Trichlorfon, a salmon lice pesticide, and the effect on metabolism and inflammation in Atlantic salmon (*Salmo salar*, L.)

An *in vitro* study of the effects of trichlorfon on metabolic and immune gene responses in liver cells and head kidney leukocytes when exposed to lipopolysaccharide and polyinosinic:polycytidylic acid

Thesis for the degree

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Table of Contents

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Abstract

Since the beginning of the salmon farming industry in Norway, the substantial fish health and welfare and economic costs caused by the parasitic salmon lice (*Lepeophtheirus salmonis*) has increased in tandem with the expanding production of salmon. In 1974, the first reported attempt at repelling the parasite was performed with the organophosphorus pesticide (OPP) trichlorfon (TCF). As *L. salmonis* established itself as one of the biggest biological challenges of commercial aquaculture industry, the quantitative use of pharmaceutical treatments used against salmon lice has escalated and proven to negatively affect both non-target species and the treated fish itself.

In this study, isolated liver cells and head kidney (HK) leukocytes of 11 Atlantic salmon (*Salmo salar,* L.) post-smolts were exposed to TCF in concentrations of 25, 10 and 1 µM (1.3, 0.5 and 0.25 µL) for 48 hours and 100 µg/mL lipopolysaccharide (LPS) and 50 µg/mL polyinosinic:polycytidylic acid (poly I:C) for 24 hours *in vitro*, to examine the toxic effects of TCF and if it affects metabolic or immunogenic responses when under simulated bacterial (LPS) and viral (poly I:C) infection. Untreated cultures were included as controls.

Transcription of the inflammatory markers *CD83* and *Cox-2* in isolated liver cells and HK leukocytes in addition to inflammatory markers *IL-1β* and *TNF α* in leukocytes, was significantly affected by LPS, further verifying the suitability of the *in vitro* model used in this experiment. Expression of the immunorelated metabolic genes was not significantly affected by any treatment. The gene expression of the oxidative stress (OS) related gene *Bcl-2* was down regulated in leukocytes by poly I:C alone and poly I:C with TCF. Similarly, gene expression of the apoptotic related gene *caspase3* was significantly upregulated in leukocytes cultured with poly I:C, suggesting an antiviral involvement. The expression of antiviral response genes *Mx*, significantly elevated in leukocytes by poly I:C, and *viperin*, significantly induced in both liver cells and leukocytes by poly I:C, confirms their role in the antiviral immune response of *S. salar*. No genes in this study were significantly affected by TCF.

This study solidifies application of this *in vitro* model in observing effects of LPS and poly I:C on specific metabolic and inflammatory related genes in Atlantic salmon. While not confirming the toxic effects of TCF on *S. salar* and subsequently, non-target organisms, this study could be viewed as an indicator of the toxicity of TCF.

Abbreviations

Abbreviation	*Denotation*
AChE	Acetylcholinesterase
ANOVA	Analysis of variance
APP	Acute Phase Protein
ARP	Acidic ribosomal phosphoprotein
Bcl-2	B-cell lymphoma 2
BW	Body weight
Caspase3	*C*ysteinyl *a*spartate *s*pecific *p*rote*ase* 3
CD83	Cell Differentiation Cluster 83
cDNA	Complementary DNA
CLP-m	Chlorpyrifos-methyl
Cox-2	Cyclooxygenase 2
Cq	Cycle quantification (value)
CYP1a	Cytochrome P450 1A
DAMP	Damage-associated molecular pattern
DNA	Deoxyribonucleic acid
EF1α	Elongation factor 1-alpha
FAO	Food and Agriculture Organization of the United Nations
FR	Free radicals
HK	Head kidney
IFN γ	Interferon gamma
Ig	Immunoglobulin
IL-1β	Interleukin-1 beta
IL-8	Interleukin-8
K	Control
LC_{50}	Lethal Concentration (of a given chemical in a medium with 50% mortality during observation period)
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
Mx	Interferon-induced GTP-binding protein Mx
NAC	None amplification control
NTC	Non-template control

OPP	Organophosphorus pesticide
OS	Oxidative stress
P	Probability value
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
Poly I:C	Polyinosinic:polycytidylic acid
PRR	Pathogen recognition receptor
qPCR	Quantitative real time polymerase chain reaction
RIN	RNA Integrity Number
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RPL13	Ribosomal protein L13
RT	Reverse transcriptase
RT-PCR	Reverse transcription polymerase chain reaction
SD	Standard deviation
SSAT	Spermidine/spermine-N^1-acetyltransferase
TCF	Trichlorfon
TLR3	Toll like receptor 3
TNF α	Tumor necrosis factor alpha
Viperin	*Vi*rus *i*nhibitory *p*rotein, *e*ndoplasmic *r*eticulum-associated, *in*terferon-inducible

1. Introduction

1.1 A brief history of pharmaceutical treatments in commercial aquaculture against salmon lice (*Lepeophtheirus salmonis*)

Considering the growing population and food production's increasing carbon emissions, the UN raises concerns about future food security without compromising our climate[1,2]. Norway, with its long coastline ideal for the farming of Atlantic salmon (*Salmo salar*, L.) producing 1.28 million tons in 2018[3], is already a key contributor in providing more environmental carbon-friendly protein for human consumption[4]. Nonetheless, the increasing rate of production raises questions in relation to resource usage and the challenges of external factors such as climate change affecting salinity and temperature of the oceans, and increasing rate of diseases and pathogens[4].

While the growing number of aquatic produce offers exciting opportunities for scientific research, economic endeavors and culinary explorations, the rate of pathogens in aquaculture have also risen to threatening levels, both environmentally and in terms of fish welfare[5-9]. Salmon cage culture is requiring the use of substantial quantities of pharmaceuticals[6,10,11] to help manage disease outbreaks and the persistence of economically and welfare disrupting pathogens[11,12].

A significant testament to this is the sea lice; parasitic copepods, causing substantial economic losses and compromising fish health and welfare in farmed salmon production[13-15]. Probably the most economically important copepod species, *Lepeophtheirus salmonis* has gained the most attention and research, and is regarded as a highly consequential biological problem of aquaculture today[15]. The increased amount of sea lice related issues has escalated the use of pharmaceutical treatment in salmon farming the last years (fig. 1.1)[16], but in 2020, the Norwegian Veterinary Institute[15] reported that the trend in Norway is a decrease in the use of medicinal treatment against sea lice and an increase in the use of cleaner fish.

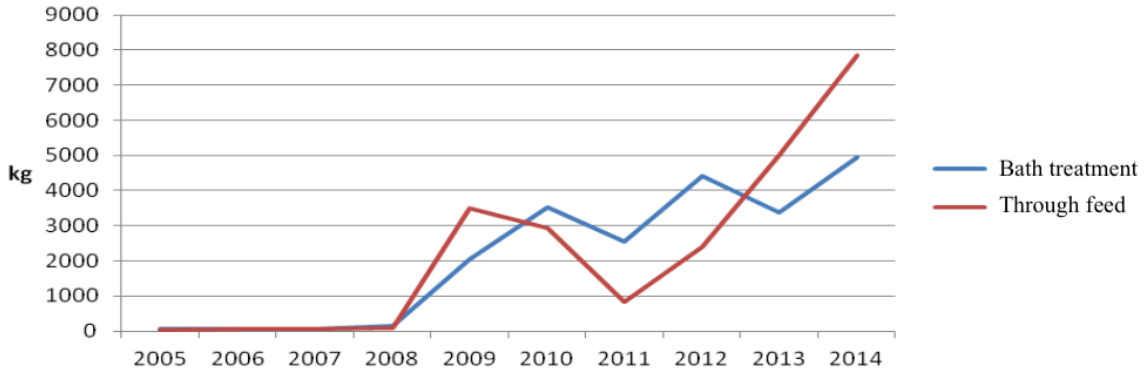

Figure 1.1: Use of pharmaceutical treatment against sea lice (kg, active substance) from 2005-2014, reprinted from "Effects of aquaculture emissions on special marine biotopes, red listed habitats and species' Knowledge status", by IMR[16].

The first pharmaceutical treatments against sea lice were introduced in Norway in the mid-1970's[5,17,18] where salmon farming had its commercial conception[12,19]. To control the increasing sea lice level in newly established salmon farm cages[20], veterinarians started utilizing organophosphorus pesticides (OPPs). The earliest notable treatment was Neguvon™ (with the active ingredient trichlorfon (TCF)) in 1974[17-19,21,22], and in the mid-80's the use of pesticides used against sea lice containing TCF was close to 30 tons[23]. Over a decade later, Nuvan™ (with the active ingredient dichlorvos) was introduced, followed by Salmosan™ (with the active ingredient azamethiphos) from 1994 to 1999[21,23,24], and reintroduced in 2008[25].

1.2 Environmental consequences of pharmaceutical treatments

The main tactic to negate sea lice has been the use of pharmaceuticals applied via *in situ* immersion treatments by means of baths and through feed. Using bath treatments, the most common procedure is to completely enclose the salmon cage with a surrounding tarpaulin. With the tarpaulin in place, the pharmaceutical is applied with a correct dosage and minimum exposure time to ensure effectiveness. Post treatment the tarpaulin is removed, and the pharmaceuticals are released into the surrounding environment. This leaves a residual concentration of pharmaceutical detritus, exposing non-target species[11,26,27]. Similar effects happen when medication is distributed through feed; uneaten pellets dissolving in the medium deploying residue in the surroundings[28]. The consequences such pharmaceuticals could have for non-target species has been a cause of concern since the 1970's[29], remaining a consistent issue in the following decades[30-32] and is still a relevant topic today[16,25,33].

Early experiments[34] exposing the OPP dichlorvos (the main degradation product of TCF) on co-locations between salmon and scallops (*Pecten maximus*) showed no observed effects in mortality or behavior, but later studies[11,35] suggests direct mortalities of non-target species in addition to sublethal effects like reduced reproductive capacity in species within proximity of production areas. TCF has also been shown[36] to affect the non-target species giant freshwater prawn (*Macrobrachium rosenbergii*), in which the cytotoxicity negatively impacted the immune response. The geographical areas favored for salmon farming are often equally preferable for other types of commercial aquaculture and fishing activities for human consumption. Cultivation of mussels, oysters and lobster or natural habitats and spawning grounds for cod[37] and other species may be present in the vicinity of salmon farms, underlining the possible socioeconomic and environmental impacts the release of pharmaceuticals could have in the marine environment.

1.3 Trichlorfon

The OPP TCF is widely used as an insecticide against terrestrial insects and fish parasites[38]. Each active ingredient used against sea lice has specific physical-chemical characteristics that defines its toxicokinetic or toxicodynamic action, dilution rates and environmental persistence, making it necessary to explore these effects individually.

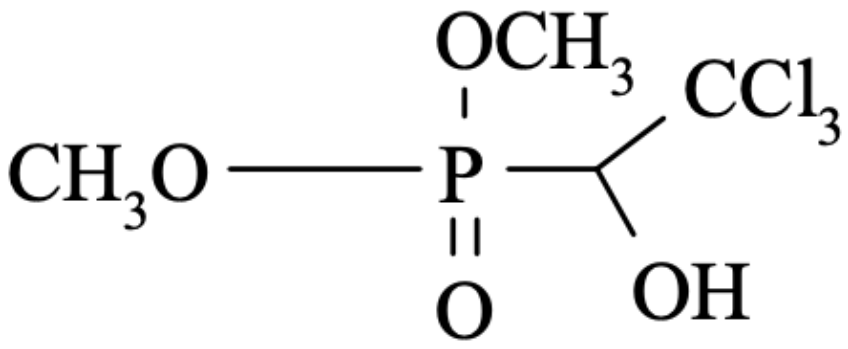

Figure 1.2: Trichlorfon, dimethyl (RS)-2,2,2-trichloro-1-hydroxyethylphosphonate, ($C_4H_8Cl_3O_4P$).

A potent neurotoxin, the toxicity of TCF is caused by blocking the breakdown of acetylcholine by acetylcholinesterase (AChE)[39]. Inhibiting AChE activity prevents the production of the enzyme responsible for hydrolyzing the acetylcholine neurotransmitter, causing sustained and excessive stimulation of nerve and muscle fibers, prompting spastic paralysis and death[40-45].

1.4 Fish immune system

All fish possess an immune system to combat pathogens, which breaks down into two main parts: external protection against physical invasion and internal handling of pathogens. Same as mammals, teleost's have both innate (non-specific) and adaptive (specific) immune responses[46]. Innate and adaptive immune responses (fig 1.3)[47] are able to recognize foreign, invading surface structures, reacting quickly to trigger molecular and cellular mechanisms for antigen elimination[48,49] and the innate immune system is important for activating and determining the nature of the adaptive immune response[50].

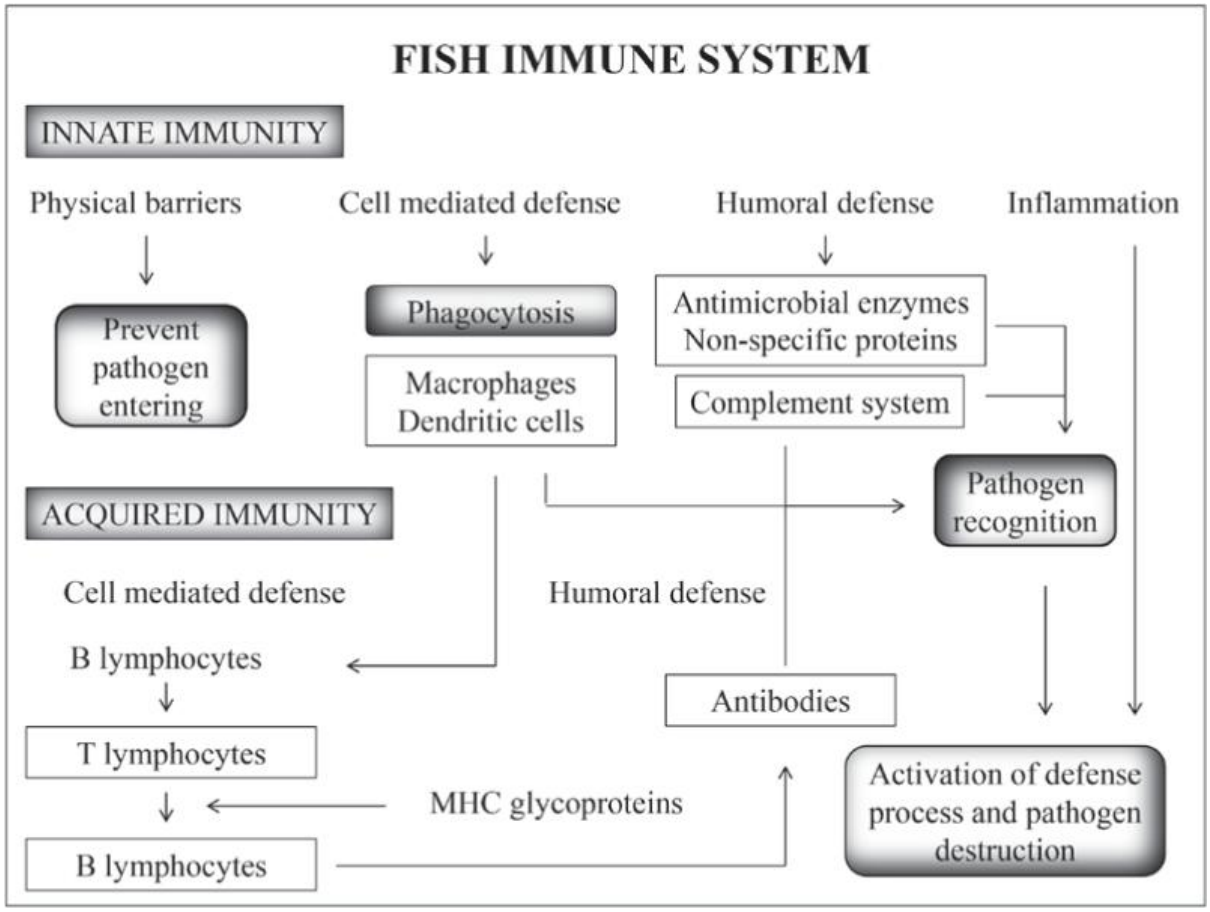

Figure 1.3: Concept of the fish immune system. reprinted from "Fish Immunology. The modification and manipulation of the innate immune system: Brazilian studies", by Biller-Takahashi & Urbinati[47].

In higher vertebrates, the immune system consists of generative and secondary lymphoid organs with specific anatomically compartments and morphology, wherein the thymus and bone marrow compose the generative lymphoid organs, with the spleen, lymph nodes and mucosal associated lymphoid tissue constituting the secondary lymphoid organs[49,51]. Fish share the generative and secondary lymphoid organs, with the exception of lymphatic nodules and bone marrow[52,53]. The principal function of these humoral and cellular immune responses

is involved in the immune defense[49]. The innate and adaptive immune systems are given activity/factor, involved cells, cellular markers and immune genes in table 1.1[54], relevant for this thesis.

Table 1.1: The innate and adaptive immune systems activity and/or factors and cellular markers, within relevancy of this study. Bcl: B-cell lymphoma, CD: Cell-differentiation cluster, Cox: Cyclooxygenase, CYP: Cytochrome P450, IFN: Interferon, Ig: Immunoglobulin, IL: Interleukin, MHC: Major histocompatibility complex, SSAT: spermidine/spermine-N^1-acetyltransferase, TLR: Toll-like receptor, TNF: Tumor necrosis factor (modified from "The Immune System Drugs in Fish: Immune Function, Immunoassay", by Kum & Sekkin)[54].

Activity/Factor	Involved cell	cDNA sequence coding for	Cellular marker
Innate immunity			
Phagocytosis	Mononuclear phagocytes	-	Antibodies, Macrophages,
	B-cells		IgM, neutrophils,
			Granulocytes
Antibacterial	Various types	Families of peptides	None
Antiviral	Leukocytes, fibroblasts	IFNs, Mx-protein, viperin	None
Enzymes	Various types incl. hepatocytes	e.g., caspases, metabolic (CYPs,	Alkoxyresorufins for CYP1
		SSAT)	
Inflammation, cytokines	Leukocytes	TNF α, Cox-2, TLRs, ILs (IL-1β),	Antibodies for TNF α
		Chemokines (IL-8)	
Adaptive immunity			
Memory, specific antibody	B-cells	Igs (IgM), Bcl-2	Antibodies to IgM, B-cells
Specific killing	T-cells	MHC_I	None
Helper activity	T-cells	MHC_{II}	Dendritic markers (CD83)
	T-helper 1/T-helper 2	IFN γ	None
	Leukocytes	ILs	None

1.5 The innate immune response

More developed than the adaptive immune system in teleost's, the innate immune system provides a powerful first line of defense against infection including physical barriers and cellular responses[46]. Lymphoid tissue distributed around the most exposed tissues: skin, gills and the intestine complements both the chemical and physical protection from these structures[46,48] Scales and the layers of dermis and epidermis form the armor providing defense against physical injury and disease organisms in the environment, further improved by a mainly skin-covering antifungal and antibacterial mucus, effectuated by immunocompetent cells such as leukocytes[46,55]. The innate immune response recognizes molecular structures common to pathogenic microorganisms such as lipopolysaccharides (LPS), bacterial DNA and single- and

double-strand viral RNA, by their interaction with specific receptors like toll receptors (TLRs)[51]. These recognition mechanisms induce successful pathogen removal by phagocytosis or trigger additional protective responses[46]. Cells operating in the innate response vary in properties with some having phagocytic or cytokine- and chemokine-secreting properties[51].

1.6 Inflammation

Stimuli signaling damage or infection results in inflammation, which can be beneficial or harmful depending on the type and duration of the stimuli. One category of inflammatory stimulation is pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) that activate enzymes (e.g., caspases)[56]. PAMPs are composed of structures derived from microorganisms which induces inflammation in response to infections[56]. LPS, a well-known PAMP, is found in the outer cell wall of gram-negative bacteria [57]. In mammals, viral RNA is recognized by TLRs like *TLR3*[58], which have been identified in rainbow trout (*Oncorynchus mykiss*) and Atlantic cod (*Gadus morhua*)[58]. DAMPs are derived from host cells and materials like tumor cells, and dead or dying cells[56]. Through inducing inflammatory responses caused by environments of trauma or tissue damage without requiring pathogenic infection, DAMPs enable fish to detect damage in its own tissue[56].

Similar to other vertebrates and invertebrates, fish activate their immune system after recognizing PAMPs or DAMPs, by specific germline-encoded host receptors, pathogen recognition receptors (PRRs), acting as soluble forms or being associated to membranes of immune cells and humoral innate components[56,57,59]. Cell types expressing PRRs includes innate immune cells like macrophages, monocytes, dendritic cells and mast cells, in addition to non-immune cells for instance epithelial cells and fibroblasts[56]. PAMPs and DAMPs binds to PRR, such as TLRs, and the PRR-ligand binding starts a cascade of downstream signaling resulting in transcriptional changes as well as post-translational modifications, with PRR engagement eliciting leukocyte recruitment signals[57]. The executioner cysteinyl aspartate specific protease, *caspase3*, is an endoprotease enzyme involved in regulating inflammation where it is centric for apoptosis signaling networks in catalyzing the specific cleavage of various key cellular proteins and coordinating destruction of e.g. cellular DNA fragmentation[60-62]. *Cox-2* is another inflammatory related enzyme involved in downstream

signaling operating in connection to the innate immune response, and has been suggested[63] to have physiologic- and pathologic-regulating effects on metabolism.

1.7 Acute Phase Response

Hepatocytes are the most abundant cell type in the liver and are central in the acute-phase response[64]. Following diverse stress factors like tissue injury, infection and inflammation, the cytokines secreted into the bloodstream stimulates hepatocytes to produce and release acute phase proteins (APPs)[65]. APPs are classified based on the extent of their concentration and direction change and is involved in a variety of defense activities[49]. It has been reported[66] that APPs of tilapia (*Oreochromis niloticus*) during acute inflammatory reaction was similar to those observed in humans.

1.8 Oxidative stress response

Molecular oxygen is critical for energy production, but also a potent oxidant which can lead to oxidative stress (OS)[67]. Reactive oxygen species (ROS) are continuously generated as byproducts of normal and aberrant metabolic processes that utilize oxygen and exert physiological actions, with an antioxidant system that keeps oxidizing levels acceptable, by major antioxidant enzymes like *catalase*[68]. Imbalance between increased production of ROS and reduced biological function in the antioxidant defense against ROS, can be causative for OS[67,68]. B-cell lymphoma 2 (*Bcl-2*) is a family of proteins, which in mammals are a key regulator of the intrinsic apoptotic cascade activated in B-cells under extended OS. They are involved in ROS and redox balance displaying antioxidant-like functions, including inhibiting hydroperoxide leakage[69,70]. The levels of antioxidant genes can be used to quantify OS in cells as these genes are easily induced by ROS[71]. The immune system can be compromised by OS and in turn result in macromolecular damage and cell death, in which the presence of ROS in the cells triggers reactions that can decrease cellular functions due to oxidative damage[72]. ROS molecules are also involved with e.g., phagocytosis and intercellular signaling in the immune defense[72]. PAMPs and DAMPs activating inflammation, in addition to the phagocytotic process interconnecting the innate and adaptive immune responses, produces ROS, which sequentially can lead to formation of bactericides[47].

ROS may be classified as free radicals (FR), which are highly reactive atoms or molecules like oxygen, hydrogen peroxide, superoxide anion radical and hydroxyl radical, that can non-specifically bind with other biological molecules[67,68]. FR are produced in three ways: oxidative metabolism, leukocyte respiratory burst activity and environmental factors (e.g., diet

or medicinal treatments)[72]. The presence of FR can induce continuing activation of granulocytes, macrophages and dendritic cells with a permanent ROS production caused by leukocytes respiratory burst activity and activation of innate immune responses[67,72]. Leukocyte respiratory burst activity requiring oxygen (oxidative burst) is correlated with cytokine release and inflammatory response in fish[72]. During phagocytosis, leukocytes increase their consumption of intracellular oxygen, producing ROS[67]. Oxygen is oxidized to superoxide anions, which plays a major role in the immune system, as neutrophils, monocytes, macrophages, dendritic cells and B-lymphocyte produce ROS to eliminate pathogens[72]. Without enzymatic elimination (e.g., catalases), the superoxide anon radical can lead to production of hydrogen peroxide, and formation of hydroxyl radicals, which can react with amino acids and proteins microorganisms, inactivating enzymatic activity, alter cell membrane active transport, oxidize DNA and fat molecules[73] and cause cytolysis and cellular destruction[67,68].

1.9 The adaptive immune response

If the pathogenic invasion of the fish perseveres despite the innate immune defenses, the adaptive immune response is activated. The adaptive immune system is capable of specific antigen recognition and drives the secondary immune response[46]. Adaptive immunity is highly regulated through specific mechanisms which increases with antigen exposure and creates immunological memory[51]. The adaptive system is characterized by the presence of B-cells, BCR, and T-cells, TCR, (B-cell receptors and T-cell receptors, respectively), MHC (major histocompatibility complex) antigens and Igs (immunoglobulins)[49].

Lymphocytes, specifically B- and T-cells, are the main effector cells of the adaptive immune response in teleost's[49,74]. B-cells play a major part within the humoral adaptive immune response with their main role producing high affinity Ig against foreign antigens[49]. Activated B-cells differentiates into plasma cells which secretes antibodies that recognize non-self-structures on surfaces of bacteria and virus [51,74]. T-cells are involved with cellular adaptive immunity[49]. When the membrane bound TCR is stimulated by interaction with an antigen presentation, activated T-cells can differentiate into helper T-cells, regulatory T-cells or cytotoxic T-cells[49]. Helper T-cells can activate other adaptive immune response cells, while cytotoxic T-cells eliminates infected cells by recognizing foreign structures on the surface of other host cells[49,51,74].

Another key function of B-cells, macrophages and dendritic cells [75], is to process and present antigen to activate T-cells, while the T-cells only recognizes antigen fragment bound to MHC[49]. MHC antigens code for proteins found on cell surfaces and are extremely polymorphic, meaning that the corresponding genes display a higher individual variation than any other gene family which accounts for various capabilities for specific protein presentations[49,74]. MHC antigens packed with peptides like the dendritic marker *CD83*[75], are transported to the cell membrane, and any detection of foreign proteins activates the immune response[74].

Igs composed of light and heavy glycoprotein chains make up antibody molecules[74]. As the most prevalent antibody in teleost's[49], IgM can induce effective, specific humoral responses against various antigens[48,51]. For IgM, one gene alone can generate six structural isoforms (variants)[48,51].

1.10 The head kidney

The HK is unique for teleost fish and is the central organ for immune-endocrine interactions[48] handling hematopoietic functions and producing leukocytes[52,76]. Leukocytes include macrophages, granulocytes (neutrophils, eosinophils, and basophils), thrombocytes, monocytes and lymphocytes (T- and B-cells) which are apt to eliminate pathogenic cells[77]. The HK is comprised of hematopoietic antibody-producing[52] and cytokine-producing lymphoid tissue with endocrine cells secreting cortisol, catecholamines, and thyroid hormones[48,76] and is the principal immune organ responsible for phagocytosis[78], antigen processing[79], and formation of IgM and immune memory through melanomacrophagic centers[80-82]. It is also one of the major lymphoid organs, in which myelopoiesis generally occurs[83], is considered the primary B-cell organ[84] and enables bidirectional signaling between the immune system and endocrine system[85].

1.10.1 Metabolic and immunological crosstalk

In teleost fish the thyroid tissue is located adjacent to cytokine-producing hematopoietic tissue like the HK, suggesting a paracrine interaction between the immune and thyroid system[84]. Pro-inflammatory cytokines secreted from activated macrophages in the immune system induce inflammation and chemokine release, which is communicated within thyroid tissue[84]. As stress and immune responses are energy demanding, energy is reallocated away from growth and reproduction and towards immunological processes, which suggest that functions of the immune system are dependent on metabolism regulation[84].

CYPs (cytochrome P450s) are a large superfamily of metabolic enzymes involved in the immune system capable of metabolizing substances like toxins and pharmaceuticals[86,87] with *CYP1a* shown[86] to be the most expressed CYP1 in liver of zebrafish (*Brachydanio rerio*). In a 2014 study on the HK leukocytes of *G. morhua*, Holen & Olsvik[87] suggests the involvement of *CYP1a* in inflammation and antibacterial defense signaling. *SSAT* (spermidine/spermine-N^1-acetyltransferase), a metabolic rate-limiting enzyme involved in regulation of polyamine homeostasis[88], has been shown to participate in crosstalk with other signaling pathways in *B. rerio*[89] and *S. salar*[90]. In a coculture study, Holen and Espe et al.[90] showed that arginine supplementation to immune cells and metabolic cells increased production of polyamines affecting transcription of *SSAT*, disclosing that polyamines inhabits important anti-inflammatory functions in salmon. Presenting that crosstalk between cell types changes pattern of secreted cell metabolites, glucose produced by the liver was utilized by the HK especially during the inflammation response, furthermore indicating which pathways plays major roles during metabolic stimulation and inflammation[90].

1.11 Fish cytokines

Cytokines are involved in several steps of the immune response, from instigating the innate response to the generation of cytotoxic T-cells and the production of antibodies, to adjusting immune responses through an autocrine or paracrine manner when binding to their corresponding receptor[91,92]. Cytokines are secreted proteins with activation, differentiation and growth functions which control the nature of immune responses and is produced by macrophages, lymphocytes, granulocytes, dendritic cells, mast cells and epithelial cells[51,92]. Upon induction by pathogens such as parasites, bacteria or viruses, cytokines are secreted by activation of immune-related cells[51,92]. The significant number of cytokines functionally active in teleost's can be classified[93-96] as tumor necrosis factors (TNFs), interleukins (ILs), chemokines and interferons (IFNs).

1.11.1 Pro-inflammatory fish cytokines

TNF α is a pro-inflammatory cytokine which performs critical roles in various host responses[97]. TNF-like protein activity has been shown[98] to induce apoptosis and enhance neutrophil migration and macrophage respiratory burst activity. *TNF α* mediates powerful anti-microbial responses, including apoptosis, elimination of infected cells and inhibiting intracellular pathogen replication[51,92].

A cytokine manufactured by one leukocyte and acting on other leukocytes is an interleukin. *IL-1β* was the first IL to be characterized in bony fish[92,99] and is an important mediator for enabling organisms to respond promptly to infections by inducing a cascade of reactions to inflammation[92,100]. Produced in cells mediated by PRRs that have been in contact with PAMPs or DAMPs, it acts as a pro-inflammatory cytokine[92,99,100]. The effective roles of *IL-1β* are mediated through up- or down-regulated expression of other cytokines and chemokines, and has been found to be regulated in response to various stimuli, such as LPS or poly I:C[92,99-101].

1.11.2 Chemokines

Chemokines are a superfamily of small secreted cytokines that direct the migration of immune cells to infection sites which is coordinated by binding to G-protein-linked receptors[92]. *IL-8* is an important chemokine with chemotactic activities related to the pro-inflammatory process produced in response to various stimuli like LPS, cytokines and viruses[92]. It has been suggested[102] that *IL-8*-derived peptides in salmonids have an additional antibacterial activity.

1.11.3 Interferons

The interferon system plays a major role in the innate defense against viruses[74] as interferons genes are involved in mediating cellular resistance against viral pathogens and modulating innate and adaptive immune systems[92]. IFNs are proteins that induce an antiviral state in host cells, wherein the viral infection activates IFNs of the host cell through cell recognition of viral nucleic acids[103]. This occurs when viral single- or double-stranded RNA binds to intracellular receptor proteins (e.g., *TLR3*)[74]. Poly I:C should be a powerful inducer of the IFN system as it functions as a viral mimic.

IFNs can be classified into two main groups[95]; type I and type II, in which the former is induced by viruses in most cells and is involved in innate immunity[74]. The pleiotropic (producing or having multiple effects from a single gene) cytokine *IFN γ* makes up the latter type. This IFN is involved with adaptive immunity[74], being produced by natural killer cells and T-lymphocytes in response to specific ILs or antigens[104]. *IFN γ* has been identified in teleost's, including *S. salar*[103,105,106] and its antiviral activity may be ascribed to upregulation of *Mx* and *viperin*[107,108]. The *Mx* protein is one of the most studied antiviral proteins, inhibiting replication of several virus types and has been shown[74] to be induced by IFNs, particularly *IFN γ*[108]. *Viperin* is an interferon-inducible protein which, similar to the *Mx* protein, inhibits replication in various viruses[107,109] and has shown[110] comparable antiviral properties to *IFN γ* in *G. morhua*.

1.12 Impact of trichlorfon on fish metabolism and immune response

Under normal conditions in their natural environment, *S. salar* and other species of fish are exposed to external stress factors such as predation, varying salinity and water temperature, and pathogenic agents like the sea lice *L. salmonis*, viruses and bacteria. In commercial aquaculture, farmed species like salmon, trout and carp are kept in closed cages with high fish densities, greatly increasing the proliferation and level of pressure and spreading of infection from pathogens and other stressors. If the fish is sick, there is a need to apply treatment to preserve the fish's health. Treatment can be highly stress-inducing, and when adding in the factor of a compromised immune system and decreased metabolism, often escalated by the necessary starvation before utilizing medication, the fish is left very vulnerable to pathogenic sources from their environment, along with potentially negative effects from the pharmaceuticals in use.

There have been several reports that show that TCF have harmful effects for fish[33], including decreasing phagocytosis in Nile tilapia (*O. niloticus*)[111], negative effects on hematological parameters in *O. niloticus*[111], pacu (*Piaractus mesopotamicus*)[112] and European carp (*Cyprinus carpio*)[113-115] in addition to affecting hepatocytes in *P. mesopotamicus*[112] and *C. carpio*[114]. The results from the study by Woo et al.[114] indicates that acute exposure to TCF and thermal stimulus can damage erythropoietic tissue, suggesting that anemia in pesticide-exposed fish could be caused by erythrocyte destruction in hematopoietic tissues. In this study[114], *C. carpio* also showed significant increases in plasma glucose levels, wherein increased glucogenesis may escalated metabolic demands. Damage in the liver of the fish by accumulation of TCF followed an increase in concentration[114]. An increase in the mRNA expression of *CYP1a* was also observed, indicating cytotoxic effects of TCF on hepatocytes and physiological mechanisms[114]. Effects of TCF on cultures of hepatocytes of Prussian carp (*Carassius auratus gibelio*) has also been shown[116,117], where TCF induced apoptosis and cell membrane rupture, increasing hepatocyte apoptosis rate, as well as increasing ROS and prompting *caspase3* activation[116]. Disturbance of antioxidative balance was observed based on monitoring *catalase*, among others, showing that TCF affected fish plasma anti-oxidative status resulting in hepatocyte apoptosis[117].

Other studies with various fish species exposed to TCF, have reported negative effects on the immune system[118] and OS by inducing ROS increase of *C. carpio*[119,120], *O. niloticus*[121] and silver catfish (*Rhamdia quelen*)[122,123], inflammatory response of *C. carpio*[119] and unwanted

effects from inhibition of AChE in cultured sea bass (*Dicentrarchus labrax*)[124]. On the other hand, Tokşen et al.[125] documented no mortality or adverse drug reactions associated with TCF (and azamethiphos) through feed treatment on *D. labrax* broodstock in a later study.

In the early 1990's, TCF was considered moderate risk to fish and high-risk for use on bodies of water by WHO[126]. In 2017, in a report by FAO[127] on the toxicity of TCF, it was classified as (both) highly toxic, to practically non-toxic, for freshwater fish, with a very high to moderate toxicity for marine and estuarine species (based on limited available studies and information on TCF as a pesticide). Acute toxicity tests were conducted with TCF on 12 species of freshwater fish as well as some marine fish and species[127]: LC_{50} values for *O. mykiss* and *B. rerio* for 96 h was 0.7 mg/L and 759 mg/L, respectively, LC_{50} estimates ranged from 0.23 mg/L for bluegill sunfish (*Lepomis macrochirus*) to 110 mg/L for fathead minnow (*Pimephales promelas*) while LC_{50} values ranged from 0.36 µg/L for pink shrimp (*Pandalus borealis*) to >1.0 mg/L for spot (*Leiostomus xanthurus*). Test concentrations were not analytically verified during the studies with TCF, meaning the aforementioned results should only be used as additional information[127].

There have also been notable effects from another OPP, chlorpyrifos-methyl (CLP-m), when dosed in feed to *S. salar* juveniles[128] and post-smolts[129]. CLP-m, similar to TCF and sharing the same main target toxic effects with the irreversible inhibition of AChE, displayed a relatively potent toxicity in liver phospholipids and arachidonic acid metabolism of post-smolts[129]. After lengthy dietary exposure to juveniles, CLP-m was also shown to affect mechanisms associated with protein degradation and lipid metabolism in the brain and liver[128].

To the best of my knowledge, information on the effects of TCF on the metabolism and immune response of *S. salar* are limited to non-existent. As one of Norway's biggest and most important exports, it is, both from a welfare, environmentally and economically perspective, necessary to have as complete comprehension as possible on the consequences of pharmaceutical treatments used in salmon farming.

1.13 Objective of the thesis

- Trichlorfon is an OPP widely used as a chemical treatment against terrestrial insects and fish parasites. In this project, an *in vitro model* is used to study the impact of TCF on metabolic and inflammatory gene responses of liver cells and HK leukocytes of Atlantic salmon.

- The goal of the present study is to evaluate the biological effects of pharmaceutical treatments containing TCF and observe a potential stronger or weaker response compared to a control. This is expressed by up-regulated/down-regulated genes, i.e. immunogens, oxidation genes and genes linked to simulated bacterial and viral infections (LPS & poly I:C, respectively). The results may show effects of the substance on the salmon metabolism and immune system and indicate similar effects on other non-target organisms inhabiting the surrounding ecosystems both under and around commercial fish farms.

2. Materials and methods

2.1 Fish

Liver cells and head kidney cells were isolated from 11 cultivated post-smolts Atlantic salmon with a mean BW of 300 g (including four males and seven females, none sexually mature, table 6.1) obtained from a single water tank in the Bergen Aquarium[1] on 23rd and 24th of April 2019. Liver cells and head kidney cells from each individual fish were isolated and cultured in separate wells and plates. The experimental protocol was approved by the Norwegian Board of Experiments with Living Animal.

Figure 2.1: Fish sampling in Bergen Aquarium, Norwegian Institute of Marine Research.

2.2 Culture medium

L-15 medium (Leibovitz, Sigma) was supplemented with 10% foetal bovine serum (FBS) (BioWhittaker, cat#14-801F), pen/strep (50 U/mL, BioWhittaker, cat#17-602E), 2% 2 mM glutamax™ (100x Gibco, cat#35056) and was designated complete medium (cL-15). Washed leukocytes or liver cells were re-suspended in cL-15 medium and counted using a Bürker chamber and 0.4 tryphan blue solution (BioWhittaker, cat#17-942E). Cell preparations with viability less than 75% were not processed further.

[1] Salmon for research purposes for IMR, in a display tank as part of the exhibition in Bergen Aquarium.

2.3 Isolation of liver cells

The isolations of cells were done with sterile equipment, buffers and solutions (table 6.2). Live fish were anaesthetized by tricaine mesylate (MS-222, 100 g/L, recommended amount for salmon: 80-100 mg/L), killed with a bump to the head and then cut open along the belly. A needle connected to a tube and a peristaltic pump was inserted into blood vessels leading into the exposed liver. To perfuse the liver to remove blood cells, an EDTA-Perfusion buffer pH 7.4 – 0.09 M Hepes buffer containing 1.4 M NaCl (Sodium chloride 1.06404.1000, Merck KGaA), 0.067 M KCl and 0.03 M EDTA (Ethylenediaminetetraacetic acid disodium salt dihydrate, ED2SS-500g, Sigma Aldrich), by a Gilson Minipuls®3 with a flow rate of 4 mL/min, was used. The liver turned yellowish indicating that the liver is free of blood and should be inserted with collagenase-perfusion buffer pH 7.4 (0.1% collagenase type IV isolated from *Clostridium histolyticum* was dissolved in the 0.09 M Hepes buffer as used for perfusion, C2139-100 mg, Sigma Aldrich). Collagenase is used to separate the liver cells inside the liver. This solution should be injected until the liver feels "soft".

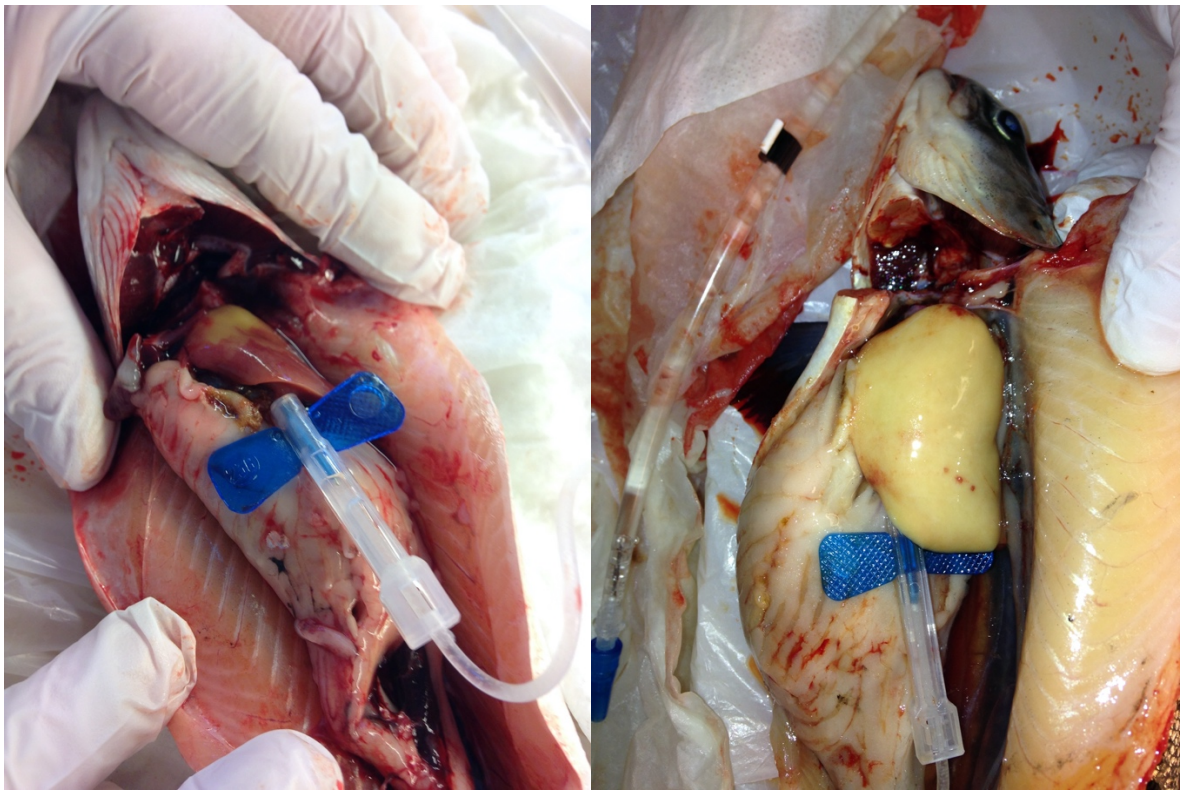

Figure 2.2: Liver perfusion.

Subsequently the liver was extracted and sliced into pieces and inserted into a PBS solution (PBS buffer: 0.002 M KH_2PO_4, 0.02 M Na_2HPO_4, 0.03 M KCl and 0.14 M NaCl, pH 7.4). Using two forceps, the tissue was disrupted and torn apart in the solution. With a 10 mL

sterile syringe (BD Emerald, Ref 307731) without needle the cell solution was sifted through a 100 µM mesh Falcon® cell strainer (Ref 352360) to remove particular matter and leaving cells dispersing through the pores into sterile 50 mL centrifuge tubes (Ref 62.547.254, 114x28mm, PP, Sarstedt AG & Co, Germany). All of the liver solution was put into tubes omitting lumps.

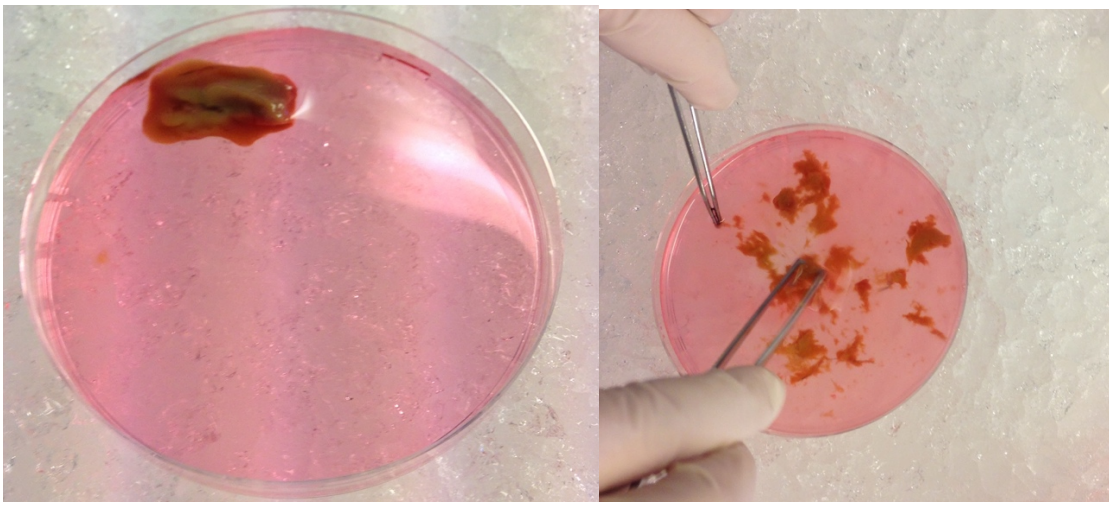

Figure 2.3: Disruption of liver tissue.

The tubes were thereafter filled with 50 mL PBS and centrifuged at 50 G, 5 min, 4°C The cells were concentrated at the bottom of the tubes and the overlaying supernatant was discarded. The isolated cells were harvested in 10 mL 10% phosphate-buffered saline buffer. The tubes were filled with the PBS solution and the washing procedure repeated three times. After discarding the supernatant, complete L-15 medium (containing FBS 50 mL, 5 mL glutamax and 5 mL antibiotic solution) were added, with the amount of L-15 medium added to the cells (20 mL) depending on the amount of cells within the solution.

2.4 Isolation of head kidney leukocytes

From the same fish used to extract liver cells, the head kidney was removed and added to a sterile isolation buffer (9 g NaCl/L and 7 g EDTA/L, pH 7.2) and then stored in a petri dish on ice to maintain the tissue on low temperature. The tissue was torn apart and disrupted using two forceps and then aspirated with a 5 mL sterile syringe (BD Emerald, Ref 307731) without needle to sift through a mesh 40-100 µm Falcon cell strainer.

Figure 2.4: Sampling and disruption of head kidney tissue.

The cells were subsequently transferred to 50 mL centrifugal tubes and washed by centrifugation in a Hettich Zentrifugen 320R, at 400 G, 5 min, 4°C. The resulting cell suspension containing both erythrocytes and leukocytes, were resuspended in the isolation buffer. The diluted cell suspension was carefully layered on top of 50 mL tubes, containing 10 mL of Percoll-gradient solution (Percoll™, 17-0891-01, GE Healthcare). The gradient tubes were centrifuged at 800 G, 5 min, at room temperature in a Hettich Zentrifugen 320R, in order

to separate the erythrocytes from the leukocytes through the difference in density between the cell types and the Percoll-gradient. The cell layer in the interface containing leukocytes was collected with a Pasteur pipette and the cells were pelleted by centrifugation at 600 G, 5 min, 4°C. Two additional washing step in the isolation buffer was performed before resuspending the cells in 10 mL cL-15.

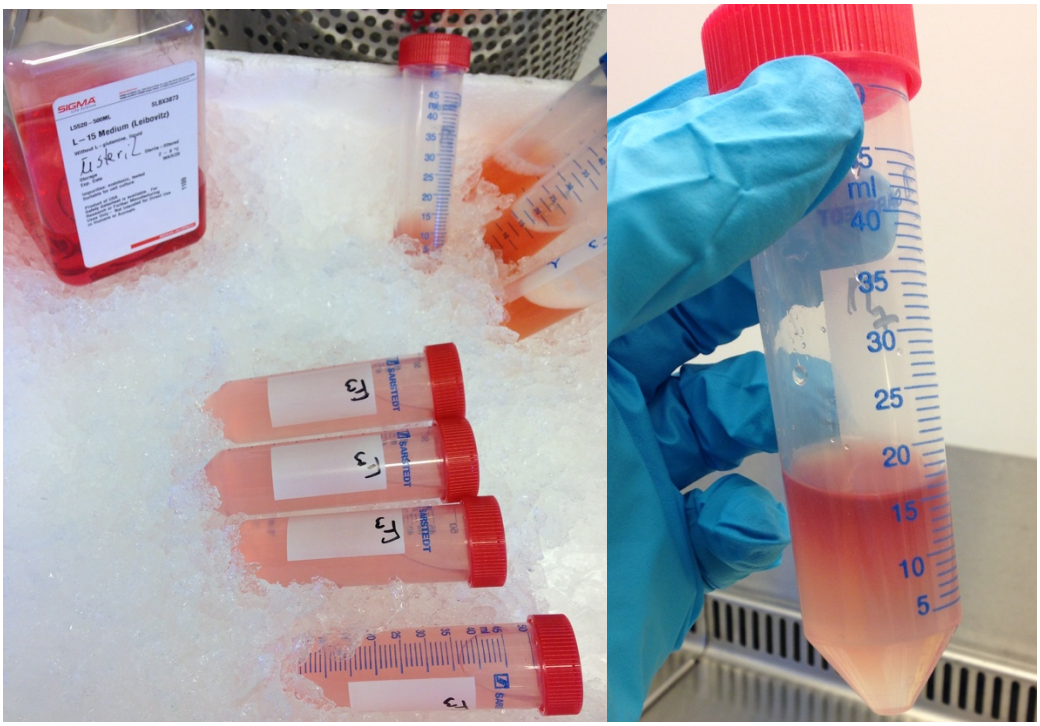

Figure 2.5: Cell suspension carefully layered in equal amounts on top of 50 mL tubes filled with 10 mL Percoll-gradient solution.

2.5 Cell culture calculation in Bürker chamber

Cells were counted using a Bürker chamber, with 20 µL of cells in a homogenous cell suspension + 0.4% trypan blue (BioWhittaker, cat#17-942E). The counting chamber and coverslip were washed with 70% ethanol and wiped with lens paper. Cell counting was initiated within five minutes of mixing. Cells with intact cell membranes does not absorb trypan blue and are not colored. Cells with compromised cell membranes absorbed trypan blue and the blue cells is thus counted as dead cells. The suspension was applied between the chamber and coverslip. Both living and dead cells were counted in 16 B-squares, counting the cells in the middle and two sides of each square (figure A1). When counting 16 squares: the number of cells counted is multiplied by the dilution factor and multiplied by 10 000 to get the number of cells pr mL. Optimally at least 200 cells should be counted. The cell viability = number of living cells x 100% / total number of cells. The viability of the isolated cells was assessed, and

the viability of the head kidney and liver cells was above 75%. The number of counted cells were used to calculate the amount of cell suspension needed for each well. This was done to assure the same number of cells in all the wells.

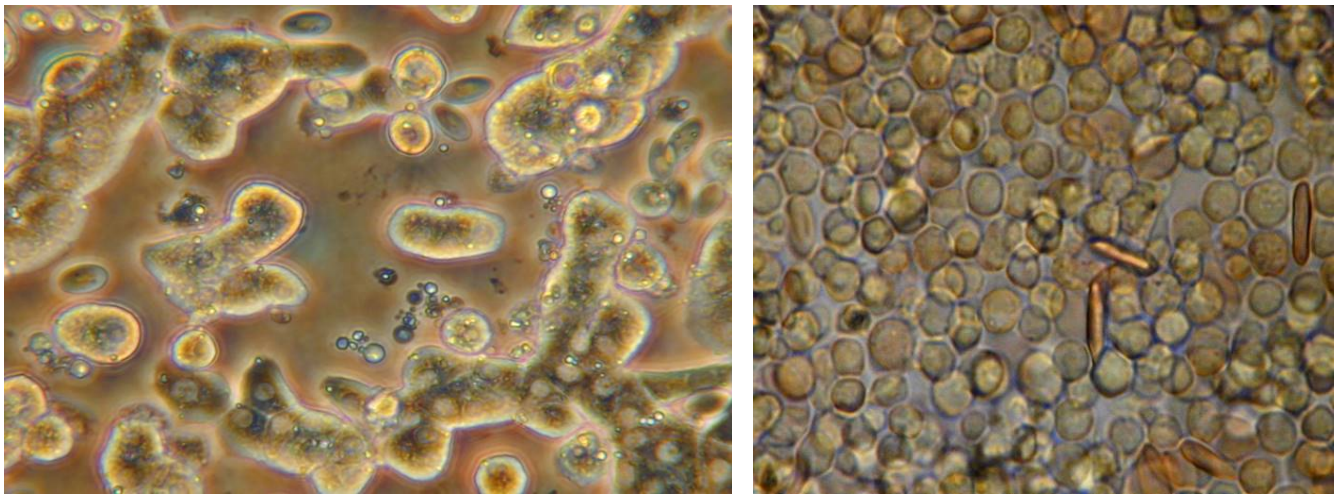

Figure 2.6: Microscopic photograph of isolated liver cells (left) and isolated leukocytes (right).

2.6 Laminin coating of cell culture wells for liver cell culturing

Wells of 6 well culture plates were coated with laminin (1-2 µg/cm^2, Sigma L2020) for 24h in room temperature. The laminin solution was subsequently removed, and the wells were allowed to dry for 1h at room temperature before adding the liver cell suspensions.

2.7 TCF, LPS and poly I:C

Trichlorfon (TCF, dimethyl (RS)-2,2,2-trichloro-1-hydroxyethylphosphonate, ($C_4H_8Cl_3O_4P$), PESTANAL R Article 45698, Sigma Aldrich) was used as main additive stressor to the cell cultures of liver cells and leukocytes. 100 mg TCF was dissolved in 5 mL dimethyl sulfoxide (DMSO, ($(CH_3)_2SO$, 08418-100 mL, Sigma Aldrich) to get exact concentrations (25 µM, 10 µM and 1 µM). These concentrations are derived from a pilot trial to prevent the use of immunotoxic concentrations on the cells.

Lipopolysaccharide, the major component of the outer membrane of Gram-negative bacteria and localized in the outer layer of the membrane, was derived from *Pseudomonas aeruginosa* (LPS, cat# L-7018-10 mg/1 mL L-15) and utilized as bacterial mimic *in vitro*.

Polyinosinic:polycytidylic acid (poly I:C, cat# P1530-25 mg/5 mL L-15) is structurally similar to double-stranded RNA and was utilized to simulate a viral infection *in vitro*. LPS and poly I:C was acquired from Sigma Aldrich.

2.8 Cell cultures

Approximately $1.5x10^7$ and $0.85x10^6/cm^2$ of head kidney leukocytes and liver cells, respectively, isolated cells from the same fish, were added to separate 6 well culture plates (Costar, cat#3335) and cL-15 medium was added to a final volume of 2 mL. The cells were plated in standard cL-15 medium on the day of cell isolation. Selected wells were added 25 µM, 10 µM and 1 µM TCF for a total of 48h exposure time. After these cell cultures had rested for 24h in an incubator (Sanyo Incubator) at 9°C in the dark in a normal atmosphere, selected wells received 100 µg/mL LPS and 50 µg/mL poly I:C. Untreated cultures were included as controls. The wells with and without treatment were incubated for an additional 24h in the incubator. For each fish, cell culturing conditions and treatments are described in table 6.3.

Figure 2.7: Isolated cells in a 6 well culture plate with cL-15 medium.

2.9 Harvesting of cell cultures

Head kidney cells were harvested by centrifugation at day 3. The pellets left after this step cells were collected separately and homogenized 3-4 times in 600 µL RTL-plus buffer (RNeasy Plus kit ®Qiagen) using a syringe and were subsequently frozen at -80°C before RNA extraction. As the liver cells grow as a monolayer attached to the laminin, these cells were added 600 µL RTL-Plus buffer directly into the cell layer after removing the cell culture supernatant. The harvested cells were frozen at -80°C before RNA extraction.

2.10 RNA extraction

RNA extraction was performed under sterile conditions to avoid sample contamination. Total RNA was extracted using RNeasy ®Plus Mini kit (Qiagen), according to the manufacturer's instructions. The lysate was slowly thawed on ice before isolation. 600 µL of ethanol (70%) was added to the lysates to promote a selective binding of RNA to the RNeasy membrane, and transferred to a gDNA spin column placed in a 2 mL collection tube. This step is to clean DNA from the sample by centrifugation (10 000 Rpm, 30s, Hettich Zentrifugen 320R) to enable RNA flow through the column. 500 µL of the buffer RPE (RNeasy Plus kit ®Qiagen) was used

twice to wash ethanol from the RNA before collecting the RNA in a new 1.5 mL collection tube using RNase-free water. The RNA was frozen at -80°C until further processed.

The concentration of RNA was assessed using the NanoDrop ND-1000 UV Spectrophotometer (NanoDropTechnologies, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA integrity was assessed using the RNA 6000 Nano LabChip® kit (Agilent Technologies, Palo Alto, CA, USA) following the instructions from the supplier. The liver RNA samples had 260/280 nm absorbance ratios of 2.0 ± 0.15 and 260/230 nm ratios of 2.4 ± 0.2. Head kidney RNA samples had 260/280 nm absorbance ratios of 2.0 ± 0.1 and 260/230 nm ratios of 2.4 ± 0.4. The quality of 10 randomly selected liver RNA samples and 24 randomly selected head kidney RNA samples were analyzed based on the RNA Integrity Number (RIN) with RNA range from 1-10. Samples with RIN value $\geq$ 7.7 was considered adequate for use in RT-PCR. Samples with poor RIN values indicates degradation of the total RNA in the sample. RIN values in all of the selected liver RNA samples and all of the selected head kidney RNA samples had values > 7.7 which indicated that both the liver and head kidney RNA samples were suitable for RT-PCR/qPCR (table 6.4).

2.11 Reverse transcription polymerase chain reaction (RT-PCR)

The enzyme reverse transcriptase was used to convert the RNA template into the more stable cDNA (complementary DNA) for use in quantitative PCR. Four separate cDNA plates were made, two for liver RNA and two for head kidney RNA. For the two liver plates a randomly selected pool from the 71 samples of liver RNA and for the two head kidney plates a randomly selected pool from the 70 samples of head kidney RNA, a standard curve was made with six serial dilutions from 1000-31.25 ng and run in triplicates into 96-well PCR plates (VWR, AB-06000). The remaining samples were diluted individually with sterile RNase free water into a concentration of 30 ng/µL and set up in duplicates in the 96-well PCR plates (10 µL/well). A RT-reaction mix (table 6.5) was prepared with the kit TaqMan reverse transcription reagents containing Multiscribe Reverse Transcriptase (50 U/mL) and added 20 µL/well RT-reaction mix to the diluted RNA samples in the two 96-well PCR plates to a total volume of 30 µL/well. Two negative controls were included to verify noncontaminated kits and RNase free water: a none amplification control (nac) without enzymes, and a non-template control (ntc) with RNase free water replacing RNA. Full 96-well plate setup for the four cDNA plates are showed in figure 6.2-5. The RT reaction was performed with a CFX96™ Thermal Cycler (Bio-RAD system) starting with an incubation step for 10 min at 25°C, continuing with RT reaction at

48°C for 60 min by using oligo dTprimers (2.5 µM) in 30 µL total volume, and finally with 5 min inactivation at 95°C (table 6.6). The PCR plates were stored at -20°C. The PCR primer genes, sequences and functions are listed in table 6.7.

2.12 Quantitative polymerase chain reaction (qPCR)

The cDNA plates synthetized from the RT-reaction functioned as templates for the qPCR. By measuring cDNA amplification and fluorescence, a relative quantification of the target gene could be obtained. Gene expression was quantified with qPCR on the Light Cycler 480 (Roche Applied Sciences, Basel Switzerland). The 30 µL volume in the cDNA plate was diluted with ddH_2O to a final volume of 60 µL per well. To ensure homogenized samples, the PCR plates was centrifuged for 1 min, 1000 G and afterwards vortexed for 5 min, 1300 Rpm. To create the qPCR 384 wells Real Time plates, a pipetting robot (Automated Laboratory Workstation, BIDMEK 4000, Beckman Coulter) transferred 2 µL RNA/well from the cDNA plate and 8 µL qPCR mix (table 6.8) to each well. Finished Real Time plates were covered with optical adhesive covers; without touching the film, and centrifuged for 2 min, 1500 G, before running qPCR with a CF384™ Real-Time system (Bio-RAD system, C1000 Touch Thermal Cycler) on the following program: 5 min activation and denaturizing step at 95°C followed by 45 cycles of 10s denaturizing step at 95°C, 20s annealing step at 60°C and a 30s synthesis step at 72°C, followed by a melt curve analysis and cooling to 4°C. The qPCR program is described in table 6.9.

The Bio-RAD CFX MAESTRO system was used to determine a normalization factor from the four reference genes and used to calculate mean normalized expression for the target genes. The stability of the reference genes was calculated by the Bio-RAD system, wherein four reference genes; RPL13, EF1α, β-actin and ARP were included. Cq values of each target gene from the qPCR were used to calculate the normalized gene expression with its respective mean Cq value, Cq value standard error of the mean (SEM), expression SEM and corrected expression SEM. From this, a gene expression normalization factor was made for each sample. Cq values from the qPCR were imported into excel where interpolate normalization, relative quantities and standard deviations were calculated.

2.13 Statistical analysis

Data comparing gene expression responses between culture conditions were subjected to one-way analysis of variance (ANOVA) using an ANOVA procedure in Statistica ver. 13.1 software (StatSoft, TIBCO Software Inc., Palo Alto, CA, USA). The experiment was designed

to use one-way factorial ANOVA design with treatment x selected gene as varying factors. Differences between treatments within culture conditions were determined by Tukey's post hoc test and the Student Newman-Keuls test at $P < 0.05$. All data were tested for homogeneity of variance by Levene's test. Data identified as non-homogeneous were subjected to a non-parametric analysis by multiple comparison of mean ranks. Data are presented as mean with standard deviation (SD) with a significance level of 95%. Figures were modeled in GraphPad Prism 8 (GraphPad Software, La Jolla, CA, USA).

3. Results

3.1 Immunorelated metabolic gene expression (*CYP1a*, *SSAT*)

Transcription of the detoxification marker *CYP1a* was not significantly up- or down regulated in cultured liver cells (fig 3.1.1) or in cultured HK leukocytes regardless of treatment (fig. 3.1.2). The transcription of the metabolic rate-limiting enzyme *SSAT* gene was not significantly up- or down regulated in cultured liver cells (fig. 3.1.3) or in cultured HK leukocytes (fig. 3.1.4) regardless of treatment, although *SSAT* was down regulated in HK.

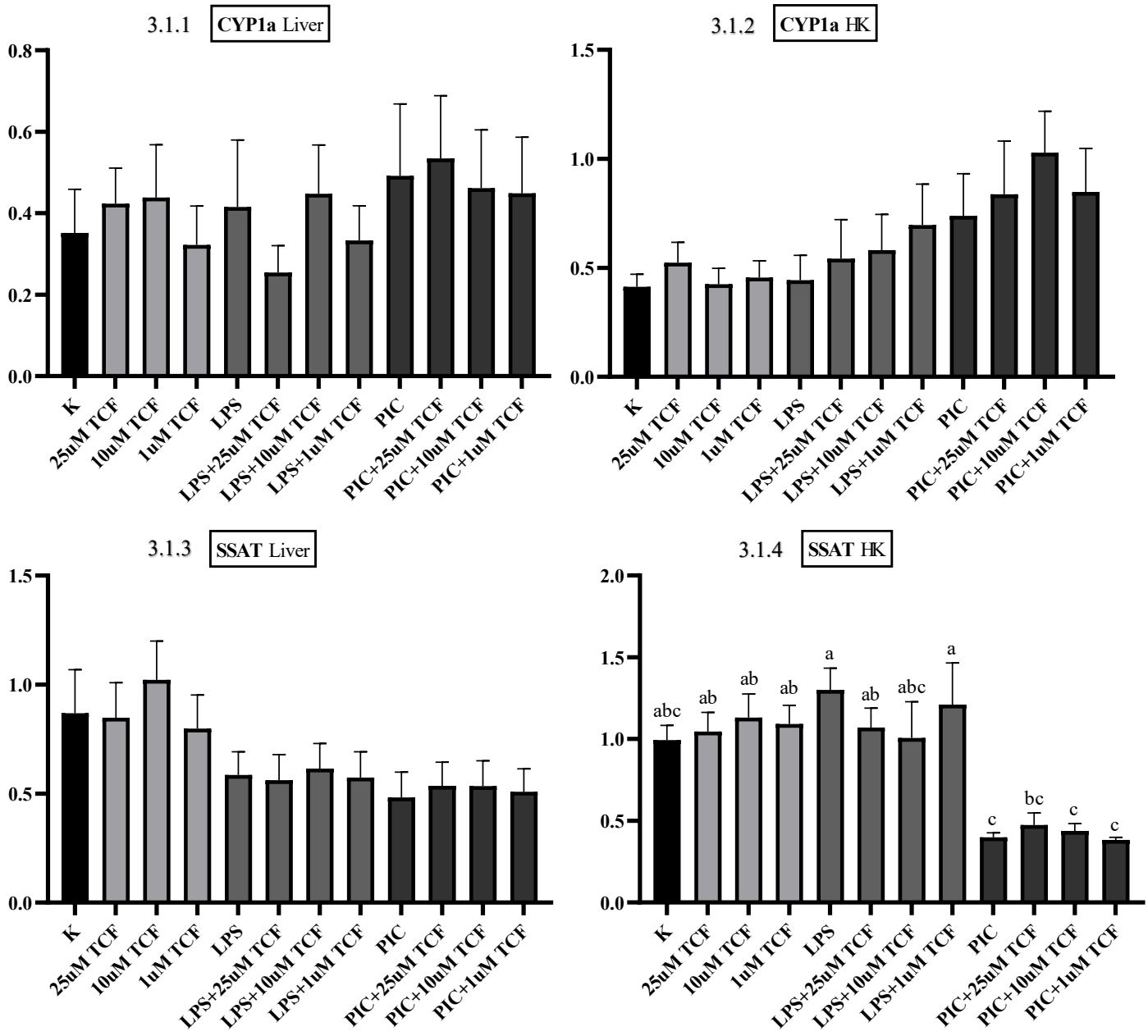

Figure 3.1: Immunorelated metabolic enzymes CYP1a and SSAT transcription in isolated salmon cells and HK leukocytes exposed to TCF, LPS or poly I:C (n = 6). Different letters designate significant different responses a ≠ b ≠ c, while ab is comparable to both a and b responses. 3.1.1) CYP1a transcription was not significantly affected

in liver cells regardless of treatment. 3.1.2) CYP1a transcription was not significantly affected in HK leukocytes regardless of treatment. 3.1.3) SSAT transcription was not significantly affected in liver cells regardless of treatment. 3.1.4) SSAT transcription was not significantly affected in HK leukocytes regardless of treatment.

3.2 Oxidative stress related gene expression (*Bcl-2*, *catalase*)

Of the OS related genes, transcription of *Bcl-2* was not significantly up- or down regulated in cultured liver cells (fig. 3.2.1) regardless of treatment, while in HK leukocytes (fig. 3.2.2) cultured with poly I:C alone and cells cultured with poly I:C with 25, 10 & 1 µM TCF, *Bcl-2* transcription was significantly down regulated compared to non-treated cells (K) ($p = 0.0001$, $p = 0.0002$, $p = 0.0001$ and 0.0001, respectively). TCF had no effect. The transcription of the OS related gene *catalase* was not significantly up- or down regulated in cultured liver cells (fig. 3.2.3) or in cultured HK leukocytes (fig. 3.2.4) regardless of treatment.

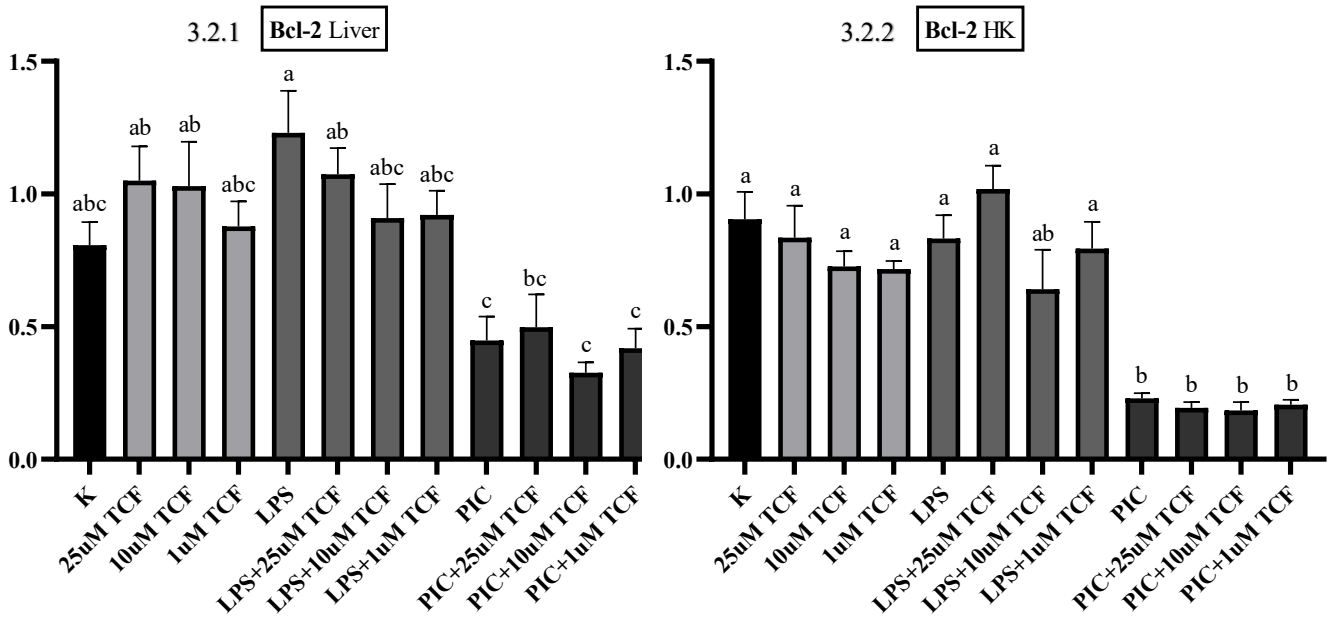

Figure 3.2: Oxidative stress related genes Bcl-2 and catalase transcription in isolated salmon cells and HK leukocytes exposed to TCF, LPS or poly I:C (n = 6). Different letters designate significant different responses a ≠ b ≠ c, while ab is comparable to both a and b responses. 3.2.1) Bcl-2 transcription was not significantly affected in liver cells regardless of treatment. 3.2.2) Poly I:C alone and poly I:C + TCF (25, 10, 1 µM) significantly down regulated Bcl-2 transcription compared to control (K) ($p < 0.05$). 3.2.3) Catalase transcription was not significantly affected in liver cells regardless of treatment. 3.2.4) Catalase transcription was not significantly affected in HK leukocytes regardless of treatment.

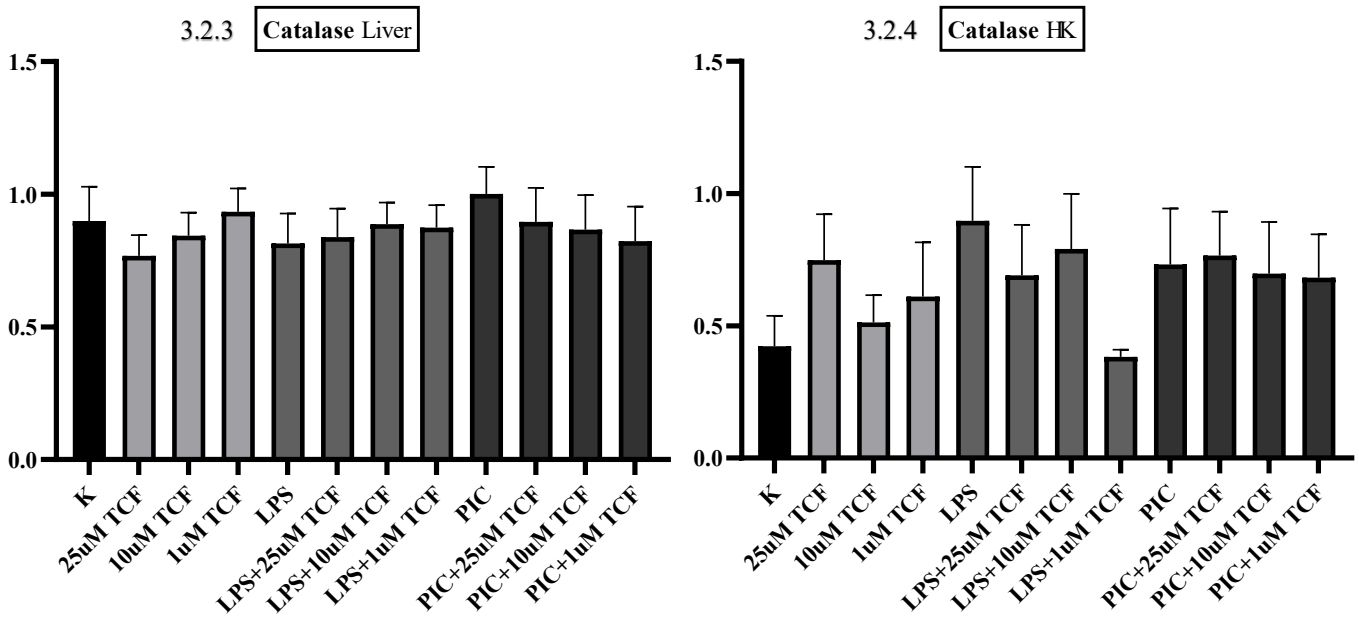

Fig. 3.2 (continued).

3.3 Inflammation marker gene expression (*CD83*, *Cox-2*, *IL-1β*, *IL-8*, *IFN γ*, *TNF α*)

CD83 transcription was not significantly up- or down regulated in liver cells (fig. 3.3.1) regardless of treatment, while *CD83* transcription was significantly upregulated in HK leukocytes (fig. 3.3.2) cultured with LPS and 10 µM TCF compared to non-treated cells (K) ($p = 0.0084$). TCF had no effect. Transcription of the eicosanoid pathway gene *Cox-2* was significantly upregulated in liver cells (fig. 3.3.3) cultured with LPS alone and when cultured with 10 & 1 µM TCF compared to non-treated cells (K) ($p = 0.0010$, $p = 0.0089$ and $p = 0.0154$, respectively), with transcription also being significantly upregulated in HK leukocytes (fig. 3.3.4) cultured with LPS alone and when cultured with 25 or 1 µM TCF compared to non-treated cells (K) ($p = 0.0306$, $p = 0.0036$ and $p = 0.0227$, respectively). TCF had no effect on either cell type. Transcription of the pro-inflammatory gene *IL-1β* was not significantly up- or down regulated in liver cells (fig. 3.3.5) regardless of treatment. In HK leukocytes (fig. 3.3.6) cultured with LPS alone and when cultured with 10 or 1 µM TCF *IL-1β* transcription was significantly upregulated compared to non-treated cells (K) ($p = 0.0088$, $p = 0.0049$ and $p = 0.0049$, respectively). TCF had little to no effect. Chemokine *IL-8* transcription was not significantly up- or down regulated in cultured liver cells (fig. 3.3.7) or in cultured HK leukocytes (fig. 3.3.8) regardless of treatment. Transcription of the pleiotropic cytokine *IFN γ* was not significantly up- or down regulated in cultured liver cells (fig. 3.3.9) or in cultured HK leukocytes (fig. 3.3.10) regardless of treatment. Transcription of the pro-inflammatory

cytokine *TNF α* was significantly upregulated in liver cells (fig. 3.3.11) cultured with LPS and 10 & 1 µM TCF compared to non-treated cells (K) ($p = 0.0303$ and $p = 0.0191$, respectively). *TNF α* transcription was also significantly upregulated in HK leukocytes (fig. 3.3.12) cultured with LPS and 10 µM TCF compared to non-treated cells (K) ($p = 0.0072$). TCF had little to no effect on either cell type.

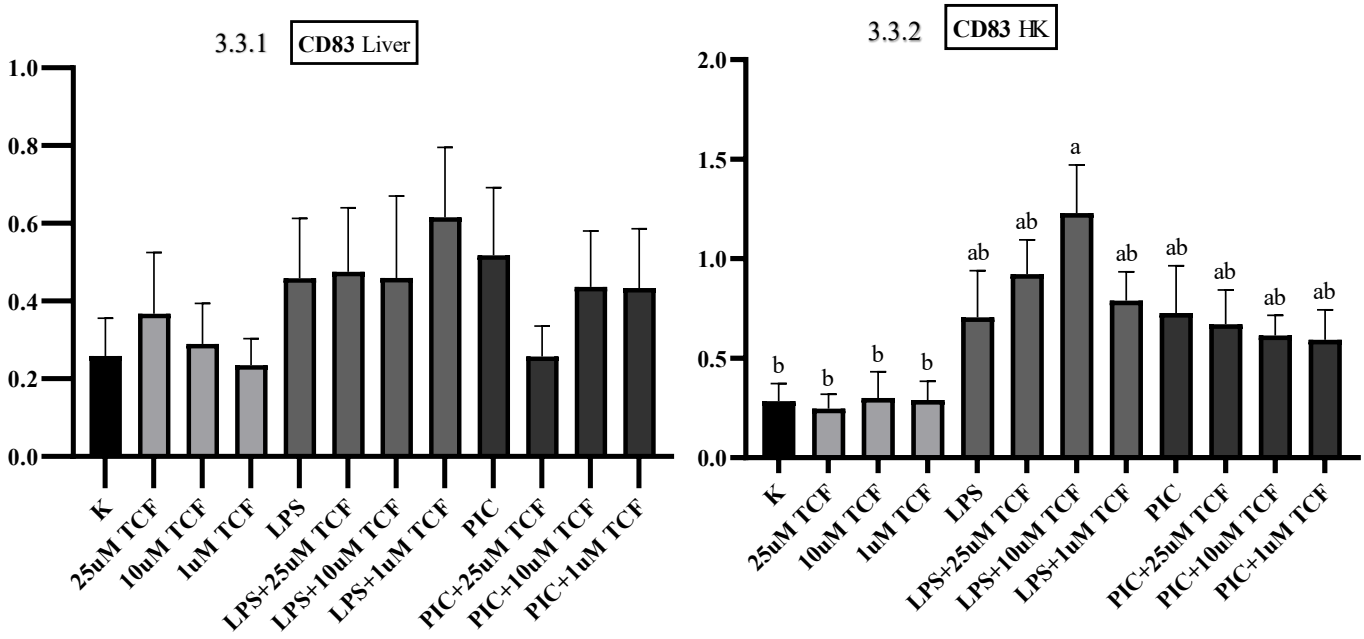

Figure 3.3: Inflammation markers CD83, Cox-2, IL-1β, IL-8, IFN γ and TNF α transcription in isolated salmon liver cells and HK leukocytes exposed to TCF, LPS or poly I:C (n = 6). Different letters designate significant different responses a ≠ b ≠ c, while ab is comparable to both a and b responses. 3.3.1) CD83 transcription was not significantly up- or down regulated in liver cells regardless of treatment. 3.3.2) In HK leukocytes cultured with LPS + TCF (10 µM), CD83 transcription was significantly induced compared to control (K) ($p < 0.05$). 3.3.3) Cox-2 transcription was significantly induced in liver cells cultured with LPS alone and LPS + TCF (10, 1 µM) compared to control (K) ($p < 0.05$). 3.3.4) Cox-2 transcription was also significantly induced in HK leukocytes cultured with LPS alone and LPS + TCF (25, 1 µM) compared to control (K) ($p < 0.05$).

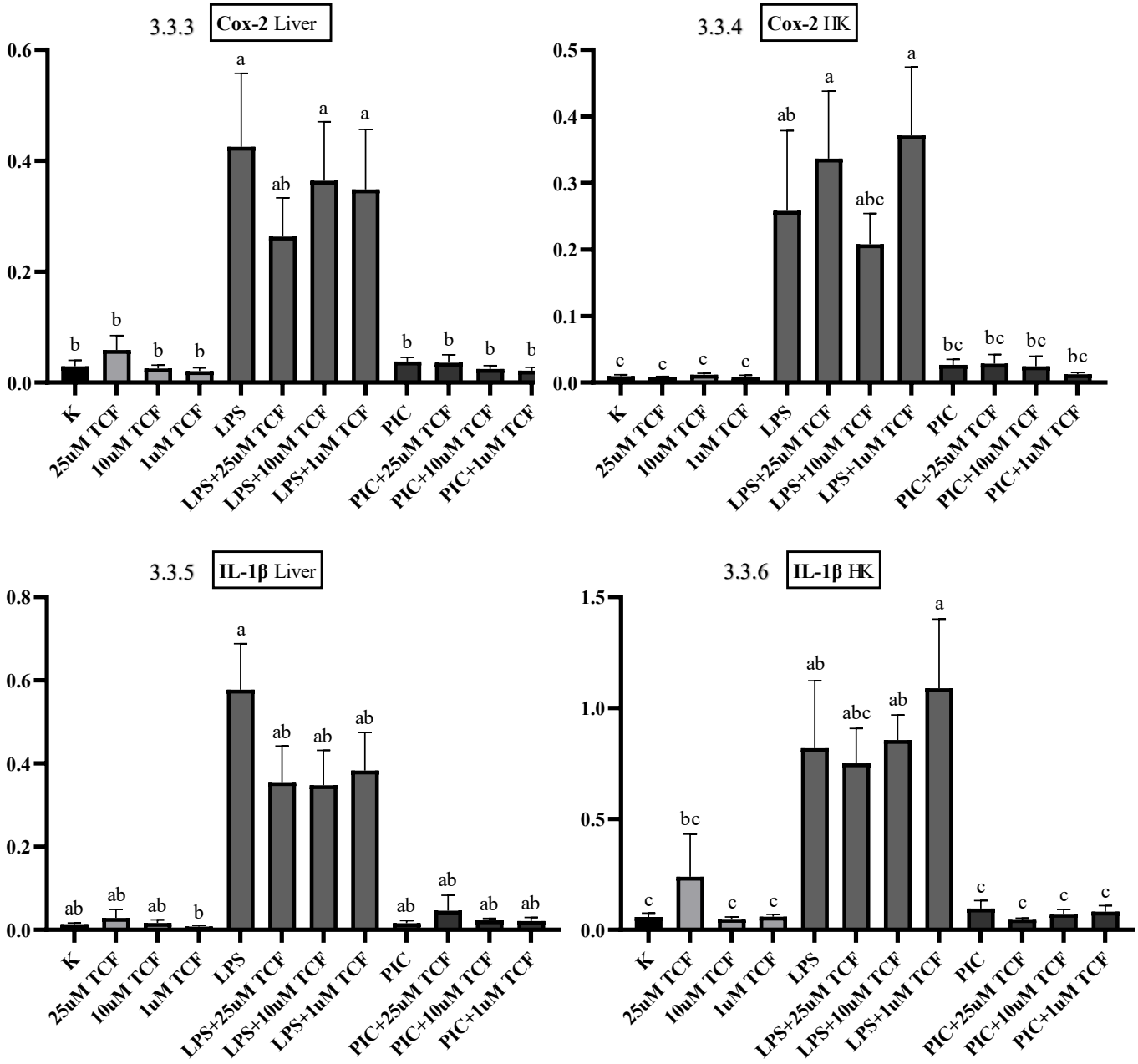

Fig. 3.3. (continued). 3.3.5) IL-1β transcription was not significantly up- or down regulated in liver cells regardless of treatment. 3.3.6) IL-1β transcription was significantly induced in HK leukocytes cultured with LPS alone and LPS + TCF (10, 1 μM) compared to control (K) ($p < 0.05$). 3.3.7) IL-8 transcription was not significantly up- or down regulated in liver cells regardless of treatment. 3.3.8) IL-8 transcription was not significantly up- or down regulated in HK leukocytes regardless of treatment. 3.3.9) IFN γ transcription was not significantly up- or down regulated in liver cells regardless of treatment. 3.3.10) IFN γ transcription was not significantly up- or down regulated in HK leukocytes regardless of treatment. 3.3.11) Transcription of TNF α was significantly induced in liver cells cultured with LPS + TCF (10, 1 μM) compared to control (K) ($p < 0.05$). 3.3.12) In HK leukocytes cultured with LPS + TCF (10 μM), transcription of TNF α was significantly induced compared to control (K) ($p < 0.05$).

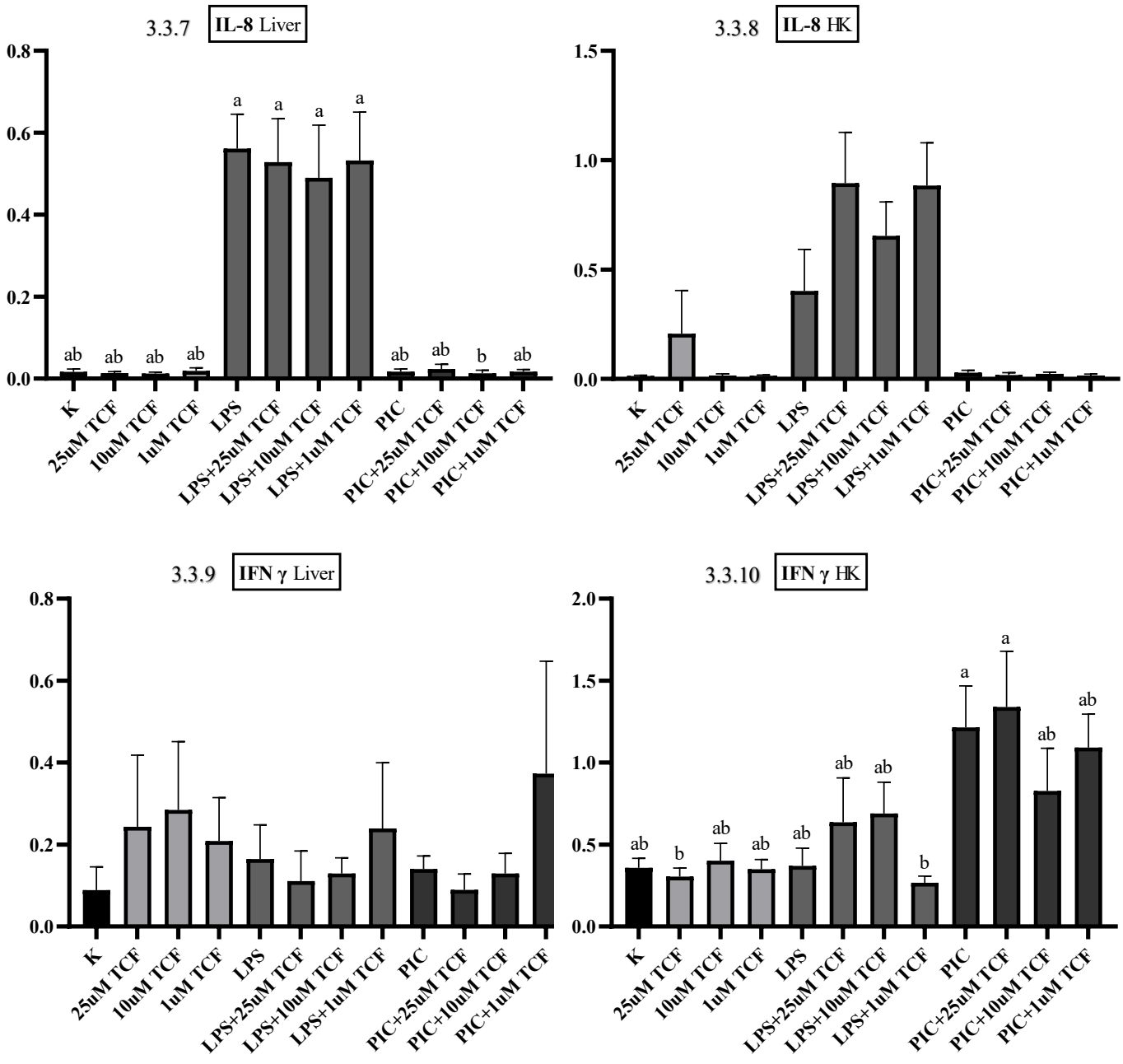
3.3.7 IL-8 Liver
0.8
0.6
0.4
0.2
0.0
ab ab ab ab a a a a ab ab b ab
K
25uM TCF
10uM TCF
1uM TCF
LPS
LPS+25uM TCF
LPS+10uM TCF
LPS+1uM TCF
PIC
PIC+25uM TCF
PIC+10uM TCF
PIC+1uM TCF
3.3.8 IL-8 HK
1.5
1.0
0.5
0.0
3.3.9 IFN γ Liver
0.8
0.6
0.4
0.2
0.0
3.3.10 IFN γ HK
2.0
1.5
1.0
0.5
0.0
ab b ab ab ab ab ab b a a ab ab

Fig. 3.3. (cont.).

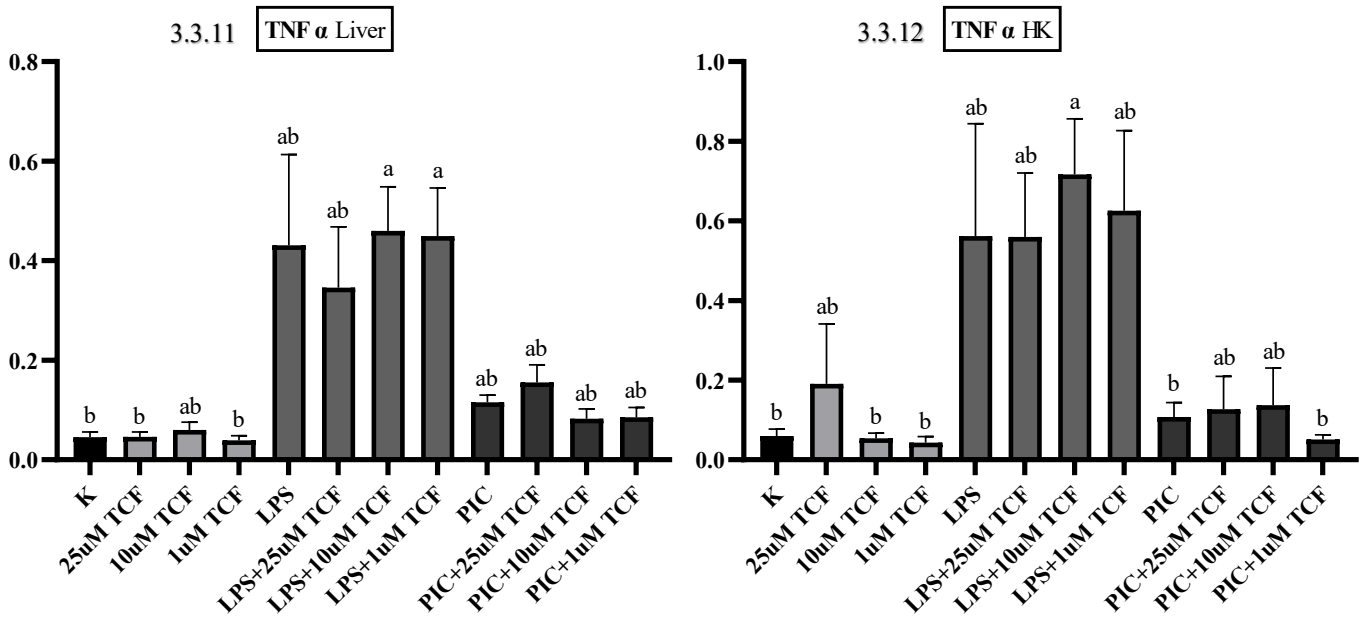

Fig. 3.3. (cont.).

3.4 Apoptotic related gene expression (*Caspase3*)

Transcription of the apoptotic related gene *caspase3* was not significantly up- or down regulated in liver cells (fig. 3.4.1) regardless of treatment. *Caspase3* transcription was significantly upregulated in HK leukocytes (fig. 3.4.2) cultured with poly I:C alone and poly I:C cultured with 10 µM TCF compared to non-treated cells (K) ($p = 0.0180$ and $p = 0.0113$, respectively). TCF had little to no effect.

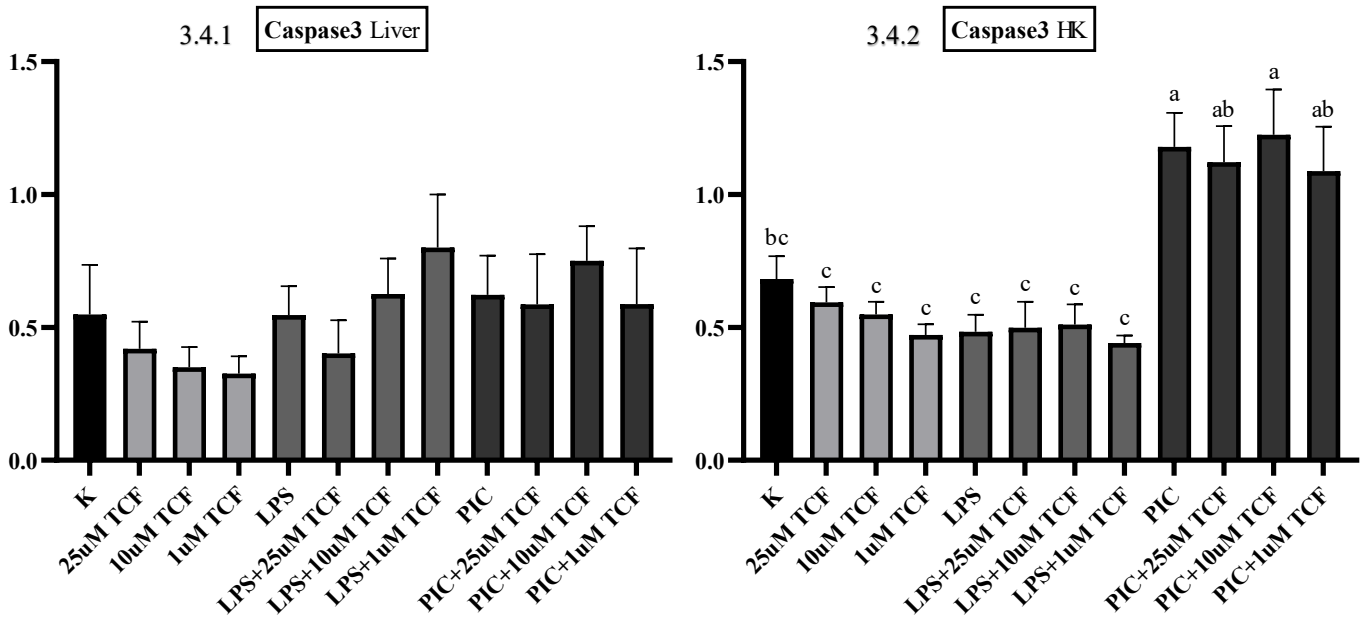

Figure 3.4: Apoptotic related gene caspase3 transcription in isolated salmon cells and HK leukocytes exposed to TCF, LPS or poly I:C (n = 6). Different letters designate significant different responses a ≠ b ≠ c, while ab is comparable to both a and b responses. 3.4.1) Caspase3 transcription was not significantly affected in liver cells regardless of treatment. 3.4.2) Poly I:C alone and + TCF (10 µM) significantly induced transcription in HK leukocytes compared to control (K) ($p < 0.05$).

3.5 Cellular antiviral response gene expression (*Mx*, *TLR-3*, *viperin*)

Transcription of the antiviral gene *Mx* was not significantly up- or down regulated in liver cells (fig. 3.5.1) regardless of treatment. *Mx* transcription was significantly upregulated in HK leukocytes (fig. 3.5.2) cultured with poly I:C alone and cells cultured with poly I:C with 25, 10 & µM TCF compared to non-treated cells (K) ($p = 0.0001$, $p = 0.0001$, $p = 0.0001$ and $p = 0.0001$, respectively). TCF had no effect. Transcription of the intracellular receptor involved in antiviral mechanics *TLR3* was not significantly up- or down regulated in liver cells (fig 3.5.3) or in HK leukocytes (fig. 3.5.4) cultured with LPS or poly I:C alone or cultured together with TCF, all concentrations, compared to non-treated cells (K). Transcription of the antiviral gene viperin was significantly upregulated in liver cells (fig. 3.5.5) cultured with poly I:C and 25 µM TCF compared to non-treated cells (K) ($p = 0.0477$). In HK leukocytes (fig. 3.5.6) cultured with poly I:C alone and poly I:C with 25, 10 & µM TCF, viperin transcription was significantly upregulated compared to non-treated cells (K) ($p = 0.0001$, $p = 0.0001$, $p = 0.0001$ and $p = 0.0001$, respectively). TCF had little to no effect on either cell type.

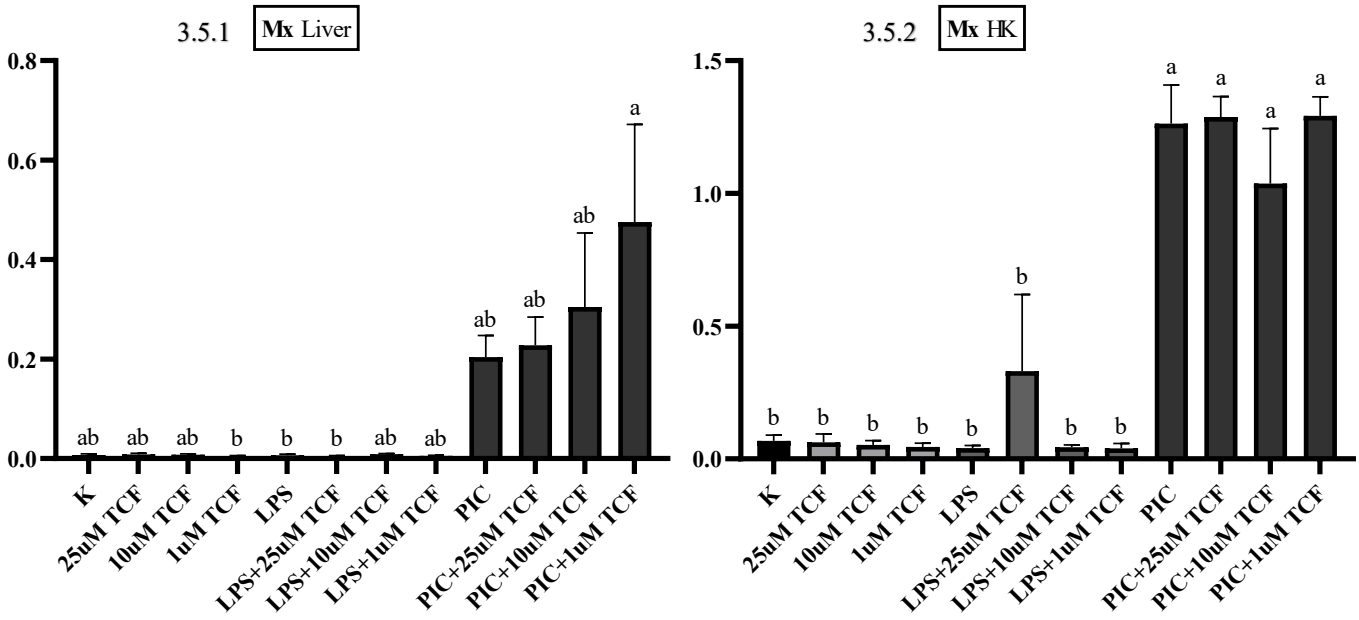

Figure 3.5: Cellular antiviral response genes Mx, TLR3 and viperin transcription in isolated salmon liver cells and HK leukocytes exposed to TCF, LPS or poly I:C (n = 6). Different letters designate significant different

responses a ≠ b ≠ c, while ab is comparable to both a and b responses. 3.5.1) Mx transcription was not significantly up- or down regulated in liver cells regardless of treatment.

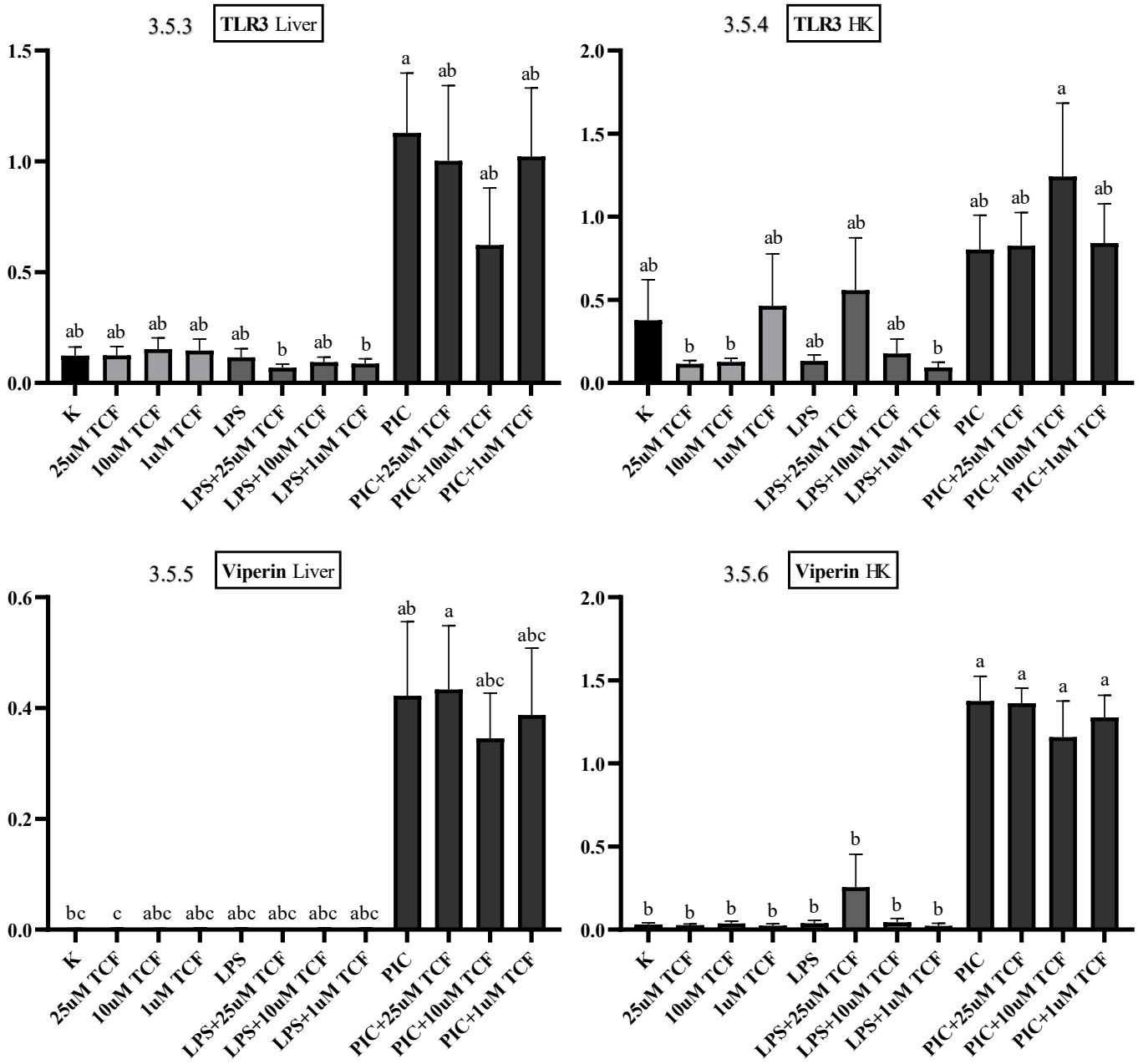

Fig. 3.5 (continued). 3.5.2) Poly I:C alone and + TCF (25, 10, 1 μM) significantly induced Mx transcription in cultured HK leukocytes compared to control (K) ($p < 0.05$). 3.5.3) TLR3 was not significantly up- or down regulated in liver cells regardless of treatment. 3.5.4) TLR3 was not significantly up- or down regulated in HK leukocytes regardless of treatment. 3.5.5) Liver cells cultured with poly I:C + TCF (25 μM) significantly induced viperin transcription compared to control (K) ($p < 0.05$). 3.5.6) Viperin transcription was significantly induced in HK leukocytes poly I:C alone and + TCF (25, 10, 1 μM) compared to control (K) ($p < 0.05$).

4. Discussion

As the planet's population is increasing in tandem with our food supply starting to grow scarce[1,2], commercial aquaculture and particularly the farming of Atlantic salmon, *Salmo salar* L., presents itself as a more carbon-friendly way of producing healthy protein for human consumption. However, with an escalating production of fish, the occurrence of bacterial and viral diseases and parasitic infections also increases. Since the conception of salmon farming in the 1970's[12,19], the use of pharmaceutical treatment in commercial aquaculture have been rising to unsustainable levels, both environmentally and in terms of welfare for non-target species in close proximity of salmon farms[5-9]. As the first agent applied against salmon lice, *Lepeophtheirus salmonis*, the organophosphorus pesticide trichlorfon was a success in repelling the parasite[5,17,18]. TCF is a neurotoxic insecticide widely used against terrestrial insects and fish parasites by inhibiting AChE activity[39] causing paralysis and death of the parasite[40-45]. With the ongoing growth in the commercial aquaculture industry, the use of pharmaceuticals is expected to follow suit, and the effects of chemical agents like TCF negatively impacting non-target species and the treated fish itself will continue to be a challenge in the future.

In this current study, isolated liver cells and HK leukocytes from post-smolts of *S. salar*, were exposed to the OPP TCF with or without the addition of LPS and poly I:C, to observe effects of TCF on metabolic and inflammatory immune responses. To evaluate the biological effects of pharmaceutical treatments containing TCF, a potential stronger or weaker response compared to a control had to be observed. This is expressed following a qPCR by up-regulated/down-regulated genes through, i.e., immunogens, oxidation genes and genes linked to simulated bacterial and viral infection (LPS & poly I:C, respectively).

To the best of my knowledge, no studies on the effects of TCF on the metabolic and immunogenic responses of *S. salar* exists, leaving no basis to compare consistency of any direct similarly effects of TCF on *S. salar*. The *in vitro* cell model used in this study have been utilized and published several times with different treatments. This method has proven to be accurate with responses found in the fish itself, allowing the use of inexpensive and sustainable experiments with fewer individuals. Utilizing cell models is useful when exposing fish cells to LPS, poly I:C or toxins, yet it will not be quite the same as *in vivo* or *in situ* trials.

4.1 Effects of LPS and poly I:C treatment on metabolic and immune gene transcriptions

LPS is used *in vitro* as a bacterial mimic for observation of bacterial infections on cellular level. Genes in this study involved in inflammatory and antibacterial immune responses was expected to be affected by LPS. Poly I:C is used *in vitro* to mimic viral infections on cells. Genes in this study involved with antiviral responses was expected to be affected by poly I:C. Isolated liver cells and HK leukocytes were cultured with 100 µg/mL LPS and 50 µg/mL poly I:C for 24h at 9°C, with and without TCF, with untreated cultures included as controls for comparison (fig. 3.1-3.5).

4.1.1 Inflammatory marker genes

Exposure to LPS significantly upregulated transcriptions in isolated liver cells of the pro-inflammatory genes *Cox-2* and *TNF α*, while significantly upregulating transcriptions in isolated HK leukocytes of the pro-inflammatory genes *CD83*, *Cox-2*, *IL-1β* and *TNF α*. As these cytokines act to mediate resistance to bacterial infections, the results suggest the suitability of the *in vitro* model used in the current study. Interestingly, none of these genes were significantly affected by the presence of poly I:C, dissimilar from previous studies by Holen et al.[58], where HK leukocytes isolated from *G. morhua* displayed immune responses to bacterial or viral mimics operating through different pathways. Fierro-Castro et al.[130] has shown that genes related to the innate immune response is upregulated in the HK macrophages isolated from *O. mykiss* exposed to poly I:C. The cells were exposed to LPS and poly I:C for 4h and 24h at 18°C, displaying a peak of immune-related gene expression after 4h to 100 µg/mL poly I:C[130].

Martins et al.[131] cultured isolated salmon liver cells and HK leukocytes with 100 µg/mL LPS for 24h, resulting in significantly upregulated transcription of *CD83*, *Cox-2*, *IL-1β* and *IL-8* in liver cells, and *Cox-2*, *IL-1β*, *IL-8* and *TNF α* in HK leukocytes. Cell cultures of liver cells and HK leukocytes was also added 50 µg/mL poly I:C for 24h, upregulating *Mx* and *viperin*[131], coinciding with the results in the current study. Here, HK leukocytes were exposed for 24h to 50 µg/mL poly I:C at 9°C, in which the difference in responses from the HK cells of *O. mykiss* and *S. salar* could be explained by a high variance in responses between individual fish, differences between species *O. mykiss* and *S. salar*, time of exposure or dosage. Martins et al.[131] cultured identical cell types from the same species of fish, with the same dosage and time of exposure, which resulted in similar results to the current study.

The inflammatory marker gene *CD83* is a specific marker for dendritic cells in mammals[75], and has been suggested[132] to correlate with surface expression of MHC II in *O. mykiss*. Donate et al.[133] showed *CD83* being upregulated in sea bream (*Pagrus major*) in response to LPS, and Goetz et al.[134] reported an upregulated expression of *CD83* in *O. mykiss* leukocytes using 10 µg/mL LPS for 12h *in vitro*. Abóz et al.[135] studied a fish rhabdovirus effect on IgM^+ cells in blood from *O. mykiss*, in which the rhabdovirus induced upregulation of MHC II cell surface expression on IgM^+ cells along with increased transcription of *CD83*, pointing virus-induced IgM^+ cell activation toward an antigen presenting profile. After 24h of infection, the virus caused a significant upregulation of *CD83* mRNA levels in IgM^+ B lymphocytes[135]. These studies could suggest that *CD83* and MHC II expression are somewhat related in teleost's[75]. Holen & Espe et al.[90] also showed that *CD83* was upregulated by LPS in HK leukocytes of *S. salar*. Martins et. al[131] showed *CD83* being upregulated by LPS in *S. salar* liver cells as well, but not in HK leukocytes. In the present study, LPS induced a significant transcription of *CD83* in HK leukocytes, showing similar responses to earlier studies.

Cox-2 is an inflammatory related enzyme involved in downstream signaling operating in connection to the innate immune response suggested to have physiologic- and pathologic-regulating effects on metabolism[63]. Another inflammation gene marker, *IL-1β* is a pro-inflammatory cytokine[92,99,100] important in enabling an inflammation response to infections through a cascade signaling[92,100] and its effective roles are mediated through up- or down-regulated expression of other cytokines and chemokines[92,99-101]. The chemokine *IL-8* is part of the cytokines directing immune cells to infection sites[92] and is involved with the pro-inflammatory process produced in response to various stimuli like LPS, cytokines and viruses[92]. It has been suggested[102] that *IL-8*-derived peptide in salmonids have an additional antibacterial activity. In a study by Holen et al.[58] HK leukocytes of *G. morhua* were exposed to 100 µg/mL LPS and 50 µg/mL poly I:C at 9°C overnight. LPS significantly upregulated *Cox-2* (16.3-fold), *IL-1β* (25.8-fold) and *IL-8* (10.5-fold), with poly I:C having no effects in these genes' expression. Holen & Olsvik[87,136] later reported again that HK leukocytes of *G. morhua* exposed to 100 µg/mL LPS significantly induced transcription of *Cox-2*, *IL-1β* and *IL-8* which coincides with other reports[92,99-101] of *IL-1β* and *IL-8* being regulated in response to various stimuli (like LPS or poly I:C). Holen & Espe et al.[90] also presented results showing HK leukocytes of *S. salar* cultured with 100 µg/mL LPS induced transcription of *Cox-2*, *IL-1β* and *IL-8*. Stenberg et al.[137] cultured salmon HK leukocytes with 100 µg/mL LPS for 24h and reported a significant upregulated expression of *Cox-2* and *IL-8*. In the study by Martins et

al.[131], *Cox-2*, *IL-1β* and *IL-8* were upregulated by LPS in both liver cells and HK leukocytes of *S. salar*. Presumably, *Cox-2* should be upregulated by LPS, something the results from the current study reinforces: *Cox-2* is in both isolated liver cells and HK leukocytes upregulated by LPS, upholding the genes status as a serviceable inflammation marker. In accordance to observed consensus, the present study also showed LPS significantly inducing transcription of *IL-1β* in isolated HK leukocytes, additionally affirming *IL-1β* as an applicable inflammation marker gene. Based on earlier the aforementioned studies[58,92,131,136], *IL-8* should be expected to be upregulated by LPS, but the current study resulted in no significant effects in either HK leukocytes or liver cells. The differences are not significant, probably due to a considerable variance in response between the individual fishes.

The pleiotropic type II interferon, *IFN γ*, is involved with adaptive immunity in fish[74] and has been identified in *S. salar*[103,105,106]. *IFN γ* is produced in response to specific viral, interleukin or antigen signals[74,104], and induced by other anti-viral genes like the *Mx* protein or *viperin*[107-109]. Zou et al.[105] identified *IFN γ* in *O. mykiss* and showed *in vitro* that *IFN γ* expression were induced in HK leukocytes cultured with 10 µg/mL and 100 µg/mL poly I:C after 4h stimulation. Chen et al.[138] observed increased mRNA levels in HK kidney and blood of grass carp (*Ctenopharyngodon idella*) infected with reovirus and stimulated by LPS and poly I:C. In the current study, poly I:C did not significantly upregulate expression of *IFN γ* in liver cells or HK leukocytes of *S. salar*, but as can be seen in fig. 3.3.10, there is a notable effect on HK leukocytes, although not significant. Martins et al.[131] also reported no significant effects from *IFN γ* in salmon liver cells and HK leukocytes. Given that the high variance of responses could be accredited to differences between individual fish, this should suggest, in accordance with other studies, that poly I:C functions as an inducer of the IFN system.

The pro-inflammatory cytokine *TNF α* is involved with several host immune responses[97] including inducing apoptosis, anti-microbial response, and macrophage respiratory burst activity and inhibiting intracellular pathogen replication[51,92,98]. Hong et al.[139] verified the key role of *TNF α* in the inflammatory cytokine network by exposing 4d-old primary macrophages from HK leukocytes of *O. mykiss* to 25 µg/mL LPS and 50 µg/mL poly I:C for 4h, 8h and 24h. Expression of *TNF α* were significantly increased after LPS and poly I:C stimulation, with LPS being more potent than poly I:C, but interestingly peaking at an earlier stage well below 4h, indicating *TNF α* as an early response gene[139]. Hong et al.[139] also showed that LPS significantly upregulated expression of *IL-1β* after both 30 min and 24h. In the results from the

current study, LPS significantly induced *TNF α* expression in both isolated liver cells and HK leukocytes, in accordance to the study by Martins et al.[131], wherein LPS inducing *TNF α* expression in salmon HK leukocytes at the same concentration and incubation time. Comparable results were also reported by Stenberg et al.[137] with LPS significantly inducing transcription of *TNF α* in salmon HK leukocytes with the same concentration and exposure time. This indicates the inflammatory role of *TNF α* in the inflammatory system, especially in response to bacterial stimulation.

4.1.2 Immunorelated metabolic genes

CYPs are metabolic detoxification enzymes capable of metabolizing substances like toxins and pharmaceuticals[86,87] with *CYP1a* suggested[87] to be involved with inflammation and bacterial defense signaling. *SSAT* is a metabolic rate-limiting enzyme involved in regulation of polyamine homeostasis[88] and has been shown to participate in crosstalk with other signaling pathways in *B. rerio*[89] and *S. salar*[90]. It has been suggested[90,140] that polyamines affect transcription of *SSAT*, indicating its important anti-inflammatory functions in salmon. As the metabolic detoxification enzyme *CYP1a* and rate-limiting enzyme *SSAT* are suggested[86,87,90,140] to be involved with crosstalk between cell types and related to the immune response of fish, a significant response from immunostimulants LPS and poly I:C should be expected. Holen et al.[58] and Holen & Olsvik[87,136] reported a significant upregulation of *CYP1a* expression in *G. morhua* HK leukocytes cultured with LPS (32.5-fold) and a significant upregulation of *CYP1a1*, but no significant effect from poly I:C. For the *SSAT* gene, Holen & Espe et al.[90] reported no significant transcription in liver cells or immune cells of *S. salar* cultured with LPS. In the study by Martins et al.[131] LPS poly I:C did not induce significant effects of *SSAT* in salmon liver cells and HK leukocytes. The current study revealed no significant expression of *CYP1a* or *SSAT* in neither liver cells nor HK leukocytes. With no significant effects, this could hint at a lesser role of detoxification and metabolic enzymes in the immune response of *S. salar*, but the high variance of difference can also possibly be accredited to the responses of individual fish.

4.1.3 Oxidative stress related genes

Oxidative stress within tissue or cells elicits reactions from the fish immune system, profoundly affecting fish health, which in turn makes antioxidants key health-benefiters. Reactive oxygen species are byproducts generated from metabolic processes utilizing oxygen and exerting physiological actions[68]. OS often occurs when there is an imbalance between increased

production of ROS and reduction in the antioxidant defense against ROS [67,68]. To keep cell oxidizing levels in check, major antioxidant enzymes like *catalase* is used as regulators, e.g. catalyzing the conversion of H_2O_2 to water and molecular oxygen[68]. *Bcl-2* proteins in mammals functions as regulators of the intrinsic apoptotic cell death cascade when under OS and has shown antioxidant-like properties[69][70]. As the OS related genes are induced by ROS it should be possible to measure OS in fish[71]. Martins et al.[131] did not report any significant effects of *Bcl-2* or *catalase* in either salmon liver cells or HK leukocytes. Interestingly, results from the current study showed that *Bcl-2* was downregulated by poly I:C in both isolated liver cells and HK leukocytes, while *catalase* was not significantly affected regardless of treatment or cell type. Holen & Olsvik[87] reported a significant down regulation of *catalase* in HK leukocytes of *G. morhua* exposed to 100 µg/mL LPS, which, together with the down regulation of *Bcl-2* in this study, might indicate that the reduced expression of the OS related genes is caused by lower production of ROS when the cells are exposed to LPS or poly I:C[87]. The results presented in the current study may reinforce the idea of the effects of poly I:C impact on salmon cellular stress response.

4.1.4 Apoptotic related genes

Caspase3 is an apoptotic related enzyme involved in inflammation regulation through pathogen-associated molecular patterns and is essential for apoptosis signaling networks, and in the last stage of apoptosis[56,60-62,140]. *Caspase3* have been shown to play important parts in apoptotic signal pathway in *B. rerio*[141] and large yellow croaker (*Pseudosciaena crocea*)[142] and the responses of *caspase3* to viral infections have also been reported[143]. Li et al.[144] cultured HK macrophages from Japanese flounder (*Paralichthys olivaceus*) *in vitro* overnight with 20 µg/mL LPS or poly I:C for 4h, 8h, 12h, 24h, 36h or 48h. *Caspase3* was reported[144] to be upregulated (1.8-fold) at 24h and 48h after LPS and poly I:C exposure, respectively. Martins et al.[131] reported a significant down regulation of *caspase3* induced by LPS in HK leukocytes of *S. salar*, but no significant effects from poly I:C in either liver cells or HK leukocytes. In the current study, transcription of *caspase3* was significantly upregulated in HK leukocytes cultured with poly I:C, in accordance to previous reports of the antiviral involvement of *caspase3*, but interestingly differing from the study by Martins et al.[131].

4.1.5 Antiviral response genes

As antiviral response genes[58,107-109], it is likely to assume that poly I:C would have significant effects on *Mx*, *TLR3* and *viperin*. The antiviral activity of *IFN γ* is suggested[107,108] to be

induced by upregulation of the antiviral *Mx* protein and *viperin*. The antiviral genes are involved with inhibiting replication of several virus types[74,107,109,110], in which *viperin* has been shown[110] to have comparable poly I:C induction properties to the likes of *IFN γ* in *G. morhua*. Zhou, Zhang & Sun[145] studied the effects of 20 µg/mL poly I:C for 24h on HK leukocytes of *P. olivaceus in vivo* and reported that the expression levels of *Mx* were comparable and significantly higher than those in untreated control fish. In the study by Martins et al.[131], their results show poly I:C inducing a significant upregulated transcription of *Mx* in salmon HK leukocytes, and *viperin* in both salmon liver cells and HK leukocytes, strongly indicating *Mx* and *viperin* as effective antiviral marker genes. As for the results in the present study, poly I:C had the exact same effects on *Mx* and *viperin* in salmon liver cells and HK leukocytes[131]: *Mx* in HK leukocytes cultured with poly I:C was significantly upregulated, displaying the antiviral properties of the *Mx* protein in accordance with Martins et al.[131], while *viperin* was significantly induced by poly I:C in both isolated liver cells and HK leukocytes. This further solidifies the role of *Mx* and *viperin* in antiviral responses in teleost immune responses.

Another antiviral-involved gene is *TLR3*, a toll-like receptor protein expressed in the membrane of B-cells, macrophages and dendritic cells, participating in inflammatory responses by binding to viral RNA through pathogen-associated molecular patterns[58,74]. Abóz et al.[135] found that *TLR3* in *O. mykiss* IgM^+ cells were significantly upregulated in response to being exposed 50 µg/mL poly I:C and a rhabdovirus after 24h and 48h of incubation. The same concentration of poly I:C has also been shown[58] to significantly induce transcription of *TLR3* in HK leukocytes of *G. morhua*. It should be expected that poly I:C induces *TLR3* transcription, as the intracellular toll-like receptor is involved with cellular antiviral responses and poly I:C is a synthetic double stranded RNA[58,74,131]. Both Martins et al.[131] and Stenberg et al.[137] reported a significant upregulation of *TLR3* in salmon HK leukocytes cultured with the same concentration poly I:C with the same exposure time, supporting the consensus of the antiviral properties of *TLR3*. In the results of the current study, *TLR3* is upregulated in both liver cells and HK leukocytes cultured with poly I:C, although not significantly, indicating the antiviral properties associated with *TLR3*. With no significant differences from the expected *TLR3* transcription-inducing poly I:C, this result is probably due to a too substantial SD between the samples.

4.2 Effects of trichlorfon on metabolic and immune gene transcriptions

Atlantic salmon in commercial aquaculture are kept in closed cages with high fish densities, exposing them to a higher level of pressure and spreading of bacterial, viral and parasitic infections. Stress inducing procedures like pharmaceutical treatments compromises the immune system of the fish, affecting their defense response against potential pathogenic agents, which in turn could make the fish weaker and more receptive for additional infections and diseases. Furthermore, the pharmaceuticals used for treatment could also have direct negative effects on the fish.

In this study, isolated liver cells and HK leukocytes were incubated *in vitro* for 48h at 9°C, cultured with TCF in concentrations of 25 µM, 10 µM and 1 µM, with untreated cultures included as controls for comparison (fig. 3.1-3.5). LPS and poly I:C were added after 24h, halfway through the incubation period. The results show no significant up- or down regulations of any genes in either isolated liver cells or HK leukocytes cultured with any concentration of TCF.

Multiple reports[33,118] have shown negative effects from TCF on fish, exhibiting toxicity in species like *O. niloticus*[111,121], *P. mesopotamicus*[112], *C. carpio*[113-115,119,120], *C. gibelio*[116,117,146], *R. quelen*[122,123], *D. labrax*[124], *O. mykiss*[127], *B. rerio*[33,127] and striped catfish (*Pangasionodon hypophthalmus*)[147]. In *S. salar*, the toxicity of the TCF similar organophosphate CLP-m has been shown in juveniles[128] and post-smolts[129], which could suggest that TCF would also have negative effects on salmon. Experiments with the toxic effects of TCF on *S. salar* has to the best of my knowledge only been reported once previously by Brandal & Egidius[17], who observed blindness in farmed salmon treated orally with TCF, which in turn prompted the change to apply TCF as bath treatments.

4.2.1 Inflammatory marker genes

Exposure to TCF alone in isolated liver cells and HK leukocytes did not significantly affect any of the inflammatory genes *CD83*, *Cox-2*, *IL-1β*, *IL-8*, *IFN γ* and *TNF α* in this study, suggesting that the concentrations of TCF used might be too low. Significant effects of LPS or poly I:C cultured together with TCF in any concentration could most possibly be attributed to antibacterial or antiviral responses.

4.2.2 Immunorelated metabolic genes

As CYPs are involved with metabolizing toxins and pharmaceuticals[86,87], the detoxification enzyme *CYP1a* should be expected to be significantly affected, especially in liver cells, in response to the toxicity of TCF. Woo et al.[114] exposed *C. carpio* to TCF *in vivo* applying TCF in concentrations of 0, 0.5, 1.0, 2,0 and 4.0 mg/L at 15°C and 25°C, resetting the concentrations every two days to maintain a constant exposure period, for 14 days. In their study, Woo et al.[114] observed that TCF and low temperature stress induced significant increases in the mRNA expression of *CYP1a* in the liver, indicating the cytotoxic effects of TCF on hepatocytes. Sinha et al.[147] characterized expression of *CYP1b* in liver of *P. hypophthalmus*, with TCF in concentrations of 0.01, 0.1 and 0.5 mg/L for 6h, 24h, 96h, 7 days, 14 days, 28 days and 56 days. Results from their[147] study showed a significant effect on *CYP1b* after 7 & 14 days with concentrations of 0.1 and 0.5 mg/L. Although not the same gene as *CYP1a*, *CYP1b* is part of the same family of cytochromes and can similarly be utilized as an indicator for toxicity of TCF in fish. In the present study, *CYP1a* and *SSAT* was not significantly affected in either liver cells or HK leukocytes, however a small upregulation of *CYP1a* cultured with TCF in concentrations of 25 µM in both cell types could indicate a possible cytotoxic effect of TCF in *S. salar*. *SSAT* was upregulated in both liver cells and HK leukocytes, implying that TCF did have a small impact on metabolic immune response. The lack of significant effects of TCF on *CYP1a* and *SSAT* in the present study may be attributed to a too low TCF concentration.

4.2.3 Oxidative stress related genes

As negative effects of TCF on OS in other species of fish have been reported[119-123], the oxidative stress related genes *Bcl-2* and *catalase* should be expected to be significantly affected by exposure to TCF. Xu et al.[117] reported a significant increase of *catalase* activity in hepatocytes of fish treated with 1 mg/L TCF, while *catalase* activity being significantly reduced with 0.5 mg/L TCF. The increased *catalase* activity with 1 mg/L could indicate the role of *catalase* in converting reactive oxygen species to less reactive species[117]. Considering the significant decreased *catalase* activity in the 0.5 mg/L treatment, Xu et al.[117] hypothesizes that this occurrence was a consequence of a corresponding reaction between antioxidative enzymes. Lu et al.[146] studied the effects of TCF on tissue metabolism and hepatotoxicity in *C. gibelio*, which was subjected to oral treatment of TCF in concentrations of 0.5, 1 and 2 g/kg and sampled at 1, 2, 3, 4, 8, 12, 24, 48, 72 and 96h after oral drug administration. In liver tissues, *catalase* activity was inhibited in a dose-dependent manner[146]: at 0.5 g/kg TCF *catalase* activity was significantly reduced at 48h; at 1 g/kg TCF *catalase* activity was reduced

at 24h; and at 2 g/kg TCF *catalase* activity was reduced from 12h. *Catalase* activity was also shown to be inhibited in liver of *B. rerio* by Coelho et al.[33] after exposure to 5, 10 and 20 mg/L TCF. These decreases in *catalase* activity could indicate negative effects on the antioxidant system in fish exposed to TCF. Compared to the results from the present study, *catalase* was not significantly affected in either liver cells or HK leukocytes, although a small upregulation of *catalase* in HK leukocytes, could indicate that TCF affected *catalase* activity and OS. *Bcl-2* was not significantly affected in cells cultured with TCF alone, but a small upregulation in liver cells cultured with 25 µM & 10 µM TCF alone could further imply that TCF had some effect on OS and possibly triggering the anti-apoptotic gene.

4.2.4 Apoptotic related genes

Hepatocyte apoptosis increased by TCF in *C. gibelio* was reported in an *in vitro* study by Xu et al.[116], where hepatocytes was cultured with 0, 0.01, 0.1 and 1 mg/L TCF at 25°C for 24h. Their[116] results showed that TCF induced a significant increase in hepatocyte apoptosis in cells cultured with 0.01, 0.1 and 1 mg/L TCF. TCF also increased hepatocyte *caspase3* activity in cells cultured with 0.01, 0.1 and 1 mg/L TCF, triggering hepatocyte apoptosis[116]. Xu et al.[117] also exposed *C. gibelio* to TCF *in vivo*, using concentrations of 0, 0.5, 1, 2 and 4 mg/L TCF dosed in pellets for 30 days. Apoptosis rate of hepatocytes was significantly increased in hepatocytes of fish treated with 1, 2 and 4 mg/L TCF, showing similar results of apoptosis in *C. gibelio* hepatocytes as the *in vitro* study by Xu et al.[116]. In the present study, TCF alone had no significant effect on *caspase3* in either liver cells or HK leukocytes. Applying a higher concentration of TCF should be considered in future studies to potentially observe apoptotic effects in *S. salar*.

4.2.5 Antiviral response genes

Exposure to TCF alone in isolated liver cells and HK leukocytes did not significantly affect any of the antiviral response genes *Mx*, *TLR3* or *viperin* in this study, suggesting that the concentrations of TCF used could be too low, or that TCF simply does not inhibit or increase antiviral responses in *S. salar*. Significant effects of poly I:C cultured together with TCF should be attributed to antiviral responses, as TCF in any concentration together with poly I:C did not show significant difference from each other, in either liver cells or HK leukocytes.

Conclusion

- The results from this study further solidifies the *in vitro* model utilized here to examine the effects of LPS and poly I:C on specific genes involved in metabolic and inflammatory responses of *S. salar*, to be applicable in observing the effects of simulated bacterial and viral infections, respectively, on genes in liver cells and HK leukocytes.

- This study does not confirm the effects of TCF in concentrations applied in this study on the metabolic and inflammatory response genes of *S. salar*. However, the results could be viewed as an indication of the toxicity of TCF and other organophosphates with similar toxic attributes, as toxic effects have been reported in other species of fish.

- As there were no significant results from TCF on any of the genes in this study, this study will not be able to provide solidification regarding the toxicity of TCF on non-target organisms in proximity of salmon fish farms, although there have been reports of the toxicity of TCF and other pharmaceutical organophosphates on non-target species.

Future perspectives

- Future studies should examine the effects of TCF on metabolic and inflammatory immune responses of *S. salar* in higher concentrations *in vitro* and consider experimenting with the effects of TCF on *S. salar in vivo*. External factors like temperature and salinity of the fish environment should also be taken more into consideration, in regard to the possible interactions these factors might have with the toxicity of TCF.

- In future studies involving the toxic effects of TCF on *S. salar*, a bigger focus should also be placed on specific correlations in metabolic and inflammatory gene response pathways, such as specific inflammation pathways, AChE activity, acute phase response, hematopoietic functions and oxidative stress response.

- In this study, no experiments were conducted to examine the effect of TCF *in situ* or *in vivo* with *S. salar* infected with *L. salmonis*. This is something that should be investigated further, not only to observe the effectiveness of TCF as an OPP against salmon lice, but also to detect any possible TCF resistance in *L. salmonis*.

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6. Appendix

Table 6.1: Body weight and sex of fish, sampled.

Sample #	Mass (g)	Sex
F^{1}	174.0	M
F^{2}	327.0	F
F^{3}	214.2	F
F^{4}	404.6	M
F^{5}	287.3	F
F^{6}	398.0	M
F^{7}	248.0	F
F^{8}	314.7	F
F^{9}	304.7	F
F^{10}	214.1	F
F^{11}	384.0	M

Table 6.2: Solutions used for cell isolation.

Solution	Content
Stock perfusion medium (SPM)	141.9 g NaCl
	2.5 g KCl
	12 g Hepes buffer
	500 mL ddH_2O, pH 7.4
Perfusion medium with EDTA	20 mL SPM
	2.22 g EDTA disodium salt dihydrate
	360 mL ddH_2O, pH 7.4
Perfusion medium with collagenase	10 mL SPM
	90 mL ddH_2O, pH 7.4
	100 µL 1 M $CaCl_2$
	100 mg Collagenase
1.5 M NaCl	87.6 g NaCl
	ddH_2O until 1.0 L
Stock Isotonic Percoll (SIP)	1 part 1.5 M NaCl
	9 parts Percoll
Percoll-gradient solution, density 1.08 g/mL	*50 mL*
Approx. 51% Percoll	28.5 mL SIP
	21.4 mL L-15 media
Complete L-15 medium for cell culture	*500 mL*
10% Foetal Bovine Serum (FBS)	440 mL Leibovitz, L-15
	50 mL FBS
1% Glutamax	5 mL Glutamax (10x)

1% Antibiotic Antimycotic	5 mL Antibiotic Antimycotic (10x)
Isolation buffer	9 g NaCl
	7 g EDTA
	1 L ddH_2O, pH 7.2
Phosphate-buffered saline buffer (PBS)	0.002 M KH_2PO_4
	0.02 M Na_2HPO_4
	0.03 M KCl
	0.14 M NaCl, pH 7.4

Table 6.3: The cell culture wells for each fish with cell type, culture conditions, TCF (25 µM + 10 µM + 1 µM), LPS (100 µg/mL) and Poly I:C (50 µg/mL) additions.

Treatment #	Liver cells	Head Kidney cells
1	K	K
2	LPS	LPS
3	25 µM TCF	25 µM TCF
4	10 µM TCF	10 µM TCF
5	1 µM TCF	1 µM TCF
6	LPS + 25 µM TCF	LPS + 25 µM TCF
7	LPS + 10 µM TCF	LPS + 10 µM TCF
8	LPS + 1 µM TCF	LPS + 1 µM TCF
9	Poly I:C	Poly I:C
10	Poly I:C + 25 µM TCF	Poly I:C + 25 µM TCF
11	Poly I:C + 10 µM TCF	Poly I:C + 10 µM TCF
12	Poly I:C + 1 µM TCF	Poly I:C + 1 µM TCF

Table 6.4: RIN values and [RNA] to appurtenant samples from figure A1-4 and treatments for isolated head kidney and liver cells.

	Sample #	Treatment	RIN	[RNA]
Head kidney	2019-536/26	LPS	9.3	351.58
	2019-536/28	10 µM TCF	9.2	298.01
	2019-536/31	LPS + 10 µM TCF	9.0	342.02
	2019-536/34	Poly I:C + 25 µM TCF	9.3	310.90
	2019-536/37	K	8.6	364.49
	2019-536/40	10 µM TCF	8.2	329.82
	2019-536/43	LPS + 10 µM TCF	9.9	195.48
	2019-536/48	Poly I:C + 1 µM TCF	9.7	295.02
	2019-536/54	LPS + 25 µM TCF	9.3	1160.45
	2019-536/56	LPS + 1 µM TCF	9.6	603.37
	2019-536/59	Poly I:C + 10 µM TCF	8.8	950.91
	2019-536/61	K	8.9	592.84

	2019-536/63	25 µM TCF	9.2	594.27
	2019-536/66	LPS + 25 µM TCF	9.1	588.06
	2019-536/68	LPS + 1 µM TCF	8.8	580.65
	2019-536/70	Poly I:C + 25 µM TCF	8.2	752.40
	2019-573/1	K	9.6	574.02
	2019-573/3	25 µM TCF	9.1	459.72
	2019-573/5	1 µM TCF	10	337.87
	2019-573/8	Poly I:C + 1 µM TCF	9.9	312.51
	2019-573/12	10 µM TCF	7.7	224.78
	2019-573/15	Poly I:C + 10 µM TCF	9.6	104.20
	2019-537/1	K	9.7	1722.55
	2019-537/5	1 µM TCF	9.8	1438.76
Liver	2019-537/9	Poly I:C	9.6	1420.60
	2019-537/12	Poly I:C + 1 µM TCF	9.8	1207.25
	2019-537/16	10 µM TCF	9.5	1248.31
	2019-537/25	K	9.6	1161.80
	2019-537/34	Poly I:C + 25 µM TCF	9.6	1181.87
	2019-537/39	25 µM TCF	9.2	1346.51
	2019-537/44	LPS + 1 µM TCF	9.3	1577.46
	2019-537/49	K	9.3	1532.68
	2019-537/58	Poly I:C + 25 µM TCF	9.1	1300.81
	2019-537/67	LPS + 10 µM TCF	9.0	1711.53

Table 6.5: Reaction mix for RT-PCR.

	Reagents	Volume (30 µL)	Concentration
Non enzymatic reagents	ddH_2O	1.3	
	10x TaqMan RT buffer	3.0	1x
	25 mM $MgCl_2$	6.6	5.5 mM
	10 mM dNTP mix	6.0	500 µM per dNTP
	50 µM oligo $d(T)_{16}$	1.5	2.5 µM
Enzymes	RNase inhibitor (20 U/µL)	0.6	0.4 U/µL
	Multiscribe Reverse Transcriptase (50 U/µL)	1.0	1.67 U/µL

Table 6.6: RT-PCR conditions.

Reaction program steps	Temperature (°C)	Time (min:sec)
Incubation	25	10:00
RT	48	60:00
RT Inactivation	95	5:00
End	4	-

Table 6.7: PCR primer sequences, functions and accession numbers.

Gene	Forward primer (5' – 3') Reverse primer (5' – 3')	Function	Accession no.
ARP	GAAAATCATCCAATGCTGGATG CTTCCCACGCAAGGACAGA	Reference gene	AY255630
β-actin	CCAAAGCCAACAGGGAGAA ACGAGCTAGAAGCGGTTTCG	Reference gene	BG933897
EF1α	TGCCCCTCCAGGATGTCTAC CAGCGTGATAGACTCGTTC	Reference gene	AF321836
RPL13	CCAATGTACAGCGCCTGAAA CGTGGCCATCTTGAGTTCCT	Reference gene	NM_001141291
Bcl-2	TGACAGATTTCATCTACGAGCGG GCCATCCAGCTCATCTCCAATC	Anti-apoptotic gene	NM_001141086
Caspase3	ACAGCAAAGAGCTAGAGGTCCAACAC AAAGCCAGGAGACTTTGACGCAG	Apoptotic gene	DQ008070
Catalase	CCAGATGTGGGCCGCTAACAA TCTGGCGCTCCTCCTCATTC	Stress response	Est04a09
CD83	CAAACTGGTCCAGACAGGGT CAGCGTGATAGACTCGTTC	Dendritic marker	DQ339141
Cox-2	GGAGGCCTACTCCAACCTATT CGAACATGAGATTGGAACC	Eicosanoid pathway	AY848944
CYP1a	TGGAGATCTTCCGGCACTCT CAGGTGTCCTTGGGAATGGA	Detoxification enzyme	AF364076
IFN γ	AAGGCGGTCTCGTTAAGT GCGGCATTACTCCATCCTAA	Cytokine pathway	AJ84811
IL-1β	GTATCCCATCACCCCATCAC GCAAGAAGTTGAGCAGGC	Cytokine pathway	NM001123582
IL-8	GAGCGGTCAGGAGATTTGTC TTGGCCAGCATCTTCTCAAT	Cytokine pathway	NM_001140710
Mx	TGCCATGCAACGTTGACATTG GCCTAATGTCCTTTCCCCTTCAG	Viral marker	NM_001165344.1
SSAT	TCGTGGCGGAAGTCCCCAGT GCCGATGCCAAACCCCCTGT	Protein coding gene	NM_00297.02
TLR3	GTTTCATGGTCAATTACAGTAGG TGGTTAATGAGTGCAATAGTGG	Toll like receptor	CB499949
TNF α	GGCGAGCATACCACTCCTCT TCGGACTCAGCATCACCGTA	Cytokine pathway	AY848945
Viperin	TCCTTGATGTTGGCGTGGAA GCATGTCAGCTTTGCTCCACA	Viral marker	NM_001140939

Table 6.8: SYBR® Green qPCR mix for Light Cycler 480.

Reagents	Volume per sample (µL)
ddH_2O	2.8
Forward primer (5' – 3') (50 µM)	0.1
Reverse primer (5' – 3') (50 µM)	0.1
TaqMan universal PCR Master Mix (2x) (SYBR® Green)	5

Table 6.9: qPCR SYBR® Green program.

Reaction program steps	Temperature (°C)	Time (min:sec)	Operation
Pre-incubation	95	05:00	Denaturation and activation of FastStart Taq DNA polymerase
Amplification			*45 cycles, 3 steps*
Denaturation	95	00:10	Separating DNA strands
Annealing	60	00:10	Primer binds to DNA strand
Elongation	72	00:10	Synthesis of double stranded DNA
Melting point analysis			*1 cycle, 3 steps*
Denaturation	95	00:10	
Annealing	65	01:00	
Melting	97		
Cooling	40	00:10	

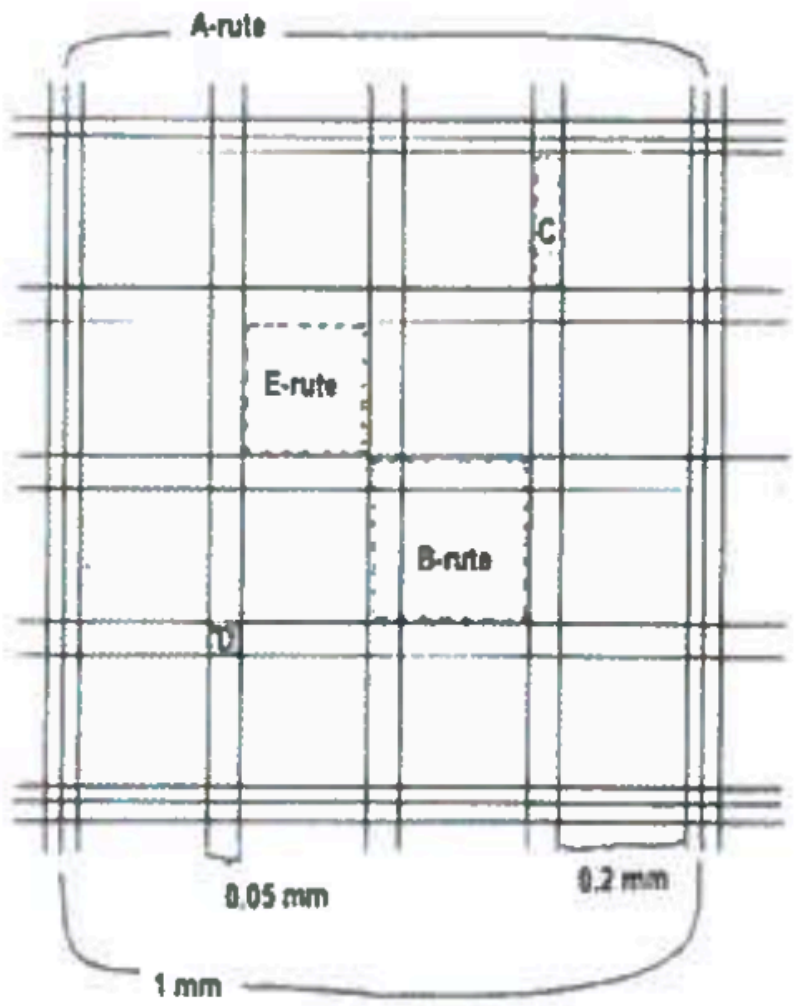

Figure 6.1: Sketch of an A-rute (A-square), in which a Bürker counting chamber consists of 9 A-squares on each side of the trench. Volume indications:

1 A-square:	1	mm^2	x	0.1 mm	=	0.1	mm^3.
1 B-square:	1/16	mm^2	x	0.1 mm	=	0.00625	mm^3.
1 C-square:	1/100	mm^2	x	0.1 mm	=	0.001	mm^3.
1 D-square:	1/400	mm^2	x	0.1 mm	=	0.00025	mm^3.
1 E-square:	1/25	mm^2	x	0.1 mm	=	0.004	mm^3.
1 C+D-square:	1/80	mm^2	x	0.1 mm	=	0.00125	mm^3.

	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve
A	1000 ng	1000 ng	1000 ng	500 ng	500 ng	500 ng	250 ng	250 ng	250 ng	125 ng	125 ng	125 ng
	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve	2019-537/1	2019-537/1	2019-537/2	2019-537/2	2019-537/3	2019-537/3
B	62.5 ng	62.5 ng	62.5 ng	31.25 ng	31.25 ng	31.25 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng
	2019-537/4	2019-537/4	2019-537/5	2019-537/5	2019-537/6	2019-537/6	2019-537/7	2019-537/7	2019-537/8	2019-537/8	2019-537/9	2019-537/9
C	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng
	2019-537/10	2019-537/10	2019-537/11	2019-537/11	2019-537/12	2019-537/12	2019-537/13	2019-537/13	2019-537/14	2019-537/14	2019-537/15	2019-537/15
D	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng
	2019-537/16	2019-537/16	2019-537/17	2019-537/17	2019-537/18	2019-537/18	2019-537/19	2019-537/19	2019-537/20	2019-537/20	2019-537/21	2019-537/21
E	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng
	2019-537/22	2019-537/22	2019-537/23	2019-537/23	2019-537/24	2019-537/24	2019-537/25	2019-537/25	2019-537/25	2019-537/25	2019-537/27	2019-537/27
F	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng
	2019-537/28	2019-537/28	2019-537/29	2019-537/29	2019-537/30	2019-537/30	2019-537/31	2019-537/31	2019-537/32	2019-537/32	2019-537/33	2019-537/33
G	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng
	2019-537/34	2019-537/34	2019-537/35	2019-537/35	2019-537/36	2019-537/36	2019-537/26	2019-537/26			500 ng	
H	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng			nac	ntc

Figure 6.2: 96-well PCR liver plate 1. Standard curve/dilution curve in triplicate from 1000-31.25 ng. 10 µL/well samples in wells with 20 µL/well RT-reaction mix in duplicate and two negative controls: nac (none amplification control), ntc (non-template control): no reverse transcriptase.

	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve
A	1000 ng	1000 ng	1000 ng	500 ng	500 ng	500 ng	250 ng	250 ng	250 ng	125 ng	125 ng	125 ng
	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve	Dilution Curve	2019-537/37	2019-537/37	2019-537/38	2019-537/38	2019-537/39	2019-537/39
B	62.5 ng	62.5 ng	62.5 ng	31.25 ng	31.25 ng	31.25 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng
	2019-537/40	2019-537/40	2019-537/41	2019-537/41	2019-537/43	2019-537/43	2019-537/44	2019-537/44	2019-537/45	2019-537/45	2019-537/46	2019-537/46
C	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng
	2019-537/47	2019-537/47	2019-537/48	2019-537/48	2019-537/49	2019-537/49	2019-537/50	2019-537/50	2019-537/51	2019-537/51	2019-537/52	2019-537/52
D	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng
	2019-537/53	2019-537/53	2019-537/54	2019-537/54	2019-537/55	2019-537/55	2019-537/56	2019-537/56	2019-537/57	2019-537/57	2019-537/58	2019-537/58
E	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng
	2019-537/59	2019-537/59	2019-537/60	2019-537/60	2019-537/61	2019-537/61	2019-537/62	2019-537/62	2019-537/63	2019-537/63	2019-537/64	2019-537/64
F	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng
	2019-537/65	2019-537/65	2019-537/66	2019-537/66	2019-537/67	2019-537/67	2019-537/68	2019-537/68	2019-537/69	2019-537/69	2019-537/70	2019-537/70
G	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng	500 ng
	2019-537/71	2019-537/71	2019-537/72	2019-537/72							500 ng	
H	500 ng	500 ng	500 ng	500 ng							nac	ntc

Figure 6.3: 96-well PCR liver plate 2. Standard curve/dilution curve in triplicate from 1000-31.25 ng. 10 µL/well samples in wells with 20 µL/well RT-reaction mix in duplicate and two negative controls: nac (none amplification control), ntc (non-template control): no reverse transcriptase.

A	Dilution Curve 1000 ng	Dilution Curve 1000 ng	Dilution Curve 1000 ng	Dilution Curve 500 ng	Dilution Curve 500 ng	Dilution Curve 500 ng	Dilution Curve 250 ng	Dilution Curve 250 ng	Dilution Curve 250 ng	Dilution Curve 125 ng	Dilution Curve 125 ng	Dilution Curve 125 ng
B	Dilution Curve 62.5 ng	Dilution Curve 62.5 ng	Dilution Curve 62.5 ng	Dilution Curve 31.25 ng	Dilution Curve 31.25 ng	Dilution Curve 31.25 ng	2019-573/1 500 ng	2019-573/1 500 ng	2019-573/2 500 ng	2019-573/2 500 ng	2019-573/3 500 ng	2019-573/3 500 ng
C	2019-573/4 500 ng	2019-573/4 500 ng	2019-573/5 500 ng	2019-573/5 500 ng	2019-573/7 500 ng	2019-573/7 500 ng	2019-573/8 500 ng	2019-573/8 500 ng	2019-573/9 500 ng	2019-573/9 500 ng	2019-573/10 500 ng	2019-573/10 500 ng
D	2019-573/11 500 ng	2019-573/11 500 ng	2019-573/12 500 ng	2019-573/12 500 ng	2019-573/14 500 ng	2019-573/14 500 ng	2019-573/15 500 ng	2019-573/15 500 ng	2019-536/1 500 ng	2019-536/1 500 ng	2019-536/2 500 ng	2019-536/2 500 ng
E	2019-536/3 500 ng	2019-536/3 500 ng	2019-536/4 500 ng	2019-536/4 500 ng	2019-536/6 500 ng	2019-536/6 500 ng	2019-536/7 500 ng	2019-536/7 500 ng	2019-536/8 500 ng	2019-536/8 500 ng	2019-536/13 500 ng	2019-536/13 500 ng
F	2019-536/14 500 ng	2019-536/14 500 ng	2019-536/16 500 ng	2019-536/16 500 ng	2019-536/17 500 ng	2019-536/17 500 ng	2019-536/18 500 ng	2019-536/18 500 ng	2019-536/19 500 ng	2019-536/19 500 ng	2019-536/20 500 ng	2019-536/20 500 ng
G	2019-536/25 500 ng	2019-536/25 500 ng	2019-536/26 500 ng	2019-536/26 500 ng	2019-536/28 500 ng	2019-536/28 500 ng	2019-536/29 500 ng	2019-536/29 500 ng	2019-536/30 500 ng	2019-536/30 500 ng	2019-536/31 500 ng	2019-536/31 500 ng
H	2019-536/33 500 ng	2019-536/33 500 ng	2019-536/34 500 ng	2019-536/34 500 ng	2019-536/36 500 ng	2019-536/36 500 ng	2019-536/37 500 ng	2019-536/37 500 ng	2019-536/38 500 ng	2019-536/38 500 ng	500 ng nac	ntc

Figure 6.4: 96-well PCR head kidney plate 1. Standard curve/dilution curve in triplicate from 1000-31.25 ng. 10 µL/well samples in wells with 20 µL/well RT-reaction mix in duplicate and two negative controls: nac (none amplification control), ntc (non-template control): no reverse transcriptase.

A	Dilution Curve 1000 ng	Dilution Curve 1000 ng	Dilution Curve 1000 ng	Dilution Curve 500 ng	Dilution Curve 500 ng	Dilution Curve 500 ng	Dilution Curve 250 ng	Dilution Curve 250 ng	Dilution Curve 250 ng	Dilution Curve 125 ng	Dilution Curve 125 ng	Dilution Curve 125 ng
B	Dilution Curve 62.5 ng	Dilution Curve 62.5 ng	Dilution Curve 62.5 ng	Dilution Curve 31.25 ng	Dilution Curve 31.25 ng	Dilution Curve 31.25 ng	2019-536/39 500 ng	2019-536/39 500 ng	2019-536/40 500 ng	2019-536/40 500 ng	2019-536/41 500 ng	2019-536/41 500 ng
C	2019-536/43 500 ng	2019-536/43 500 ng	2019-536/44 500 ng	2019-536/44 500 ng	2019-536/45 500 ng	2019-536/45 500 ng	2019-536/47 500 ng	2019-536/47 500 ng	2019-536/48 500 ng	2019-536/48 500 ng	2019-536/49 500 ng	2019-536/49 500 ng
D	2019-536/50 500 ng	2019-536/50 500 ng	2019-536/51 500 ng	2019-536/51 500 ng	2019-536/52 500 ng	2019-536/52 500 ng	2019-536/53 500 ng	2019-536/53 500 ng	2019-536/54 500 ng	2019-536/54 500 ng	2019-536/55 500 ng	2019-536/55 500 ng
E	2019-536/56 500 ng	2019-536/56 500 ng	2019-536/57 500 ng	2019-536/57 500 ng	2019-536/58 500 ng	2019-536/58 500 ng	2019-536/59 500 ng	2019-536/59 500 ng	2019-536/60 500 ng	2019-536/60 500 ng	2019-536/61 500 ng	2019-536/61 500 ng
F	2019-536/62 500 ng	2019-536/62 500 ng	2019-536/63 500 ng	2019-536/63 500 ng	2019-536/64 500 ng	2019-536/64 500 ng	2019-536/65 500 ng	2019-536/65 500 ng	2019-536/66 500 ng	2019-536/66 500 ng	2019-536/67 500 ng	2019-536/67 500 ng
G	2019-536/68 500 ng	2019-536/68 500 ng	2019-536/69 500 ng	2019-536/69 500 ng	2019-536/70 500 ng	2019-536/70 500 ng	2019-536/71 500 ng	2019-536/71 500 ng	2019-536/72 500 ng	2019-536/72 500 ng	500 ng nac	ntc
H												

Figure 6.5: 96-well PCR head kidney plate 2. Standard curve/dilution curve in triplicate from 1000-31.25 ng. 10 µL/well samples in wells with 20 µL/well RT-reaction mix in duplicate and two negative controls: nac (none amplification control), ntc (non-template control): no reverse transcriptase.