636	0,2	18,9	15	5	7	12				
T4	E104-5	S	F	2	G110	Tissue	946,14	1,98	2,27	4	0,94614	0,2	18,9	15	5	7	12				
T4	E104-5	S	F	3	G111	Tissue	1254,17	1,99	2,19	4	1,25417	0,2	25,1	21	5	7	12				
T4	E104-5	S	F	4	G112	Tissue	1199,97	2	2,3	4	1,19997	0,2	24,0	20	5	7	12				
T4	E104-5	S	M	8	G113	Tissue	918,38	1,98	2,18	4	0,91838	0,2	18,4	14	5	7	12				
T4	E104-6	S	M	1	G114	Tissue	952,73	1,99	2,09	4	0,95273	0,2	19,1	15	5	7	12				
T4	E104-6	S	M	2	G115	Tissue	967,48	1,98	2,13	4	0,96748	0,2	19,3	15	5	7	12				
T4	E104-6	S	F	3	G116	Tissue	734,22	1,96	2,06	4	0,73422	0,2	14,7	11	5	7	12				
T4	E104-6	S	F	4	G117	Tissue	839,48	1,99	2,32	4	0,83948	0,2	16,8	13	5	7	12				
T4	E104-6	S	F	5	G118	Tissue	1254,92	1,97	2,28	4	1,25492	0,2	25,1	21	5	7	12				
T4	E104-6	S	M	8	G119	Tissue	945,43	1,99	2,17	4	0,94543	0,2	18,9	15	5	7	12				

Appendix IX

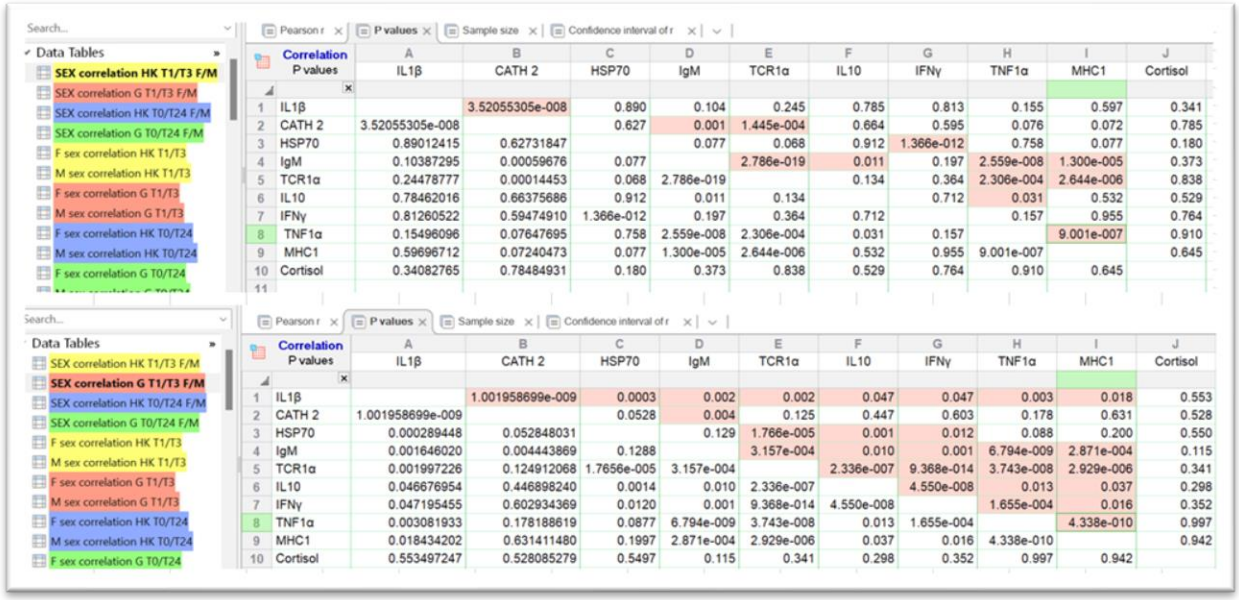


Fig 25: Screen prints from Grafpad Prism showing the significant correlations, with significance level $p<0.05$ marked in red for the smoltification experiment data.

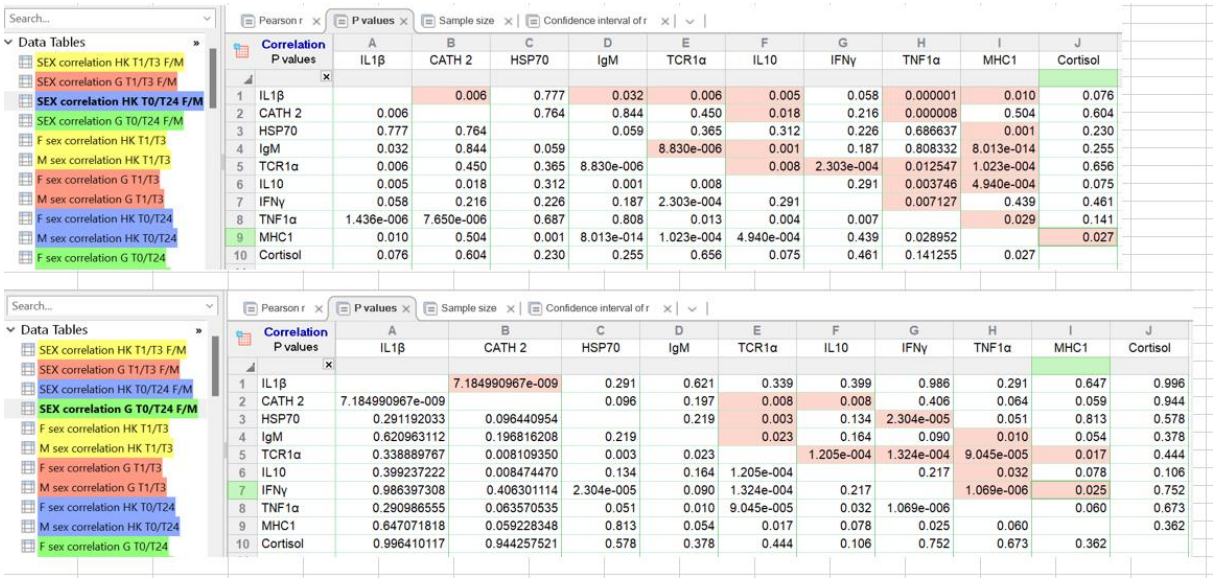


Fig 26: Screen prints from Grafpad prism showing the significant correlations, with significance level $p<0.05$ marked in red for the transport stress experiment data.

Table 4: Overview of result of person correlation tests in smoltification experiment T1-T3.

Pearson correlation T1-T3		**Explanation degree (%)**	
Significant correlating genes, correlating in both tissues		**HK**	**G**
IL1β	CATH 2	34	27
CATH 2	IgM	15	9
HSP70	IFNγ	50	8
IgM	TCR1α	67	15
IgM	IL10	8	8
IgM	TNF1α	35	35

IgM	MHC I	23	16
TCR1α	TNF1α	17	32
TCR1α	MHC I	26	25
IL10	TNF1α	6	8
TNF1α	MHC I	15	40

Table 5: Overview of result of person correlation tests in stress experiment T0-T24.

Pearson correlation T0-T24		**Explanation degree (%)**	
Significant correlation genes, correlating in both tissues		**HK**	**G**
IL1β	CATH 2	14	48
CATH 2	IL10	11	12
IgM	TCR1α	34	10
TCR1α	IL10	14	25
TCR1α	IFNγ	25	24
TCR1α	TNF1α	12	25
TCR1α	MHC I	27	10
IL10	TNF1α	16	8
IFNγ	TNF1α	14	36

Table 6: Overview of results of further pearson correlation test of significant correlations in smoltification experiment.

Pearson correlation T1-T3									
Overall significant correlations corrresponding in both tissues									
Genes		P values HK				P values G			
		SEX		GROUP		SEX		GROUP	
		Males	Females	Sterile	Controls	Males	Females	Sterile	Controls
IL1β	CATH 2	ns	****	ns	****	**	****	***	****
CATH 2	IgM	****	ns	ns	****	***	**	ns	*
HSP70	IFNγ	****	****	****	****	ns	*	ns	**
IgM	TCR1α	****	****	****	****	**	ns	ns	****
IgM	IL10	ns	*	ns	ns	**	ns	ns	**
IgM	TNF1α	****	ns	****	*	****	***	ns	****
IgM	MHC1	****	ns	**	****	***	ns	ns	****
TCR1α	TNF1α	****	*	***	ns	****	ns	*	****
TCR1α	MHC1	****	**	***	****	****	ns	ns	****
IL10	TNF1α	ns	ns	ns	ns	****	ns	ns	****
TNF1α	MHC1	****	ns	****	ns	****	ns	ns	****

Table 7: Overview of results of further pearson correlation test of significant correlations in stress experiment

Pearson correlation T0-T24									
Overall significant correlations corrresponding in both tissues									
Genes		P values HK				P values G			
		SEX		GROUP		SEX		GROUP	
		Males	Females	Sterile	Controls	Males	Females	Sterile	Controls
IL1β	CATH 2	ns	*	*	ns	****	****	***	****
CATH 2	IL10	*	ns	*	ns	*	ns	*	ns
IgM	TCR1α	****	*	***	ns	*	ns	**	ns
TCR1α	IL10	*	ns	ns	ns	**	**	**	**
TCR1α	IFNγ	**	**	*	ns	**	*	**	*
TCR1α	TNF1α	ns	*	ns	ns	***	ns	**	*
TCR1α	MHC1	****	ns	**	ns	****	ns	ns	**
IL10	TNF1α	*	ns	ns	ns	ns	ns	ns	*
IFNγ	TNF1α	****	ns	ns	ns	**	****	****	ns

Table 8: Overview of results of further person correlation test of significant correlations In smoltification experiment

Person correlation T0-T24 **Further testing for Cortisol** **Significant p value only observed in head kidney**					
		SEX		GROUP	
Genes		Males	Females	Sterile	Controls
MHC I	**Cortisol**	ns	ns	* 0,039	ns

Explanation of significance markers*

Symbol	Meaning
ns	$P > 0.05$
*	$P \leq 0.05$
**	$P \leq 0.01$
***	$P \leq 0.001$
****	$P \leq 0.0001$