Fish health and fish quality

Effects of tissue regeneration from a molecular perspective

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Cover: front: eye of brown trout, *Salmo trutta*; back: collagen molecule

Fish health and fish quality

Effects of tissue regeneration from a molecular perspective

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The thesis submitted for the degree of Doctor of Philosophy is based on a literature review and 3 scientific publications

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Accompanying papers

Paper I:

Ingerslev H.C., Hyldig G., Przybylska D, Frosch S. & M.E. Nielsen. Previous bacterial infections affect the textural quality of the fillet in rainbow trout (*Oncorhynchus mykiss*). Submitted to Aquaculture. Pages 1-18.

Paper II:

Ingerslev H.C., Ossum C.G., Lindenstrøm T. & M.E. Nielsen (2010). Fibroblasts express immune relevant genes and are important sentinel cells during tissue damage in rainbow trout (*Oncorhynchus mykiss*). PLoS ONE, 5(2), 1-9.

Paper III:

Ingerslev H.C., Lunder T. & M.E. Nielsen (2010). Inflammatory and regenerative responses in salmonids following mechanical tissue damage and natural infection. Fish & Shellfish Immunology. Pages 1-11.

Summary

Aquaculture is an expanding worldwide industry producing an increasing amount of fish every year. The quality of the fish meat is dependent upon many biological and non-biological factors. Infectious diseases are known to cause bleedings and damage of the muscle tissue that may lead to scarring after recovery, which possibly will affect some quality parameters. However, until now it has not been examined if previous infections have an impact on the sensory characteristics of the meat after slaughter. Further, the underlying molecular mechanisms involved in regeneration of muscle tissue are poorly described in fish. The present work in this thesis focused on: 1) examination of potential changes in the quality regarding texture of the muscle tissue in rainbow trout (*Oncorhynchus mykiss*) after previous infection with the bacterial pathogens *Yersinia ruckeri* and *Vibrio anguillarum*; 2) characterisation of potential immune functions of fibroblasts and the importance of this in relation to tissue regeneration; 3) creation of a model to study local, sterile tissue damage in the muscle tissue of rainbow trout and comparison of this to infection of Atlantic salmon (*Salmo salar*) by the bacterium *Moritella viscosa*, the causative agent of 'winter ulcer' in Norway.

In order to reach these objectives, sensory analysis and ELISA was used. Further, quantitative real-time RT-PCR was used in order to measure the expression of genes coding for immunological factors and tissue regeneration.

The results of these studies showed that previous infections by *Yersinia ruckeri* and *Vibrio anguillarum* gave rise to subsequent changes regarding textural quality parameters in fresh fish meat, while no differences were seen for cold-smoked meat from the same fish. The texture in previous infected fish was less flaky and less oily, but had a higher toughness and fibrousness in comparison to control fish. These observations could be explained from the molecular studies. Herein, local inflammatory responses characterised by up-regulation of IL-1β and IL-8 was seen in mechanically damaged and *M. viscosa* infected fish. However, this response was much stronger in infected versus damaged fish, indicating that damaged cells initiate an immune response, but pathogenic triggering was much more potent. A further activation of the genes TGF-β, MMP-2, CTGF and myostatin-1αβ was then seen in both groups, indicating initiation of tissue regeneration. Likely as a consequence of heavier tissue damage in infected fish, the collagen-1α gene was induced in this group. Scarring or fibrosis is equal to deposition of collagen in repaired tissue. Hence, these data may explain the sensory observations from previously infected fish. Finally, the fibroblast cell-line RTHDF from rainbow trout was found to be an immune competent cell type. This was

examined since a fast up-regulation of IL-1β and IL-8 shortly after tissue damage was observed in muscle tissue from rainbow trout. These observations led to the suggestion that local resident cells in the muscle tissue are the first to initiate an inflammatory response following tissue damage. The RTHDF cell-line was found to be responsive to LPS from the surface of gram-negative bacteria, but also from damaged RTHDF cells. Hence, the data supported that theory.

Sammendrag

Akvakultur er en verdensomspændende industri, der producerer en stigende mængde fisk årligt. Kvaliteten af fiskens kød er afhængig af mange biologiske og ikke-biologiske faktorer. Infektiøse sygdomme er kendt for at kunne give blødninger og vævs ødelæggelser i muskulaturen, der kan medføre arvævsdannelse efter at fisken er kommet sig over sygdommen. Dette vil sandsynligvis efterfølgende påvirke nogle kvalitetsparametre i kødet. Indtil nu er der ikke foretaget nogle undersøgelser, der har påvist om tidligere sygdomshistorik har en efterfølgende indflydelse på sensoriske karakteristika på kødet efter slagtning. Endvidere er de molekylære mekanismer, der er involveret i regenerering af muskelvæv, kun beskrevet i ringe grad i fisk. Dette arbejde har haft til hensigt at: 1) undersøge om der kan påvises teksturmæssige kvalitetsændringer i muskelvæv fra regnbueørred (*Oncorhynchus mykiss*) som følge af tidligere infektioner med de bakterielle patogener *Yersinia ruckeri* og *Vibrio anguillarum*; 2) karakterisere potentielle immunologiske egenskaber af fibroblaster og vigtigheden af dette i relation til vævs regenerering; 3) udvikling af en model til at studere lokal, steril vævs ødelæggelse af muskulaturen i regnbueørred og sammenligning af denne med en infektion med bakterien *Moritella viscosa* i Atlantisk laks (*Salmo salar*). Bakterien forårsager det, der populært kaldes 'vintersår' i norsk opdrættede fisk.

For at opnå disse målsætninger, blev der anvendt sensoriske analyser og ELISA. Endvidere blev kvantitativ real-time RT-PCR anvendt til at måle ekspressionen af gener, der koder for immunologiske faktorer og vævs regenerering.

Resultaterne af disse studier viste at forhenværende infektioner med *Yersinia ruckeri* og *Vibrio anguillarum* resulterede i efterfølgende kvalitetsmæssige ændringer hvad angik teksturmæssige parametre i ferskt kød, men ikke i koldrøget kød fra samme fisk. Teksturen i forhenværende inficerede fisk var mindre flaget, mindre olieret, men mere sej og fibrøs i forhold til kontrolfisk. Disse resultater kan forklares ved det, der blev fundet i de molekylære studier. I disse blev der efter mekanisk skade og infektion med *M. viscosa* fundet en lokal, inflammatoriske reaktion karakteriseret ved opregulering af IL-1β og IL-8. I modsætning til mekanisk skadede fisk, var denne reaktion langt kraftigere i inficerede fisk. Dette viser, at skadede og ødelagte celler er i stand til at initiere et immunrespons, men at patogener er mere potente i forhold til at aktivere dette respons. En efterfølgende aktivering af generne TGF-β, MMP-2, CTGF og myostatin-1αβ blev observeret i begge grupper af fisk, hvilket indikerer en begyndelse på vævs regenerering. Sandsynligvis som en konsekvens af en voldsommere vævs

ødelæggelse i inficerede fisk, var collagen-1α genet induceret i denne gruppe. Arvævsdannelse eller fibrose er ensbetydende med en akkumulering af collagen i repareret væv. På baggrund af dette kan disse data muligvis forklare de sensoriske observationer i fisk, der tidligere har været syge. Endvidere blev det fundet, at fibroblast cellelinjen RTHDF fra regnbueørred viste sig at være en immun kompetent celletype. Dette blev undersøgt på baggrund af observationer som følge af mekanisk vævs ødelæggelse, hvor opregulering af IL-1β og IL-8 blev målt kort tid herefter i muskelvæv fra regnbueørred. Disse observationer førte til den idé, at lokalt forekommende celler i muskelvævet er de første til at initiere et inflammatorisk respons som følge af vævs ødelæggelse. RTHDF cellelinjen blev fundet at respondere på LPS fra overfladen på gramnegative bakterier, men også i forhold til strukturer fra ødelagte RTHDF celler. Disse resultater var med til at styrke den teori.

1. General introduction

Aquaculture is an expanding worldwide industry and the amount of produced fish and number of species is increasing every year. Since 1970, the production has annually increased by almost 9% and fish farming is the fastest growing food industry worldwide. Today, the sector accounts for about half of the world's food fish (FAO, 2008). Some highly economical important species are the salmonids rainbow trout (*Oncorhynchus mykiss*) and Atlantic salmon (*Salmo salar*), which are mainly produced in Europe and America. During production of these species in aquaculture, occasionally it happens that they become infected or mechanical damaged due to e.g. high stocking densities and handling. Many of the pathogens causing these infections harm the fish by giving rise to bleedings in the musculature and these have subsequently been suggested as associated with quality changes at slaughter. Those subjects and tissue regeneration in fish in general, have so far not been examined very intensive. In this work we aim to examine these issues in the muscle tissue of rainbow trout and Atlantic salmon. In the following, the term fillet will be used when relating the muscle tissue to food. Firstly, previously infected rainbow trout reared under farming conditions will be examined using sensory analysis in a search for textural changes in the muscle tissue following disease. Further, tissue regeneration in a molecular perspective will be studied *in vivo* by gene expression in mechanical damaged and infected salmonids and *in vitro* in fibroblasts.

1.1 What is (fish) quality?

Defining quality is complicated because it covers many aspects and can be considered from many perspectives such as the product itself, processing and the consumer (Meiselman, 2001). In this study, the product is the fish fillet. Recently, quality of food has been described as a nominal hierarchy of interrelated levels, which contain properties that can be measured (Bremner, 2000). Measurable parameters of the product can be sensory parameters, chemical composition, physical properties, level of microbial and toxicological contaminants (Molnar, 1995). Some of these parameters have been shown to be under influence from welfare of the fish, stress at slaughter, the fat content and the composition of this, texture, content of connective tissue, coloration and discoloration from bloodstains, marbles and melanin (Aubourg et al., 2002; Lakshmisha et al., 2008; Ozbay et al., 2006; Riebroy et al., 2007; Rodriguez et al., 2007; Torstensen et al., 2005; Waagbo et al., 1993). Those parameters may be considered as biological factors with pre-harvest influence on the post-harvest quality. Post-harvest

factors with influence on the quality are among others processing procedures, storage temperature, thawing procedures and length of storage. These factors influence parameters like water content, firmness and taste of the fillet.

1.2 Diseases and quality

As mentioned previously, the quality of the fish fillet is under influence of many different factors. Several reports from the field indicate changes in the tissue structure of the fillet following disease in infected or previously infected fish, which may influence the quality (Lunder et al., 1995). Many different bacterial pathogens give rise to bleedings in the skin and musculature, which in some cases influences the later tissue architecture. As an example, the gram-negative bacterium *Moritella viscosa* causes scarring in the fillet in seawater leading to downgrading of the fish from 'superior quality' to 'production fish' resulting in a lower sales price on the market (Salte et al., 1994). Other common bacterial pathogens such as *Yersinia ruckeri*, *Vibrio anguillarum*, *Aeromonas salmonicida*, *Flavobacterium psycrophilum* and *F. columnare* cause bleedings in gills, fins, skin and musculature, but the potential impact on the post-harvest fish meat quality are so far unknown (Poppe and Bergh, 1999). With respect to parasites, the myxosporean parasite *Kudoa sp.* is known to cause softened texture 'myoliquefaction' of the filet in hake, herring and mackerel due to high levels of protease activity (Moran et al., 1999; Zhou and Li-Chan, 2009). Further, the parasitic crustacean *Lepeophtheirus salmonis* 'salmon lice', is a huge problem in salmon farming in the sea since it causes high mortality, but also due to mechanical tissue damage and consequently downgrading of the fish after slaughter (Mackinnon, 1993; MacKinnon, 1997). Viral diseases are also known to contribute to tissue damage and bleedings. For instance, the Salmon Pancreas Disease (SPD) virus causes inflammation and degenerated muscle fibres in salmon leading to a heavy weight loss, and the Viral Hemorrhagic Septicaemia (VHS) virus causes heavy bleedings and necrosis in several different tissues (Mcvicar, 1987; Smail, 2000). Hence, many different pathogenic species and types seem to influence the quality of the fish meat and some seem to be potential players.

Besides the direct visible effects of disease on the fresh fish meat, signs of previous infections might as well be seen in the final processed product. Within the fish industry, a high individual variance in fluid-loss from smoked fillets has been observed and it has been speculated that this phenomenon may be attributed to previous infections in the fish pre-harvest (unpublished).

1.3 The fish muscle and skin – composition of cells and tissue types

The fish skin is the outer, protective barrier against the environment. In contrast to mammals, live cells and a mucosal surface cover all outer surfaces of the fish. A model of the skin in salmonids is shown in figure 1. Roughly, the fish skin can be divided into two layers; an outer layer called epidermis and a lower layer termed dermis. These two layers are separated by a basal membrane. An external mucosal cuticle layer consisting of mainly mucopolysaccharides and glycoproteins covers the epidermis. Dermis can further be subdivided into an outer layer of loose connective tissue and an inner layer of firmer connective tissue. For some fish species, a third layer termed hypodermis does also exist (Kryvi and Totland, 1997).

1.3.1 The mucus layer

The whole surface of the fish is covered by mucus. The function of this layer is to limit the friction between the fish and the water, but it also plays an important protective role against infections. The composition of the mucus is dependent on the fish species, but it always contains polysaccharides and glycoproteins. In addition to this, it contains a range of antimicrobial enzymes and peptides. These include alkaline phosphatase, cathepsin B, complement, transferrin, lysozyme, C-reactive protein, antibodies and beta-defensins (Casadei et al., 2009; Narvaez et al., 2010; Subramanian et al., 2007; Zhao et al., 2008)

1.3.2 Epidermis

The epidermal layer is morphologically very different between fish species and varies in thickness from 3-4 and up to 10-12 layers of epithelial cells. The mucosal layer is synthesised and secreted from goblet cells within the epidermis. The main structural component of the epidermal layer is malpighian cells, which are also termed keratocytes (Whitear, 1970). These are epithelial cells containing filaments, they are motile which enables the them to establish a new layer of cells very fast following tissue damage (Asbakk and Dalmo, 1998). In addition they are phagocytic and capable of engulfing latex beads *in vitro* (Asbakk, 2001; Asbakk and Dalmo, 1998). Other cells that may be represented in the epidermis include club cells, granule cells, lymphocytes and macrophages (Kryvi and Totland, 1997; Roberts, 2001a).

1.3.3 Dermis

The dermis layer below the basal membrane consists of two sub layers (Seegers and Meyer, 2009). The upper layer of dermis, stratum spongiosum, consists of a loose network of connective tissue, reticulin fibres, fibroblasts, the pigment-containing chromatophores, mast cells and the scales. The scales are flexible, calcified collagen plates, which lie within pockets.

The stratum compactum layer of the dermis is deeper positioned and is tighter than stratum spongiosum due to a higher content of fibres. It consists mainly of cross-linked collagen fibres highly organised into a matrix. Between this layer and the muscle fibres is hypodermis positioned. This layer is characterised by the presence of adipose tissue and loose connective tissue (Seegers and Meyer, 2009).

1.3.4 The muscle and supporting tissue

The muscle tissue is of the skeletal type and this exists as red and white fibre types, where the white muscle fibres dominate in the fillet from most fish species. These are produced by the fusion of mononucleated myoblasts to form multinucleated muscle fibres (Chong et al., 2009). Both types of muscle fibres are organised in segments termed myotomes separated by connective tissue, which is very important in maintaining the muscular structure and function. The major constitution of connective tissue is collagen and this exists in several different forms. So far type-I and type-V have been recognised in fish and the genes for the different subunits of type I have been sequenced in rainbow trout (Mizuta et al., 2005; Nishimoto et al., 2005; Saito et al., 2001; Sato et al., 1988; Yata et al., 2001).

1.3.5 Additional cell types in the muscular tissue

Other cell types than muscle fibres are also present in the muscular tissue. Fibroblasts are important cells and part of the connective tissue by functioning as synthesisers of collagen and precursors of extracellular matrix. Further, they are also very important in maintaining a structural framework in the tissue and have a significant role in tissue regeneration (Glaros et al., 2009; Khaw et al., 1994). Recently it has been shown that they also have immune regulating properties and work as sentinel cells in the muscular tissue, indicating a very broad range of functions for this cell type (Ingerslev et al., 2010; Smith et al., 1997). In addition to this important type of cell, the vascularisation also contributes to supplementary cell types by the presence of endothelial cells and blood cells. However, the vascularisation and blood supply is very limited in the white

musculature in comparison to the red type. Under normal conditions the presence of traditional immune cells is almost absent. On the other hand, under abnormal physiological conditions like infection or tissue damage, a range of different cells is also present in the muscular tissue. Neutrophils, macrophages, basophils and lymphocytes are attracted to the site of infection / damage in order to elicit an immune response and clean up affected tissue for pathogens, cell debris and necrotic cells (Martin and Leibovich, 2005).

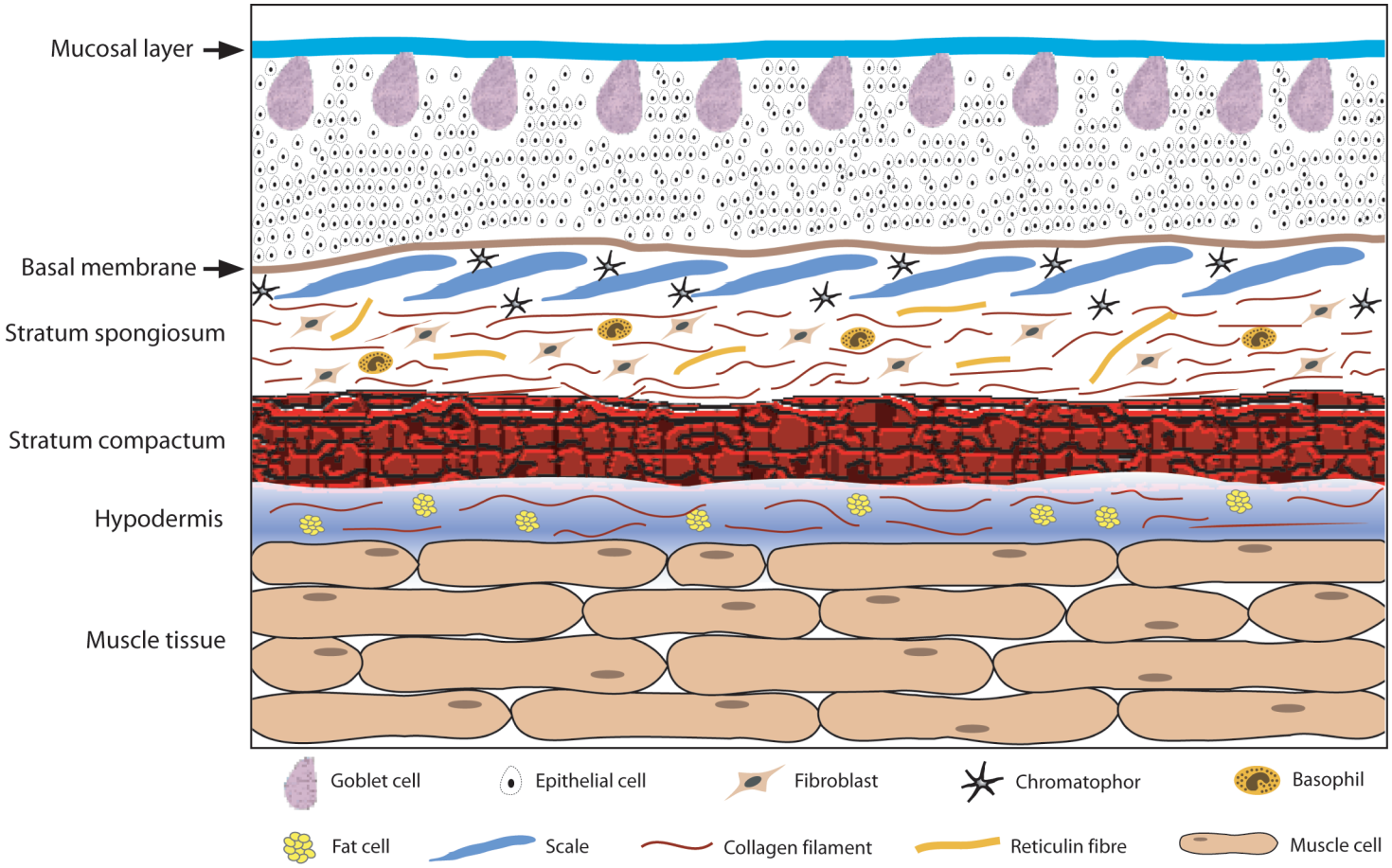

Figure 1. A model of the skin and musculature in salmonids showing the most common cell types present under normal physiological conditions. The outer surface of the skin is covered by mucus, which is secreted from the mucus producing goblet cells in the epidermis. The epidermal layer is separated from the dermis by the basal membrane. Dermis is composed of the two layers stratum spongiosum and stratum compactum. Stratum spongiosum consists of the scales, a loose network of connective tissue, reticulin fibres, fibroblasts, basophils and chromatophores. Stratum compactum is a tight compact layer with dense collagen arranged into a matrix. Hypodermis is the inner layer of the skin and composed of fat cells and loose collagen. The muscle cells can be of either white (fast) or red type (slow).

1.4 Tissue regeneration in fish

The regeneration of tissue in fish shows many parallels to what is known in mammals. The entire process in mammals from damage / infection to repaired or regenerated tissue consists of several overlapping phases including coagulation, early / late inflammation, proliferation and remodelling. A simplified model of the different steps is outlined in figure 2. Many of the same cell types and molecules are taking part in the different phases of tissue regeneration in fish. Especially from a molecular aspect, many of the processes and interacting molecules are currently not very well described in fish. However, the inflammatory response is to date fairly well described in fish, whereas the later phases are poorly described.

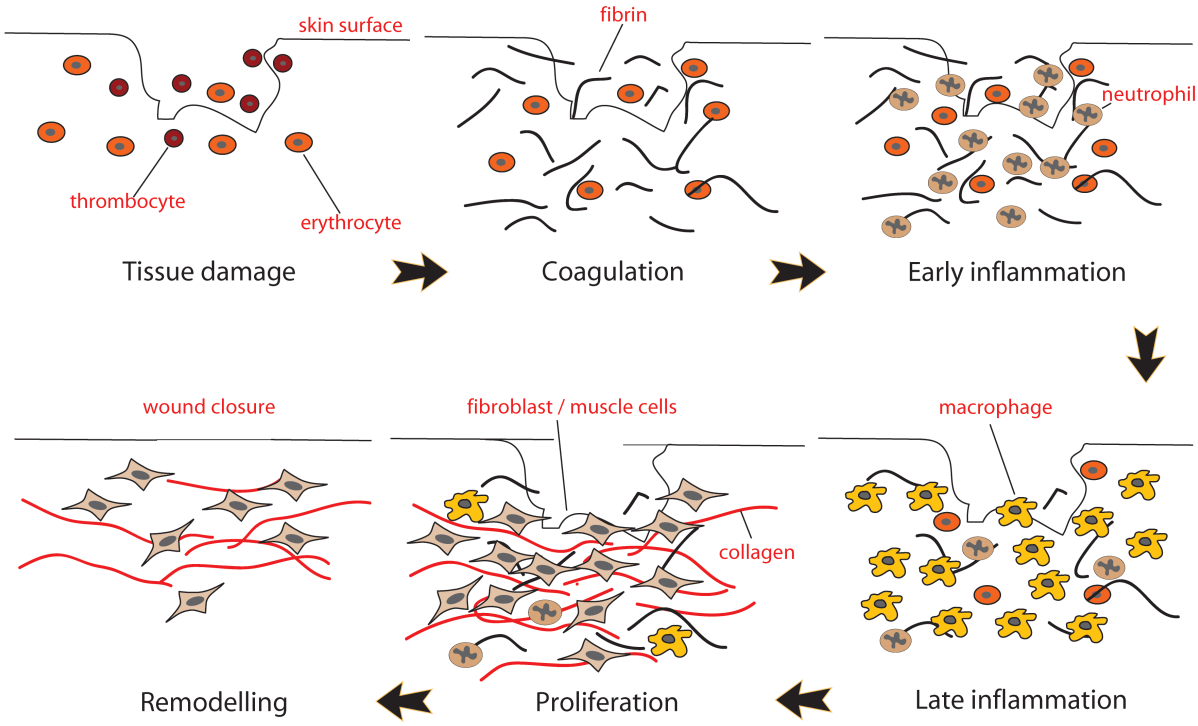

Figure 2. Tissue regeneration involves several distinctive phases. The first event following tissue damage is influx of erythrocytes and thrombocytes / platelets to the site of injury. Shortly hereafter, the coagulation cascade is activated and a clot is established from thrombocytes and fibrin. Subsequently, an early inflammatory response is initiated characterised by influx of neutrophil granulocytes. These secrete IL-8, which later lead to attraction of macrophages. Together, these cells clear the damaged tissue for dead cells and debris. Later, proliferation of fibroblasts and synthesis of extracellular matrix components take place in order to synthesise new tissue. Lastly, the wound is closed and covered by a new layer of cells and a long lasting phase of remodelling of the tissue architecture goes on.

1.4.1 The inflammatory response in relation to tissue damage

Influx of red blood cells and formation of a clot are the first events following tissue damage. Erythrocytes and thrombocytes become very abundant in the affected area. The thrombocytes together with fibrin fibers derived from fibrinogen create a matrix, which together with vitronectin and thrombospondin form a clot (Doolittle, 1990; Jiang and Doolittle, 2003). Rapidly and overlapping with this, an inflammatory reaction is established in order to fight infection and clean up the affected tissue for cell debris and necrotic cells (Gonzalez et al., 2007a; Gonzalez et al., 2007b; Martin and Leibovich, 2005). The cell types neutrophil granulocytes and macrophages are of particular importance in this regard as being highly phagocytic and producers of chemokines and cytokines. These cells are recruited to the affected tissue from the periphery and the neutrophils are the first to arrive. Arrival of neutrophils happens very fast and has been observed in the skin and musculature already two hours after mechanical tissue damage in carp (Gonzalez et al., 2007a). This recruitment relies on chemokines and cytokines locally secreted from cells at the site of damage or infection, but release of H_2O_2 from cells within the affected area has been suggested as the first signal to attract immune cells in zebrafish (*Danio rerio*) (Diegelmann and Evans, 2004; Niethammer et al., 2009). Recruitment of macrophages in high amounts is also taking place, though they arrive later than the neutrophils in the skin and musculature (do Vale et al., 2002). These circulate in the blood as monocytes, but mature and differentiate into macrophages when entering the tissue through the endothelium of the blood vessels. Afonso and co-workers (Afonso et al., 1998) showed that induced inflammation in the peritoneal cavity of rainbow trout following administration with Freund's adjuvant and formol-killed *Yersinia ruckeri* showed a fast influx of neutrophils into the inflamed area followed by a later invasion of macrophages.

Other cell types in addition to neutrophils and macrophages can also be present at the inflamed site. Important cells in this regard are the T cells dendritic cells. In fish, it is however still debated whether dendritic cells exist (Pettersen et al., 2008).

1.4.2 Inflammation and fibrosis

In general, the extent of the inflammatory response can vary a lot and may be dependent on the triggering stimuli. The inflammatory response may function as a double-edged sword in connection to fibrotic responses. By definition, fibrosis is characterised by an excessive accumulation of connective tissue during tissue repair (Diegelmann and Evans, 2004). Naturally, it is very important to have a sufficient

inflammatory response that is powerful enough to combat pathogens and clean-up of necrotic and apoptotic cells (Murphy et al., 2008). Further, some signals like the cytokines released from inflammatory cells trigger fibroblasts and keratinocytes to divide. Hence, inflammation is a necessary process and event following tissue injury and needed in connection to tissue regeneration and repair. On the other hand, an un-balanced and excessive inflammatory response may lead to a high degree of fibrosis, showing that the level of inflammation is very important for the outcome. Some cytokines are profibrotic while others are anti-fibrotic. The pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) causes high amounts of tissue destruction during inflammation, but at the same time it is an anti-fibrotic protein since it suppresses the expression of matrix genes and TGF-β induced production of CTGF and collagen (Abraham et al., 2000; Bermpohl et al., 2007). Further interferon-γ (IFN-γ) seems to exhibit a similar role, since it is an activator of macrophage function and hence the inflammatory response, but at the same time IFN-γ functions as an anti-fibrotic protein since it inhibits synthesis of collagen in fibroblasts (Gillery et al., 1992; Murphy et al., 2008).

1.4.3 Important molecules in the inflammatory response

Crucial molecules in the inflammatory response are cytokines and chemokines, which play pivotal roles as signalling molecules between cells. Activation of the cellular responses would simply not be established without these molecules. Further, they also have important roles in the later stages of tissue regeneration since they take part in controlling cells responsible for this step. Most cytokines are activating cells while some have inhibitory effects. They usually work in an autocrine or paracrine manner and more seldom in an endocrine way. Many of the genes in fish homologues to the mammalian counterparts have been cloned and described in fish (Laing et al., 2001; Zou et al., 1999). In the following some of the inflammatory markers interleukin-1β, interleukin-8 and interleukin-10 will be mentioned.

1.4.4 Interleukin-1β (IL-1β)

IL-1 is composed of two different proteins in mammals; IL-1α and IL-1β, but only the latter exist in fish. IL-1β is probably the most well described cytokine in fish. It is a pro-inflammatory molecule with multiple functions such as stimulating cell division and activation of cells, expression of other cytokines and induction of fever. The cytokine acts via the two IL-1 receptors, IL-1R1 and IL-1R2, where IL-1R2 has been described as

a decoy receptor, which inhibits the action of IL-1 (Lindenstrom et al., 2003). In inflamed sites, IL-1β enhances migration of leucocytes by the activation of the lining endothelial cells (Martin and Leibovich, 2005). A wide range of different ligands such as LPS (Brubacher et al., 2000), bacterial infections (Lovoll et al., 2009), parasites (Lindenstrom et al., 2006) and ergosan (Peddie et al., 2002) triggers the expression of IL-1β in fish. Further, acute stress in fish does also increase IL-1β expression indicating that the expression of this gene is influenced by an enormously high amount of triggers (Metz et al., 2006). IL-1β is normally induced shortly (minutes) after stimulation and may be considered as one of the first cytokines to be activated in the inflammatory response.

1.4.5 Interleukin-8 (IL-8)

IL-8 is also a key marker of inflammation in fish. IL-8 is a member of the CXC chemokine family and also known as the CXCL8 chemokine. It causes activation and chemo-attraction of neutrophil granulocytes to inflamed tissue from the blood (Jimenez et al., 2006). In order to traffic neutrophils to the inflamed tissue, IL-8 causes increased adhesion of neutrophils to the endothelium leading to migration through the endothelium (Zhang and Chen, 2002). IL-8 is inducible in rainbow trout fibroblasts following LPS stimulation (Sangrador-Vegas et al., 2002), following vaccination and infection by *Yersinia ruckeri* in rainbow trout (Raida and Buchmann, 2008).

1.4.6 Interleukin-10 (IL-10)

The cytokine IL-10 is considered to have anti-inflammatory properties. Its functional characterisation in fish is, however, not very well described. Mammalian IL-10 is expressed by Th2 cells and monocytes and down regulates Th1 related cytokines such as IL-1β, IL-6, IL-8 and TNF-α, but enhances adaptive immune responses by stimulating growth of B-cells. In fish it also seems to be related to inflammation. A wide range of cells types and tissues express IL-10 such as fibroblasts, dendritic-like cells and cells in organs like head-kidney, liver and spleen (Pettersen et al., 2008; Rizzo et al., 2008; Savan et al., 2003). IL-10 is upregulated during soybean-mediated intestinal inflammation, IPNV infection, and bacterial infection such as *Aeromonas hydrophila* in fish (Ingerslev et al., 2009; Tanekhy et al., 2009; Uran et al., 2008). In higher vertebrates, IL-10 is also linked to extracellular matrix synthesis and thereby tissue regeneration by influencing the expression of type I collagen, collagenase and stromelysin in fibroblasts, indicating its multiple functions (Moroguchi et al., 2004; Reitamo et al., 1994). Further,

IL-10 inhibits proliferation of cultured human vascular smooth muscle cells *in vitro* following stimulation with TNF-α (Selzman et al., 1998).

1.4.7 Toll-like receptors (TLRs)

Toll-like receptors are important receptors in innate immunity and inflammatory responses since they recognise certain conserved structures on the surface of pathogens, but also components from damaged and necrotic cells (Murphy et al., 2008). Further, this recognition triggers an immune response (Medzhitov, 2008). In general, signalling is initiated by activation of the adaptor molecule MyD88, which binds to the TIR domain of the TLR. This leads to further downstream signalling cascades and activation of the transcription factor NFκB, which finally stimulates expression of immune genes. In humans, ten different TLRs exist (Murphy et al., 2008). In fish, many TLRs homologue to mammalian forms have been cloned and characterised. The similarity is due to the conserved Toll-IL-1 receptor homology domain (TIR domain) (Oshiumi et al., 2003). The fishes comprise an evolutionary extremely broad group and hence some species express a repertoire of TLRs homologues while this for other species can be very different. Thus, this is a result of evolutionary development. As an example, the salmonid fishes are evolutionary older than the carp family and one consequence if this is a lack of the homologues to the mammalian TLR2 and 4 (Purcell et al., 2006; Rodriguez et al., 2005). To date, TLR3, TLR5, TLR9 and TLR22 are known to exist in salmonid fish (Ortega-Villaizan et al., 2009; Oshiumi et al., 2003; Rebl et al., 2007).

TLR3 is associated with recognition of double stranded RNA in mammals and hence viral recognition (Rodriguez et al., 2005). Here, the receptor is intracellularly positioned in the walls of the endosomes (Murphy et al., 2008). Further, it has also been connected to necrosis since the receptor is sensible to necrotic cells (Cavassani et al., 2008). In rainbow trout, it is inducible by Poly I:C and following challenge with IHNV (Rodriguez et al., 2005).

In contrast to TLR3, TLR5 is positioned in the cell membrane and is recognising flagellin on motile bacteria. In fish, TLR5 exists as a membrane bound form and a soluble form (Oshiumi et al., 2003). TLR5 is induced following infection with *Yersinia ruckeri* in rainbow trout (Raida and Buchmann, 2009) and following infection of channel catfish (*Ictalurus punctatus*) with *Edwardsiella ictaluri* (Bilodeau and Waldbieser, 2005), indicating an importance of this receptor in connection to bacterial infections in fish.

TLR9 is like TLR3 an intracellular receptor. The ligand is unmethylated CpG dinucleotides, which is contained in most bacterial cell walls. Induction of TLR9 triggers a strong inflammatory Th1 response in mice (Hemmi et al., 2000). In fish, CpG dinucleotides and recombinant interferon-γ trigger the expression of TLR9 (Cuesta et al., 2008; Ortega-Villaizan et al., 2009; Skjaeveland et al., 2008).
The TLR22 is a toll-like receptor only found in aquatic animals (Matsuo et al., 2008). The receptor has been linked to viral recognition together with TLR3 in fugu (*Takifugu rubripes*), but in contrast to TLR3, TLR22 resides on the surface of the cells and recognises larger fragments of dsRNA than TLR3. Not much work has so far been done on TLR22, but it is sensitive to poly I:C and dsRNA and is able to trigger an interferon-β (IFN-β) response in fugu (*Takifugu rubripes*) (Matsuo et al., 2008).

1.4.8 Heat shock protein 70 (hsp70)

Hsp70 belongs to the family of heat shock proteins, which are released during stressful situations, following exposure to chemical substances and heat in order to protect the cell against damage and death (Feige and Polla, 1994). Consequently, it is termed an acute phase protein. Other members of the hsp-family are hsp60 and hsp 90, which refers to the size of the protein. During sudden or acute tissue damage stress is induced, which makes it relevant to include in studies of tissue damage. Hsp70 has protective role of the cells during inflammatory reactions since it prevents reactive oxygen species (ROS) mediated DNA strand breakage, lipid peroxidation, mitochondrial and cytokine mediated damage (Jacquier-Sarlin et al., 1994). In fish, hsp70 is responsive to a wide range of substances and treatments such as hormones, chemicals, anoxia and viral infection (Deane et al., 2007; Erdogan et al., 2007; Feng et al., 2003; Purcell et al., 2004).

1.4.9 PAMPs and DAMPs

The surfaces of pathogens consist of highly conserved structures that are recognised by innate immune receptors on the surface of the host cells. After binding to the surface, downstream cascades inside the cell are triggered. Important receptors recognising the pathogens are the TLR, NLR and RLR receptors, which by a broad definition are termed pattern-recognition receptors, PRRs (Kawai and Akira, 2007; Kawai and Akira, 2008; Medzhitov and Janeway, 2000). The patterns recognised by the PRRs are termed pathogen-associated molecular patterns, PAMPs. In recent years it has been discovered that 'self' cells and molecules are capable of triggering inflammatory responses. This led to the 'danger' theory by Matzinger in 1994 (Matzinger, 1994). In this, it is proposed

that ‘any foreign molecule is capable of generating an immune response’. These molecules can be intra-cellular components of cells like mitochondria, heat shock proteins, hyaluronan and chromatin-associated proteins like HMGB1 that are released during tissue damage, infection, necrosis and apoptosis (Klune et al., 2008; Kono and Rock, 2008; Rubartelli and Lotze, 2007; Seong and Matzinger, 2004). Non-protein-like compounds such as ATP and uric acid may also be triggers (Rubartelli and Lotze, 2007). The overall broad term of these molecules is damage-associated molecular patterns, DAMPs. It is suggested that necrotic cells in comparison to apoptotic cells are more potent inducers of inflammatory responses since they, in contrast to apoptotic cells, loose their membrane integrity and thereby release their intracellular content (Kono and Rock, 2008). Often, apoptotic cells are rapidly phagocytised and hence the dead does not lead to release of intracellular substances. Further, apoptotic cells are covered by phosphatidylserine on their surface, which bind to the phosphatidylserine receptor in immune cells. This leads to suppression of both dendritic cell maturation and the production of pro-inflammatory mediators. Instead anti-inflammatory cytokines like TGF-β are turned on (Henson et al., 2001). A model for PAMP and DAMP recognition is described in figure 3.

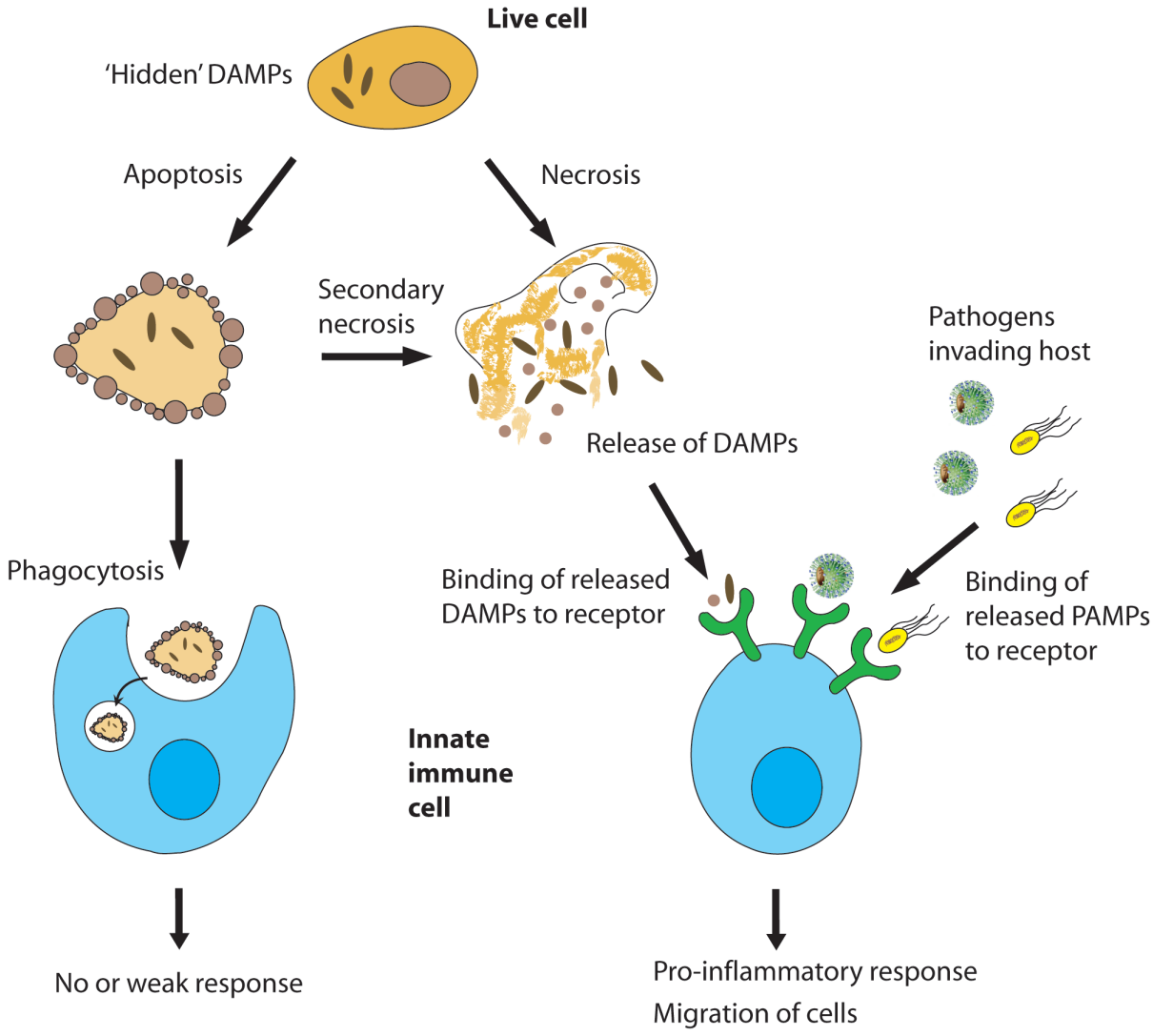

Figure 3. Recognition of PAMPs and DAMPs by immune cells. DAMPs are released from the host cells primarily by necrosis, but may also be released by apoptosis. Apoptotic cells are covered by phosphatidylserine on the surface, which binds to the phospatidylserine receptor in the immune cell initiating a negative feedback and hence suppression of inflammation or at least a weak signal. In contrast, necrotic cells leak intracellular molecules that bind to immune receptors and further activate a pro-inflammatory response and migration of cells. This, in turn, is similar to the situation when PAMPs are recognised by the same receptors and trigger inflammatory cascades.

1.4.10 The regenerative response and restoration of tissue

Whereas the inflammatory response in fish is quite well known in connection to many different conditions and infections, the later stages of tissue regeneration and tissue restoration are more poorly described from a molecular perspective. This is probably due to a traditional focus on immune responses in the fish and a lack of focus on tissue regeneration. Therefore, sequencing of genes connected to tissue regeneration in fish is staggering in comparison to genes connected to the immune system. Tissue regeneration and tissue repair are considered as two different things. Regeneration is defined as exact replacement of damaged / lost tissue. This does not occur in most

tissues of higher vertebrates like e.g. humans, but lower vertebrates such as zebra fish and salamanders can restore lost appendages such as fins and limbs, respectively. However, embryos from all-over the evolutionary spectrum are able to regenerate their cells without formation of scarring and fibrosis, so aging does also play a role in that respect (Martin and Lewis, 1992). Repair is defined as establishment of a scar and the more extreme form of this is fibrosis (Diegelmann and Evans, 2004). A model of these three different outcomes of tissue damage is shown in figure 4. Many genes are involved in the highly complex processes of tissue regeneration and repair. This requires a highly coordinated control of genes that produce regulatory and structural molecules. The process typically proceeds in cascades with influx to the damaged tissue and activation of responsible cells in a highly synchronised manner. In rainbow trout and salmon, some of the genes are sequenced while others can be found as expressed sequence tags (EST) in Genbank. Some of these key molecules and their functions are listed below.

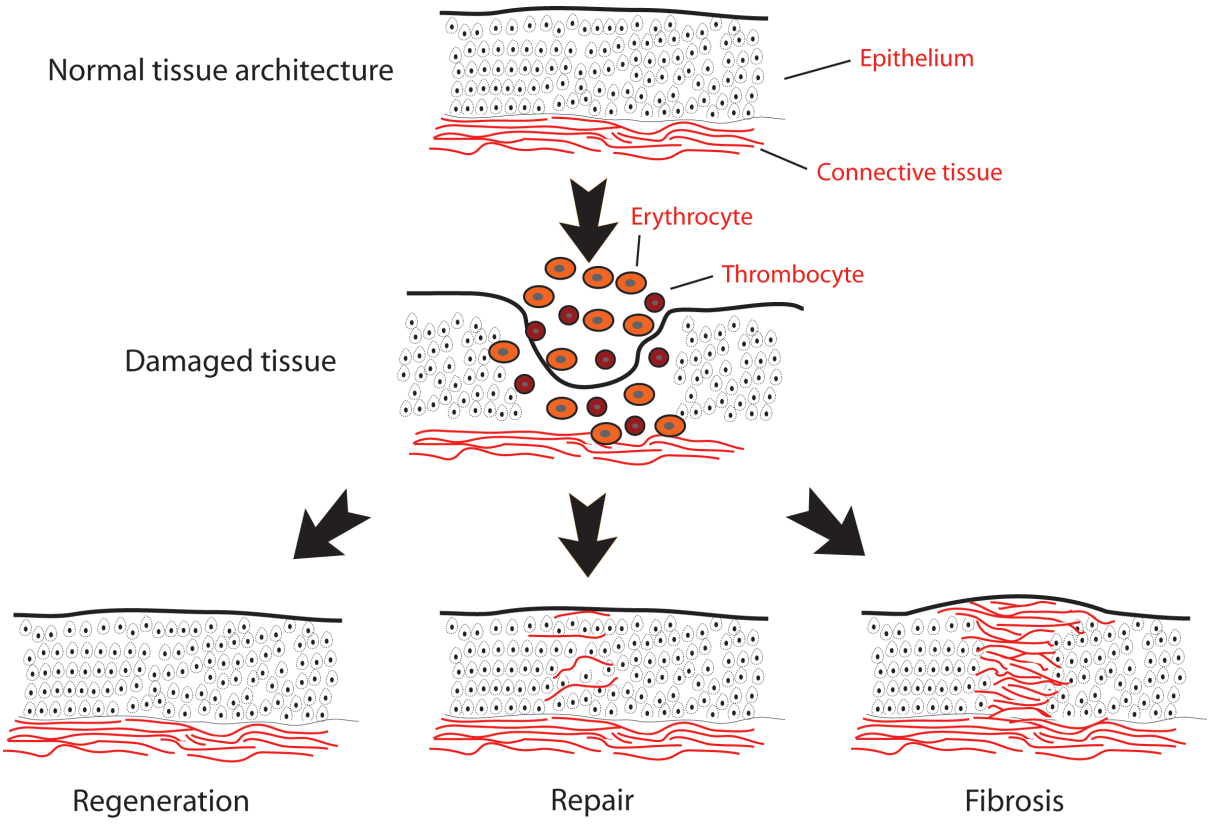

Figure 4. Tissue damage can overall result in three different outcomes. Some lower vertebrates and young individuals are able to make perfect regeneration of tissue. The normal outcome of tissue damage is however repair, which includes deposition of collagen and hence scarring. Fibrosis is the extreme result and can e.g. be an outcome of ongoing infection.

1.4.11 Transforming growth factor-β (TGF-β)

TGF-β is termed a regulatory cytokine and functionally it covers both immune and regenerative responses and has a wide range of functions. It is a very conserved molecule throughout the evolution. In mammals several isoforms of TGF-β exist and in the bony fish, three different forms seem to exist (Brubacher et al., 2000; Laing et al., 1999). The protein is primarily produced by macrophages, fibroblasts and platelets (Li et al., 2006). Regarding immune regulating properties, TGF-β inhibits T-cell and macrophage activation and together with IL-6 it causes T_H17 differentiation from naïve CD4 T-cells early in the immune response (Murphy et al., 2008). Further, the protein is involved in tolerance and silencing of dendritic cells in the intestine of higher vertebrates, which indicate regulatory capabilities of TGF-β. In fish, down-regulatory responses are also seen since administration with TGF-β decrease the respiratory burst activity of macrophages in rainbow trout (Jang et al., 1994). A wide range of treatments is influencing its expression such as triamcinolone, LPS, vaccination and parasitic damage of the skin in rainbow trout (Haddad et al., 2008; Harms et al., 2000; Lindenstrom et al., 2004; Raida and Buchmann, 2007). Further, TGF-β is important during tissue regeneration since it controls proliferation, differentiation, survival and migration of cells (Li et al., 2006). TGF-β is connected to fibrosis since it directly induces the expression of connective tissue growth factor (CTGF), which then triggers fibroblasts to proliferate at the site of wounding (Grotendorst, 1997; Kothapalli et al., 1997). However, TGF-β alone is also able to activate fibroblasts (Grotendorst, 1997). In addition to this, TGF-β induces synthesis of extra-cellular matrix components like fibronectin and type-I collagen, which further leads to fibrosis (Ignotz and Massague, 1986).

1.4.12 Collagen-1α

Collagen is produced by fibroblasts and is the main constitution of the extra-cellular matrix. It is extremely essential as 'glue' by keeping cells together and gives the proper shape to organs. In general, the collagen molecule is composed of three polypeptide chains, each of which contains one or more regions characterized by the repeating amino acid motif (Gly-X-Y), where X and Y can be any amino acid. Overall, collagens are divided into fibril-forming and non-fibril forming types (Bradbury and Rae, 1996; Vuorio and Decrombrugghe, 1990). These can again be sub-divided into several groups dependent on their structure and function. Collagens exist in many different types of tissue and organs like muscle, skin, tendon, blood vessels, nerves, lung and the fibrous

capsule of organs. About 28 different types of collagen exist in mammalian species. The most abundant protein in the body is type-I collagen, which constitutes a high percentage of the total amount of the body mass (Di Lullo et al., 2002; Gupta et al., 2006). In fish in general, the content of collagen of ordinary muscle in the dorsal part of the body range from between 1.6% to 12.4% of the total muscle protein, dependent on the fish species (Kimura et al., 1988; Sato et al., 1986). In rainbow trout and Atlantic salmon, the gene sequences of only a few types of collagen are to date available in Genbank. Of these, collagen-1α1 has been cloned from both species. Collagen-1α1 is expressed in muscle precursor cells in Atlantic salmon (Ytteborg et al.). Further, the collagen content in the musculature of fish has been shown to be connected to the texture of the fish meat, and especially type I and V collagen (Hatae et al., 1986; Sato et al., 1986).

1.4.13 Connective tissue growth factor (CTGF) / CCN2

CTGF belongs to the so-called CCN family of regulatory proteins (Connective tissue growth factor (CTGF), Cystein rich protein (Cyr61), and Nephroblastoma overexpressed gene (nov)) (Bork, 1993; Perbal, 2001). In this regard, CTGF is synonymous with CCN2. In mammals, the CCN family consists of six secreted proteins that are associated with the extracellular matrix (Leask and Abraham, 2006). CTGF is constitutively produced by fibroblasts in mammals (Holmes et al., 2003), but also in fibroblasts from rainbow trout (unpublished). In mammals, the CCN family stimulate mitosis, adhesion, apoptosis, extracellular matrix production, growth arrest and migration of multiple cell types (Leask and Abraham, 2006). It also stimulates chondrocyte proliferation, proteoglycan formation and angiogenesis. $CCN2^{-/-}$ mice exhibit impaired proliferation of chondrocytes and proteoglucan production (Ivkovic et al., 2003). Further, the function of CTGF is very closely linked to TGF-β since CTGF functions as a co-factor and binds TGF-β through the N-terminal Von-Willebrand factor domain and thereby playing an important role in TGF-β activity. Further, TGF-β induces the expression of CTGF, indicating the close relationship between these two molecules (Grotendorst et al., 1996). CTGF is induced during tissue injury and expression of the protein leads has been connected to fibrosis and scarring.

In fish CTGF does also exist. From Atlantic salmon, the gene is sequenced and accessible in Genbank, while for rainbow trout the partial gene could be found by EST screening. Only a few studies on CTGF have been done in fish. CTGF were found to be important during the early developmental stages of the notocord in zebrafish, where injection with

CTGF morpholinos caused multiple notocord defects (Chiou et al., 2006; Dickmeis et al., 2004).

1.4.14 Matrix metalloproteinase-2 (MMP-2)

Matrix metalloproteinases (MMPs) are another important group of key molecules in tissue regeneration. The MMPs belong to a family of proteins called metzincins and a further classified into at least four subfamilies: collagenases, gelatinases, stromelysins and membrane type-MMP (Massova et al., 1998; Raza and Cornelius, 2000; Stocker et al., 1995). The MMPs are typically expressed during inflammatory events like tissue injury and infection, but are also found to be upregulated during rheumatoid arthritis and cancer in humans. Different cell types including neutrophils, mesenchymal and endothelial cells express the MMPs. More than 20 different types of MMPs exist in humans and mice and they both exist as secreted and membrane bound molecules (Page-McCaw et al., 2007). Some of the most important functions of the MMPs are clearing of the extracellular matrix, generation of cleavage products, regulation of tissue architecture, activation and regulation of signalling molecules (Page-McCaw et al., 2007). Recently, it was discovered that they also are capable of recruiting leucocytes by cleavage of chemokines, and thereby they are also important during the inflammatory response (Van Lint and Libert, 2007). The direct effects of the MMPs lead to breakdown of collagen, which permit increased movement of cells within the extracellular matrix. The MMPs are also responsible for generation of new blood vessels (angiogenesis), which is very important during wound healing. Several sequence data exist for zebrafish exist in Genbank, but limited data available on MMPs in salmonid fish. In rainbow trout and Atlantic salmon, the parallels to the mammalian MMP-2 and -9 have been sequenced (Johnson et al., 2004; Saito et al., 2000). Both proteins belong to the type IV collagens / gelatinase subfamily, which cleave denatured collagens, type IV and V collagens, fibronectin and other components of the extracellular matrix. MMP-2 is termed gelatinase-A and in contrast to MMP-9, it further cleaves the interstitial type I collagen and thereby it is able to cleave the most abundant type of collagen in the connective tissue (Aimes and Quigley, 1995). The content of both type I and V collagens has been found to be important in relation to fish meat texture (Sato et al., 1986).

1.4.15 Myostatin

The mysostatin gene (growth and differentiation factor 8, GDF8) is a member of the TGF-β superfamily. The secreted protein is a negative regulator of skeletal muscle

growth and expression of the protein results in lowered muscle growth. The myostatin gene is expressed in developing and adult skeletal muscle cells and mice and cattle with a mutant in the coding sequence of the myostatin gene weigh significantly more than animals with an intact gene (McPherron and Lee, 1997). Further, the expression of the gene is decreased following heavy physical exercise in humans and increased in connection to the fibrotic and inflammatory disease muscular dystrophy in humans (Roth et al., 2003; Zanotti et al., 2007). Two different myostatin genes are cloned and characterised in rainbow trout (Rescan et al., 2001). These are termed myostatin-1 and -2, respectively, and show high homology to the mammalian protein sequences. The two genes show differential expression patterns in different types of tissue. The myostatin-1 gene is expressed in most organs and in high and equal amounts in red and white musculature. Myostatin-2 is only expressed in the brain and in muscle tissue. The expression of myostatin-2 is weaker in muscle tissue compared to myostatin-1, but much higher in red musculature compared to the white (Rescan et al., 2001). Later it was shown that two different copies of the myostatin-1 and -2 gene exist in the rainbow trout, which are termed myostatin-1a and 1b and myostatin-2a and 2b, respectively (Garikipati et al., 2007; Garikipati et al., 2006). The expression of the two myostatin-1a and 1b genes is differentially regulated, but also individually regulated at different developmental stages of the rainbow trout (Garikipati et al., 2006). A minor but significant inverse relationship between expression of myostatin and exercise-induced hypertrophy in rainbow trout has been observed, indicating that the gene expression is influenced by physiological factors (Martin and Johnston, 2005). The expression of myostatin in fish in connection to inflammation, infection and tissue regeneration has until recently not been examined.

1.5 Methods applied in the study

1.5.1 The damage model

Mechanical tissue damage of fish has previously been performed in order to mimic the inflammatory responses of ectoparasites in the skin of carp (Gonzalez et al., 2007b). In order to study locale tissue responses in the musculature, a refined model was developed in this study and examined in rainbow trout (figure 5). A device containing 25 sterile needles from 19G syringes with a thickness of 1.1 mm equally distributed on an area of 6 x 6 mm was produced and used to penetrate the skin and underlying muscular tissue. Apart sampling of tissue in the damaged area, an internal control sample from non-injured muscle tissue was taken. This allowed studies of locale tissue responses in individual fish and gave highly valuable information.

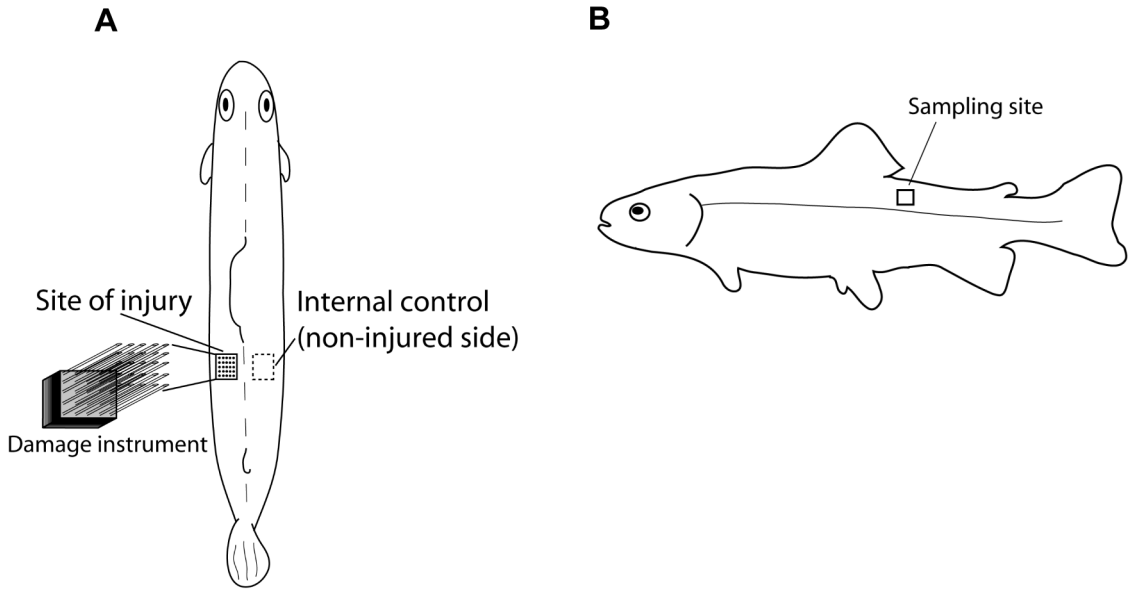

Figure 5. The mechanical damage model. The device containing 25 needles was used to penetrate the skin and musculature on the left side of the fish behind the dorsal fin. Subsequently, muscle tissue was sampled from 1) the damaged area and 2) from the opposite side of the fish relative to the site of injury (internal control). The position of the site of injury and internal control site is shown from above (A) and from the left side (B).

1.5.2 The RTHDF cell line

The fibroblast is a cell type responsible for synthesising and secretion of collagen and extracellular matrix components. It is the least specialised member of the connective tissue family, whose main function is to maintain the structural integrity of the connective tissue by secreting precursors of the extracellular matrix rich of type I and type III collagen (Alberts, 2008). Originally, it was thought that fibroblasts in wounds were derived from migrating leucocytes attracted to the wounded site, which thereafter

transformed to fibroblasts during the tissue repair (Ross et al., 1970). However, later it has been discovered that this was not true and that they originate from sources other than circulating blood cells. Instead, it was found that they mainly are of mesenchymal origin, a part of the connective tissue (Smith et al., 1997). Embryological, the mesenchyme originates from all three germ layers; endoderm, mesoderm and ectoderm. In contrast, all blood cells including the leucocytes originate from the mesoderm. In recent years it has been discovered, that fibroblasts besides their function as being important for synthesis of collagen and matrix proteins also have immune competence by the expression of a range of cytokines, chemokines and some are phagocytic (Arora et al., 2009; Chen et al., 2005; Hatakeyama et al., 2003; Hosokawa et al., 2005). In fish, several different fibroblast cell lines have to date been established. In the rainbow trout, at least the three different cell lines named RTG-2, RTG-P1 and RTHDF exist. The RTG cell lines are isolated from the gonads, whereas the RTHDF is derived from the white musculature (Ossum et al., 2004). Hence, it makes this cell line very suitable as a model for studying responses in the muscle following infection and tissue damage. The RTG-2 cell line is known to express several different immune genes, indicating that fish fibroblasts may have functions similar as the mammalians (Collet and Secombes, 2001; Zou et al., 2004).

1.5.3 Moritella viscosa – the pathogen and the potential use for studying locale tissue reactions

Moritella viscosa is a motile, gram-negative bacterium belonging to the family *Moritellaceae* and formerly it was known as *Vibrio viscosus* (Benediktsdottir and Heidarsdottir, 2007; Benediktsdottir et al., 2000). The bacterium is considered as the main causative agent of 'winter ulcer' or 'cold-water ulcer' in Atlantic salmon in Norway (Grove et al., 2008; Lunder et al., 1995). A picture of a fish infected by *M. viscosa* is shown in figure 6. Other fish species like Atlantic cod (*Gadus morhua*), Atlantic halibut (*Hippoglossus hippoglossus*) and turbot (*Scophthalmus maximus*) can also be infected by *M. viscosa* (Bjornsdottir et al., 2004; Colquhoun et al., 2004; Gudmundsdottir et al., 2006). From the first observations of fish with winter ulcers it was thought that the lesions and ulcers were of non-infectious aetiology derived from small thrombi in the blood, which gave rise to vesicles that could be mechanically disrupted by breaking the epidermis e.g. during netting and handling of the fish (Salte et al., 1994).

M. viscosa is normally a problem in seawater at temperatures below 7-8 °C and the mortality of infected fish is normally around 10% (Larsen and Pedersen, 1999). The fish

do normally recover when the temperatures increase to above 10-12 °C or if the water salinity declines to below 12-15 ‰. In culture, the growth rate is highest at 15 °C and lowest at 4 °C, but the cells are able to reach a higher density and were more stable at the low temperature (Benediktsdottir and Heidarsdottir, 2007). This may explain the temperature dependent infection rate, but immune competence of the host in connection to temperature might also play a role (Lovoll et al., 2009; Raida and Buchmann, 2007). Despite a fairly low mortality compared to many other infections, *M. viscosa* comprises an enormous problem for the aquaculture industry due to heavy wounding in the musculature of the fillet, which lead to downgrading of the fillets from 'superior quality' to 'production fish' (Salte et al., 1994). Infected fish are able to survive for long periods with heavy wounding. If an infected fish recovers, it may develop heavy scars, which leads to downgrading at slaughter. The pathological signs of the disease are initially acute/sub acute lesions with punctual swelling of the skin and loss of scales. Later, chronic lesions develop, which are characterised by open lesions and ulcers (Larsen and Pedersen, 1999; Salte et al., 1994). In this case, the skin in the ulcerated area is completely gone and the musculature is exposed directly to the water (Lovoll et al., 2009). The affected muscle tissue in this stage is typically infiltrated by immune cells and bacteria are seen in the border between the skin and the musculature (Larsen and Pedersen, 1999). Those fish that do not die of infection will start regenerating the tissue in the ulcerated area. Here, a hyper plastic epidermis without synthesis of scales will cover the musculature (Larsen and Pedersen, 1999). Infection with *Moritella viscosa* is a very useful model to study locale tissue responses due to the development of localised and punctual ulcers in the musculature. Despite a systemically spread of bacteria to some degree, the presence of ulcers are fairly localised to punctual locations in the muscle tissue (Lovoll et al., 2009). Hence, the clinical signs are different in comparison to classical gram-negative bacterial infections caused by *Vibrio anguillarum* and *Aeromonas salmonicida* (Poppe and Bergh, 1999). Sampling of muscle tissue for examining of locale tissue responses was therefore possible to perform in almost the same way as what could be done using the mechanical tissue damage device; sampling of the equivalent to the internal control was however done in different areas which were distant to ulcerated tissue.

Figure 6. Atlantic salmon infected by *Moritella viscosa*. This fish have numerous of ulcers in the skin making the muscle tissue exposed directly to the water. The shown fish had been experimentally infected for 7 days when the picture was taken.

1.5.4 Yersinia ruckeri

The gram-negative bacterium *Yersinia ruckeri* is the causative agent of 'enteric red mouth disease' and belongs to the *Enterobacteriaceae* family of bacteria. *Y. ruckeri* infects many different species of fish all over the world mainly in freshwater and causes significant economic losses in salmonid aquaculture (Tobback et al., 2007). Four main serovariants exist of *Y. ruckeri*. These are O1-O4, which can be further subdivided into O1a+b, O2a+b+c, O3 and O4 (Romalde et al., 1993). The pathological signs of the disease include subcutaneous haemorrhages primarily in the mouth and jaw region, in gills, skin, fins and exophthalmia in the eyes (Roberts, 2001b). An immersion vaccine against *Y. ruckeri* made from formalin-killed whole bacteria has been used for many years in aquaculture and provides high protection (Cossarinidunier, 1986). However, many attempts to detect increased levels of specific antibodies in the blood from fish immersion vaccinated have failed, and therefore it does not seem to correlate with protection. Recently, a new immunoglobulin isotype termed IgT has been discovered in rainbow trout and the majority of the B cells in the skin express this isotype (Zhang et al., 2009). Further, it is secreted to the mucus of the skin and may be important in mucosal immunity. Hence, on basis of this, it could be suggested that specific IgT antibodies locally produced in the skin of the fish cause the protection of the vaccine against *Yersinia ruckeri*.

1.5.5 Vibrio anguillarum

Vibrio anguillarum is a rod-shaped, motile gram-negative bacterium causing systemic infections termed vibriosis in fish. Like *Y. ruckeri*, it infects many different fish species worldwide and causes significant economical problems in aquaculture. The bacterium has a requirement for sodium ions and is commonly found in marine habitats and brackish waters with wide ranges in salinity (Larsen and Pedersen, 1999). The disease

normally occurs at high water temperatures and is a problem for farmed rainbow trout in the sea during the summer period in Danish fish farms. At least ten different serotypes have been described for *V. anguillarum*, where especially O1 and O2 infect salmonids (Roberts and Rodger, 2001). Especially in young fish, mortalities following a *V. anguillarum* infection can reach very high levels above 50%. The acute form of an infection is haemorrhagic septicaemia, but in chronically forms fish become ulcerated in the musculature and on internal organs and get erythemas around the fins (Larsen and Pedersen, 1999). The ulcerations in the skin and internal organs such as spleen, head kidney and liver are seen as petechiae and ecchymosis caused by the haemorrhages. When filleting the fish, these bleedings can be very visible. Vaccination against *V. anguillarum* is common in aquaculture using intraperitoneal vaccination, which normally gives a high antibody response (Horne et al., 1984).

1.6 Objectives of the PhD study

In this PhD study we overall aimed to examine the impacts of diseases and tissue damage on tissue regeneration and the resulting quality.

Firstly, by using sensory analysis, our goal was to examine if quality changes were detectable after recovery in conventionally farmed rainbow trout following infection with the bacterial pathogens *Yersinia ruckeri and Vibrio anguillarum*.

Further, we aimed to describe the inflammatory and regenerative responses in the musculature of rainbow trout on a molecular basis. This was performed by locally damaging the muscle tissue by introducing a new and unique sampling method. Further, in order to examine these responses, genes coding for inflammation and tissue regeneration were measured by real-time Q-PCR. Additionally, Atlantic salmon were infected with *Moritella viscosa* in order to study the responses between sterile tissue damage and infection.

Lastly, we sought to examine the potential role of fibroblasts in connection to tissue damage and infection.

Some references will be made to the mammalian system for explanation.

2. Discussion

A successful regeneration of tissue following damage or infection is extremely important and may be the determining factor for survival and further fitness of the animal. Since fish are aquatic organisms they are constantly exposed to pathogens in the water. Consequently, it is of high relevance to have a proper, outer barrier to protect the body against these organisms. Perhaps for this reason, regeneration and repair of damaged tissue is occurring very rapid in fish in contrast to higher land-living vertebrates.

The study of the potential impacts of disease on the pre-harvest quality of the fish have so far not been examined in a scientific project, but only field observations seem to indicate a correlation. Further, the processes of regeneration in a molecular perspective have so far not been described in detail in the salmonid fishes nor in fish in general. Hence, the key topics of the present PhD study were: (1) examination of the sensory effects regarding texture following mechanical tissue damage and infection in rainbow trout by the gram-negative bacteria *Yersinia ruckeri* and *Vibrio anguillarum*, (2) Expression studies of genes coding for inflammatory markers and tissue regeneration in mechanically damaged and *Moritella viscosa* infected fish and (3) Examination of the immune competence of a hypodermal fibroblast cell-line in rainbow trout.

2.1 Methodological considerations in the study

2.1.1 Quantitative real-time PCR

Several challenges meet the researcher when deciding to work with fish. When looking apart from the ever-changing aquatic environment, fish are easy to keep in high numbers on limited space and relatively cheap to obtain. In recent years many new techniques have been applied to the field giving the researcher potentials for studying processes and pathways in the fish. Despite an increased amount of tools within the research the availability of commercial antibodies within fish research is today still scarce in comparison to what exists for higher vertebrates like mouse and humans. Further, the availability of commercial recombinant proteins is also very limited, which together often hampers the ability to do functional experiments in fish. However, the continuing sequencing of new genes and the increasing amounts of expressed sequence tags (ESTs) in Genbank have to date resulted in an enormous pool of described genes

for many fish species. Especially well described are the genomes for zebrafish, fugu and stickleback, where today almost the entire genome is sequenced. Further, many of the genes from the economically important salmonid species are also fairly well described, which makes them suitable for molecular studies. Hence, after the introduction of the quantitative real-time PCR technology it was obvious to use that tool within fish research. Many genes and samples are easy to analyse in a limited amount of time making it fairly easy to obtain a lot of data. However, when looking at transcriptional regulation of genes functional results are not obtained since many post transcriptional events can occur before the protein is synthesised. Hence, a correlation between the level of messenger RNA (mRNA) and the protein level should be made with caution. On the other hand, using quantitative real-time RT PCR makes it possible to measure the very first, initial response in a cell, whereas the synthesis of proteins usually take longer time. Further, the quantification on mRNA level can be done with high precision and accuracy using real-time PCR (Forlenza, 2009). On basis of this, we chose to apply the technique in the studies.

2.2.2 The use of internal control samples

Applying the mechanical damage model and the usage of an internal control sample showed to be a powerful model to predict locale versus systemic responses in the fish (paper 2 and 3). The model clearly demonstrated that damaging a restricted area in the fish muscle also resulted in responses in that specific area; overall no significant difference in expression level between the internal control from damaged fish was seen in comparison to non-injured control fish. A question raised after the model was introduced was: are the results from the model comparable with what could be obtained from infected fish? Thus, it was chosen to study this in *Moritella viscosa* infected fish, since this bacterium gives rise to local, distinctive bleedings and tissue degeneration in the musculature. The results supported the findings from the mechanical damage study. Highest increases in expression levels were seen in the ulcerated areas compared to 'visibly non-ulcerated areas', which should correspond to the internal control sample from the mechanically damaged fish. However, not surprisingly, smaller systemic up-regulations were seen in 'visibly, non-ulcerated' tissue samples, which were due to colonisation of the bacteria from outer surfaces (route of entry) to internal organs.

2.2 Sensory evaluation of previously infected fish – can we answer our question?

For the first time, a scientific project has evaluated the impacts of disease on sensory characteristics of the fish muscle (paper 1). The two bacteria *Yersinia ruckeri* and *Vibrio anguillarum* were applied in the study since they are causing some of the most common bacterial infections in Danish fish farming (Dalsgaard, 1986; Pedersen et al., 1999). Thus, it was obvious to use these bacteria in the study. In addition, mechanical damage was applied to a separate group of fish since it was shown in paper 2 and 3 that DAMPs released following damage mediate inflammation. Hence, a comparison could be made between the impacts of infection versus sterile tissue damage under natural farming conditions. The highly fibrotic response characterised by up regulation of collagen-1α and CTGF seen for *Moritella viscosa* infected fish in paper 3 inspired to make texture as the chosen parameter for analysis in previously *Yersinia ruckeri* and *Vibrio anguillarum* infected fish. A deposition of excessive amounts of collagen in these fish du to previous infection seemed likely, and thus it would expected to influence the texture of the meat. Using this quality parameter as the only grouping parameter in the sensory analysis, some highly interesting results were obtained. Fish previously challenged by *Vibrio anguillarum* alone was the group showing differences for the most parameters in comparison to the control fish. The fresh fillet from these fish were less flaky and less oily, but had a higher toughness than control fish. A higher toughness and a higher fibrousness was also found within the group, which had been vaccinated against and challenged by *Yersinia ruckeri* followed by challenge with *Vibrio anguillarum*. Likely, a tough texture might be explained by excessive deposition of connective tissue following infection, which based on the results, apparently, seems to persist in the fish for a very long time after recovery. This interesting finding addressed the question if disease has an impact on the post-harvest quality characteristics of the fish muscle and a correlation between filet quality and disease history were established. Further, fish challenged with *Vibrio anguillarum*, but previously vaccinated against and challenged with *Yersinia ruckeri* were only affected within one single parameter in comparison to control fish, whereas non-vaccinated *Vibrio anguillarum* challenged fish were significantly different within three. Thus, these results show further, that if the immune system of the fish has been previously primed, the fish may to some degree be protected against the damaging effects of later diseases. This is likely because primed fish do not get that heavy infected and consequently, the tissue damage is then reduced. It was also demonstrated that the mechanical tissue damage had minimal effects on the texture in comparison to bacterial

challenge. A reduced inflammatory response in these fish in comparison to infected fish is the most plausible explanation to this.

2.3 Inflammatory reactions during sterile inflammation and infection in fish

By applying the mechanical damage model in the study, informative data about sterile inflammation in fish could be obtained. These data could further be compared to responses triggered by infection. Hence, a good model for examining and comparing DAMPs and PAMPs in fish was established when including the *Moritella viscosa* infection model. Inflammation is occurring as a very rapid response following damage and initiation of infection in higher vertebrates. It was shown also to be the case in this study, since a heavy up-regulation of IL-1β and IL-8 was seen already after 4 hours in the muscle tissue of mechanically damaged fish (paper 2). In paper 3 it was further revealed that sterile damage mediated inflammation can last for weeks, suggesting that DAMPs are potent triggers of inflammation in fish. All in all the expression results obtained from mechanical tissue injury and infection by *Moritella viscosa* (paper 2 and 3) is shown in figure 7. A comparison to an infection model is tricky due to the very different kinetics between the two models. Mechanical damage is initiated at 'time zero' while it takes time to build up an infection. Consequently, when tissue is damaged, 'the speeder is immediately pushed' due to release of all DAMPs at one time, while the host response to the PAMP starts from low (few bacteria present) and builds up to high (high amount of bacteria) until the infection has peaked. A dose-response relationship between the amount of LPS and the following expression of IL-1β has been demonstrated in RTS-11 macrophages from rainbow trout, indicating that the amount of bacterial products influence the strength of the immune response (Zou et al., 2000). Further, a correlation between viral load and the expression of a number of different immune genes have been observed in Atlantic salmon infected by the IPN virus (Ingerslev et al., 2009). The build up of an infection may take days dependent on a lot of factors and was also reflected in the results for IL-1β and IL-8. However, when looking at expression levels it is fair to compare the models. The inflammatory response was very much higher in infected fish in comparison to the damaged, indicating that PAMPs generate a stronger response than DAMPs in the salmonids. As a consequence of this, the suggested anti-inflammatory cytokine IL-10 was significantly elevated in the *Moritella viscosa* infected fish in order to down regulate and limit the inflammatory response. Further, TGF-β was also significantly up regulated in both experiments. The numerous different functions of TGF-β make it difficult to conclude the observed up

regulation was in relation to anti-inflammatory reactions and / or as a part of the regenerative response.

Initiation of the inflammatory response in both studies was likely induced by toll-receptor recognition of the DAMPs and further induction of NFκB transcription. Very different expression patterns were seen dependent on stimulation by DAMPs or PAMPs. During infection, toll signalling was mainly performed by TLR5, while TLR9 and -22 were important in order to trigger inflammation in connection to tissue damage. These results are further supported by the expression data from the RTHDF fibroblasts, where TLR9 was inducible following stimulation with supernatant from sonicated fibroblasts (paper 2).

2.3.1 Cross-species comparisons

It is relevant to discuss whether it is meaningful to compare results between two species. It would have been preferable to use the same species for both experiments, but obtaining Atlantic salmon mechanical damage was not an option. At the same time, the *Moritella viscosa* infection model is well established in Atlantic salmon and requires the right rearing conditions. These are seawater with a high salinity and also the potential to adjust the salt concentration and the temperature in the water in order to control the infection. Reports from the field have claimed that there are different levels in side effects from vaccines between rainbow trout and Atlantic salmon (personal communication Lars Holten Andersen). This could potentially be associated with different inflammatory responses in these two species. However, when looking at the basal expression levels of the examined interleukins, the levels are not statistically different (figure 8). This seems to be good basis in order to compare the two species. However, when comparing the expression level of the toll-like receptors, the constitutive level of TLR5 and TLR22 were significantly higher in Atlantic salmon.

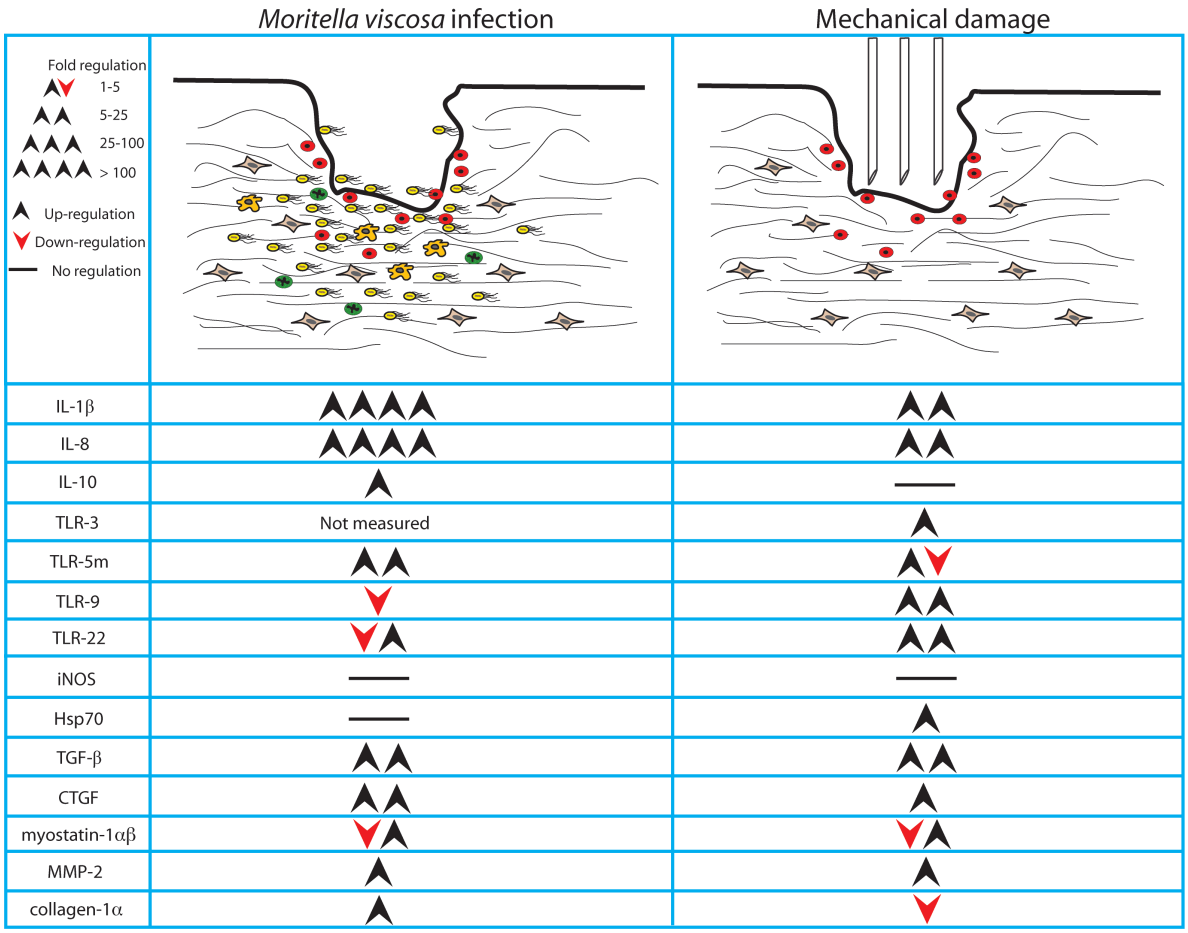

Figure 7. Summary of the expression results obtained from mechanically damaged fish in paper 2 + 3 and *Moritella viscosa* infected fish in paper 3. The expression data are grouped dependent on fold regulation in comparison to control fish. The amount of regulation shown for every single gene is not from a specific sample point, but is taken from the sample point at which the regulation was highest. A red and black arrow within the same field indicate changing expression pattern during the experimental period.

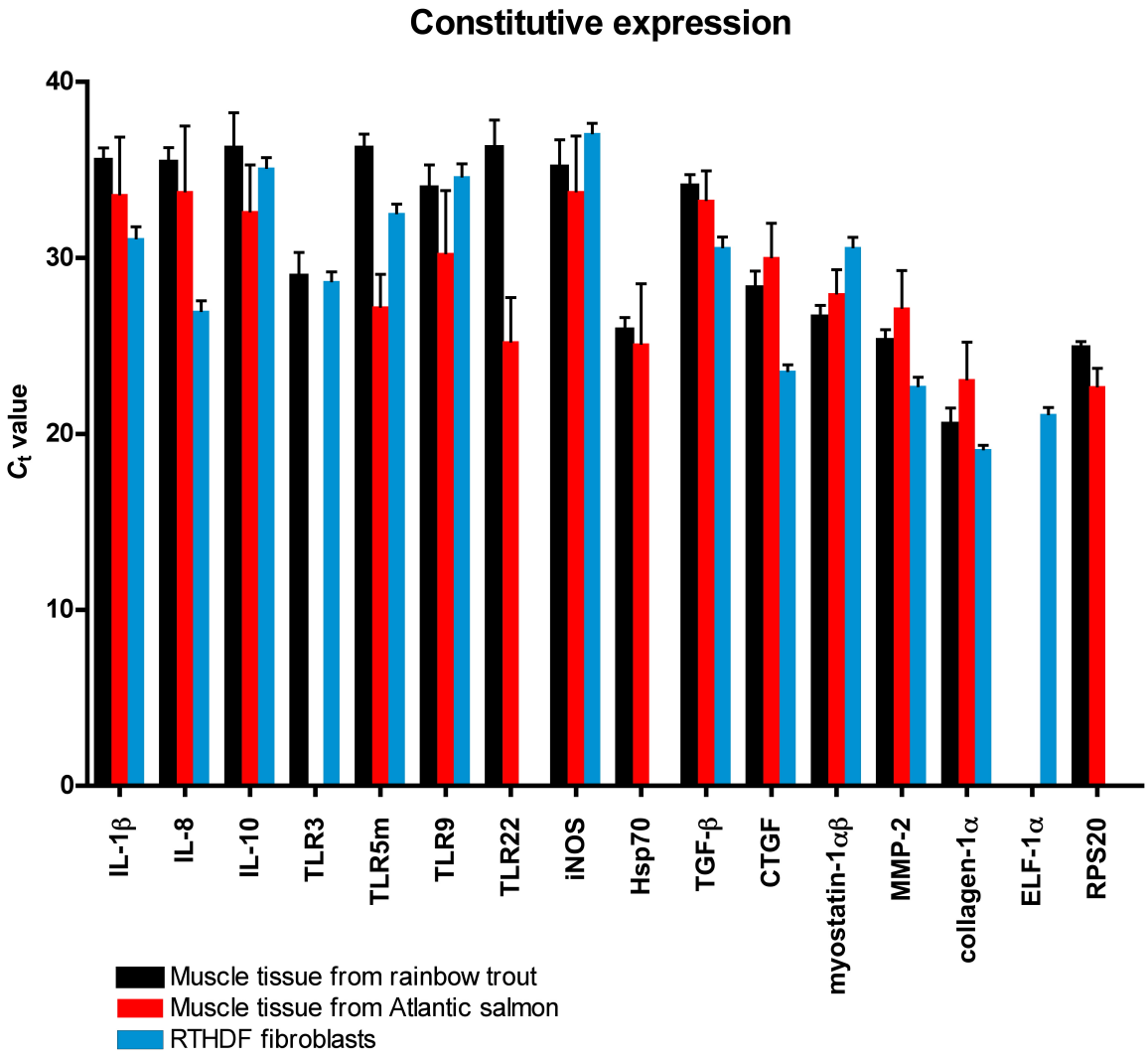

Figure 8. Constitutive expression levels in rainbow trout muscle (paper 2+3), Atlantic salmon muscle (paper 3) and RTHDF fibroblasts (paper 2) shown as C_t-values. The same threshold value was used within the same gene in order to make the results comparable. The elongation factor-1α (ELF-1α) was used as internal control for the RTHDF fibroblasts, while the ribosomal protein S20 (RPS20) was used for muscle tissue (Ingerslev et al., 2006).

2.4 Inflammation in relation to fibrosis

On basis of the strong inflammatory response seen in *Moritella viscosa* infected fish, it is thus suggested that it can explain and be connected to the quality associated problems reported from previously *Moritella viscosa* infected Atlantic salmon in the field. A fibrotic response illustrated by up regulated CTGF and collagen-1α expression was seen in both groups of fish, but it was stronger in the infected fish in comparison to the damaged fish. This induced expression may further lead to synthesis of collagen and consequently deposition of excessive connective tissue leading to scarring (Agostini et al., 1997; Heymann et al., 2009; Theiss et al., 2005). Hence, markers potentially associated with later quality changes could be CTGF and collagen-1α.

2.5 Fibroblasts in connection to inflammation and tissue regeneration

The results in paper 2 states that fibroblasts are immune competent cells due to their ability to express a range of cytokines and toll-like receptors following stimulation with LPS, sonicated fibroblasts and supernatant. On basis of this, fibroblasts from rainbow trout are suggested as having a potential to be an important sentinel cells and may function as the first line of warning signalling in the muscle tissue following infection and tissue damage. This has also been shown in higher vertebrates where fibroblasts secrete chemokines of the CXC and CC types upon stimulation by bacterial products (Smith et al., 1997). These findings support the mechanical damage results and may help to explain why the expression of the cytokine genes IL-1β and IL-8 are up regulated extremely fast following tissue damage in a type of tissue where traditional immune cells do not exist under normal physiological conditions. In addition to the data presented in paper 2, the expression of the regenerative genes presented in paper 3 were also examined (figure 9). This was in order to investigate the basal expression level of these genes in this cell type and hence their relevance according to tissue regeneration. Further, we wanted to examine if genes coding for tissue regeneration were inducible following stimulants mimicking infection and tissue damage. None of these stimulations affected the expression of either CTGF, myostatin-1αβ, MMP-2 or collagen-1α (figure 9). However, when looking at the constitutive expression level of the genes associated with tissue regeneration in the RTHDF fibroblast, they all have a higher expression level than muscle tissue in rainbow trout and Atlantic salmon except regarding myostatin-1αβ. This might indicate that they are important producers of those proteins. Why else keep up running a full-powered machinery without using the products? As seen in mechanically damaged and infected fish, the transcription of these genes is regulated. This could be explained by the presence of co-stimulatory signals *in vivo*, which are not present *in vitro*. Among these could be cytokines and proteins produced by other cell types at the inflamed site. An example of this is the CC chemokine MCP3, which have been shown to promote synthesis of collagen from fibroblasts in higher vertebrates (Ong et al., 2009). Further, interleukin-4 produced by leucocytes, have been shown to induce the amount of procollagen-I peptide and fibronectin in human conjunctival fibroblasts (Fujitsu et al., 2003). Hence, this is an example of cross-talk between different cell types and may be of high significance in order to obtain a response. Further, the strong up regulation of IL-1β and IL-8 seen in the RTHDF fibroblasts might *in vivo* facilitate chemo attraction of leucocytes that are able to produce these co-stimulatory signals. This could then be a useful explanation for

the negative results seen for the expression of the regenerative genes in the RTHDF fibroblasts, but at the same time explain why up regulations were seen *in vivo*.

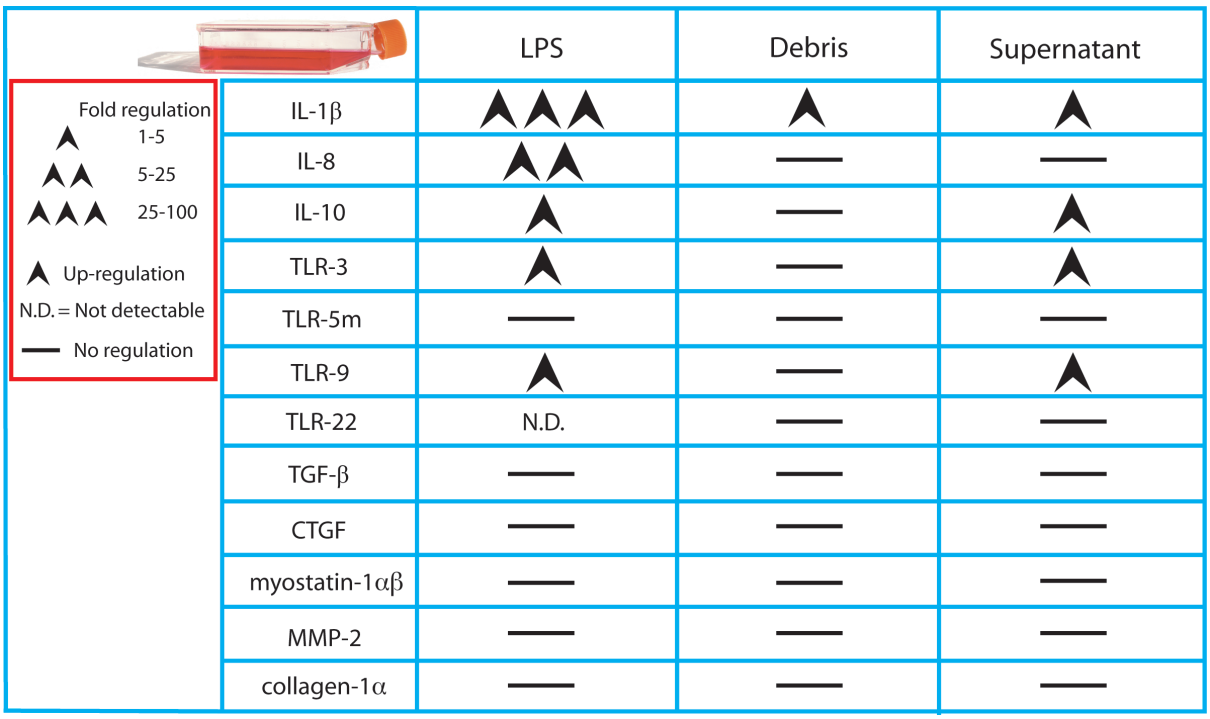

Figure 9. Quantitative real-time PCR expression of immune- and regenerative genes in the RTHDF fibroblasts following stimulation with LPS from *E. coli*, debris from sonicated RTHDF cells and supernatant. The results for IL-1β, -8, -10, TLR3, -5m, -9, -22 and TGF-β are described in paper 2, whereas the results from the remaining genes are not published data.

3. Perspectives for future research

The work conducted in my thesis has provided new insight into quality related issues in relation to previous diseases. Further, tissue regeneration in salmonid fish was examined from a molecular perspective. In continuation of these studies several interesting subjects are worth examining further and new questions would be interesting to answer. These are among others:

3.1 Functional studies of fish fibroblasts

In order to elucidate the role of the fish fibroblast further in connection to their potential as being sentinel cells signalling the first signs of danger to professional immune cells, a functional characterisation of this might be performed. This could be done in the following way: a migration assay using a macrophage cell line should be established through a gradient of supernatant from sonicated fibroblasts. This would mimic the tissue damage and show if damaged fibroblasts contain DAMPs that can attract professional immune cells. Further, fibroblasts previously stimulated by DAMPs and / or PAMPs may secrete chemokines that attract leucocytes. Hence, another migration assay using both the RTHDF fibroblasts and a macrophage cell line should be established. If the fibroblasts attract the macrophages it shows that the fibroblasts are able to 'call' professional leucocytes for assistance and may explain homing of these cells to damaged tissue or ulcers.

If fibroblasts in fish are able to phagocytise they have a potential for taking part in the clean-up process of debris and pathogens (phagocytosis) following damage and infection.

In this study it was examined if the RTHDF cells were able to phagocytise latex beads. No phagocytosis was observed in this experiment. However, despite this negative result it is still a possibility that they have the capacity to phagocytise even smaller particles. Hence, it is interesting to examine this further by establishing a new assay using nano particles.

3.2 Prolonged study of Moritella viscosa infection

The last sampling point in the *Moritella viscosa* was 14 days post infection. Hence, only the initial phase of tissue regeneration was measured. In order to follow the responses until complete recovery, later sample points are needed. This will further highlight and help to explain the significant fibrotic responses reported from the field. In addition,

biochemical measurements of the collagen content following infection will further show the influence of the infection on fibrosis. Further, obtained data should be supported by histological examinations with staining of collagen.

3.3 Do signs of disease disappear or become 'diluted' over time?

The sensory evaluation of previously infected rainbow trout with *Yersinia ruckeri* and /or *Vibrio anguillarum* showed very interesting results and supported our working hypothesis. Thus, in continuation of this, it will be interesting to examine if disease-related effects on the muscle tissue disappear or become 'diluted' over time. In this respect, it will be obvious to do sensory analysis at different time points following infection.

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Paper I

Previous bacterial infections affect the textural quality of the fillet in rainbow trout (*Oncorhynchus mykiss*)

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Previous bacterial infections affect the textural quality of the fillet in rainbow trout (*Oncorhynchus mykiss*)

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Abstract

Sensory quality parameters of fish meat post slaughter are influenced by many biological and non-biological parameters prior to slaughter. Until recently, it has not been examined if previous infections or damages in the muscle tissue influences product quality parameters in fish. Diseases and tissue damage may cause bleedings in the musculature, which further could lead to scarring and altered texture of the tissue. In this study, this was examined in rainbow trout (*Oncorhynchus mykiss*) following sterile, mechanical damage and infection by the two gram-negative bacteria *Yersinia ruckeri* and *Vibrio anguillarum*. The fish meat was then further examined as fresh and cold-smoked. The results showed that previous disease had an impact of the textural characteristics of fresh meat, while no differences were seen for cold-smoked fish. Previously diseased fish were less flaky, had a lower oiliness and a higher toughness and fibrousness in comparison to control fish. Further, it was shown that vaccination against *Vibrio anguillarum* protects the fish against disease and thus tissue damage and scarring since these fish were not affected compared to non-vaccinated fish. This paper is the first to describe a correlation between previous infections and sensory quality parameters in fish and the data provide valuable knowledge for the aquaculture industry as a broad.

1. Introduction

Aquaculture is an expanding worldwide industry and the amount of produced fish and the number of species is increasing every year (FAO, 2008). Many different biological and non-biological parameters have been shown to influence subsequent quality parameters of the farmed fish post slaughter (Ashley, 2007; Morkore et al., 2007; Pettersson et al., 2009; Poli et al., 2005; Sigurgisladottir et al., 1997). Among these is welfare and rearing, stress at slaughter, feed, the composition of the fat in the feed. The influences of these parameters and many others have been connected to textural changes of the fillet, content of connective tissue and coloration (Sigholt et al., 1997; Sigurgisladottir et al., 1997). Further, based on sensory analysis the taste and odour of the fish meat have shown to be under influence from the composition of fat in the fish feed (Torstensen et al., 2005; Waagbo et al., 1993).

In recent years, reports from the field indicate changes in the tissue structure of the fillet following disease in infected or previously infected fish (Lunder et al., 1995). Many pathogens harm their host by giving rise to heavy damages and bleedings in the musculature that may cause textural changes (Moran et al., 1999). This is illustrated from Norwegian salmon farming following infection of Atlantic salmon (*Salmo salar*) by the bacterium *Moritella viscosa*. Previous infected fish become downgraded from 'superior quality' to 'production fish' following infection, which results in a lowered sales price on the market (Salte et al., 1994). Further, mechanical damage of the skin and musculature following infection with the parasitic crustacean *Lepeophtheirus salmonis* has also been connected to quality changes (MacKinnon, 1997). So far it has not been scientifically shown whether previous infection may have an impact of sensory quality regarding the texture of the fish fillet. Hence, in this study we aim to examine this under regular farming conditions in rainbow trout (*Oncorhynchus mykiss*). Fish were infected with the common bacterial pathogens *Yersinia ruckeri* and *Vibrio anguillarum*, which give rise to bleedings in the musculature of the fish and causes serious economical problems in Danish aquaculture. Further, mechanical damage of the musculature was performed in order to mimic non-pathogenic mediated damage. 16 months after the last bacterial challenge, sensory analysis was performed on fresh and cold-smoked fillets by a trained panel of assessors in order to examine these potential effects.

2. Materials and methods

2.1. Fish and rearing conditions

Rainbow trout (0+) fry (*n*=4300) reared at Horns Herred Ørredopdræt (Skibby, Zealand) were brought to the rearing facilities at National Food Institute, Technical University of Denmark (DTU Food) in the autumn 2007. The mean size was 2.0 grams (*n*=30, sub-sampling) and the fish were non-vaccinated. The experimental facilities at DTU Food consists of two separate units A and B. Unit A which were used for control fish and stocking and consisted of two tanks with a volume of 1200 l each, which were connected to a bio-filter with re-circulated water and temperature control (Tanks 7 and 8), Unit B was used for infection of fish and was composed of four tanks with a volume of 400 l each, which were also connected to a bio-filter with re-circulated water and temperature control (Tanks 1, 2, 3 and 4). In addition to this, the facility was supplied by UV-light in order to keep the water clean of microorganisms. All rearing facilities were daily supplied with 10 percent fresh water and the content of $NH4^+$ and $NO2^-$ were measured in order to ensure good water quality. During the experimental period at DTU Food the water temperature was kept at 15 °C and the fish were exposed to 16 hours of light and 8 hours of darkness.

The following describes the procedures for obtaining the following 10 groups from the above mentioned fish: 1) control fish, 2) *Yersinia ruckeri* challenged, 3) *Yersinia ruckeri* vaccinated + challenged, 4) *Vibrio anguillarum* challenged, 5) *Vibrio anguillarum* vaccinated + challenged, 6) *Yersinia ruckeri* challenged + *Vibrio anguillarum* vaccinated, 7) *Yersinia ruckeri* challenged + *Vibrio anguillarum* challenged, 8) *Yersinia ruckeri* vaccinated + challenged + *Vibrio anguillarum* challenged, 9) *Yersinia ruckeri* vaccinated + challenged + *Vibrio anguillarum* vaccinated + challenged and 10) mechanically damaged fish.

2.2. Tagging of fish

Each of the surviving fish from groups other than control fish were individually tagged by injection of electronic PIT tags into the peritoneal cavity August 2008 (Product no. AB10400, Loligo Systems, Tjele, Denmark). Hence all groups of fish could later be

reared in the same sea cage. The individual tag numbers were read using an electronic PIT reader (Product no. AB10625, Loligo Systems, Tjele, Denmark).

At a size of approximately 100 g, all groups of fish were moved from DTU Food by commercial fish transport to new freshwater facilities for further growth in Bisserup, Denmark in September 2008. The facilities composed of four round tanks with a volume of 2000 l each, containing freshwater and connected to a bio-filter. The fish were equally distributed in the tanks upon arrival. The temperature fluctuated according to the outside temperature since the building was not isolated. Here the fish were reared until they were further transferred to seawater. In the spring 2009 all fish were transferred to one sea cage with a diameter of 40 meter and a depth of 4 meter. The position of the cage was 55°10'20'' N, 11°30'00'' E. The fish were reared in the cages until slaughter in November 2009. The average weight (g ± SD) and length (cm ± SD) of the fish at slaughter were 667.1 ± 103.9 and 36.8 ± 1.8, respectively (n = 111).

2.3. Vaccination against Yersinia ruckeri

Fish were bath-vaccinated against *Yersinia ruckeri* using the Ermogen® Vet. vaccine (Aqua Health) October 2007. The procedures were in accordance with the manufacturer's instructions by diluting the vaccine 1:10 into water and further 30 sec of bathing of the fish.

2.4. Vaccination against Vibrio anguillarum

Vaccination against *V. anguillarum* was performed May 2008 using the AquaVac™ Vibrio vet. (Schering-Plough Animal Health). The fish were intraperitoneally injected using 100 µl of vaccine per fish according to the manufacturer's instructions.

2.5. Challenge with Yersinia ruckeri

Challenge of fish with *Yersinia ruckeri* was performed March 2008 using the 99.8.60 strain (serotype O1). Bacteria were grown for 18 hours at 25 °C in LB medium with shaking until a final concentration of 10^9 * ml^{-1} bacteria. Prior to this, a challenge test using two different amounts of bacteria was set up (10^6 and 10^7 bacteria per fish) in order to determine the dose maximally 50% of the fish died. The bacteria were diluted in 1X PBS. Based on these results, 10^6 bacteria were chosen for challenge of

experimental fish. The bacteria were intraperitoneally injected (100 μl) in the fish using a syringe. When mortality was registered the fish were treated with antibiotics (see procedures below).

2.6. Challenge with Vibrio anguillarum

Challenge of fish with *Vibrio anguillarum* was performed in July 2008 using the strain 070602-1/2A (serotype O1). Bacteria were grown in LB medium containing 1% $NaCl_2$ for 24 hours at 25 °C with shaking until a final concentration of 10^9 * ml^{-1} bacteria. A challenge test was also setup for *V. anguillarum*. Based on this, a dose of 10^5 bacteria per fish was chosen for challenge of experimental fish. The bacteria were intraperitoneally injected (100 μl) into the fish using a syringe. Treatment with antibiotics was initiated 2 days post challenge (see procedures below).

2.7. Treatment of infected fish by antibiotics

Fish infected by either *Y. ruckeri* or *V. anguillarum* were fed with up to 1-1.5% of body weight per day of Aquavet feed (Biomar, product no. 84894) containing Sulfadiazine and Trimethoprim antibiotics. Feeding was started a few days after mortality was observed and continued until it had ceased according to veterinary regulations. For *Y. ruckeri*, feeding was initiated 6 days post challenge and for the following 8 days. For *V. anguillarum*, feeding was started 2 days post challenge and continued for 4 days.

2.8. Bacteriology

After challenge by *Y. ruckeri* and *V. anguillarum* plating out from the head kidney of dead fish was performed in order to confirm presence of bacteria in the blood. Plating from *Y. ruckeri* infected fish was performed on standard LB plates, while LB plates containing 5% calf blood were used for *V. anguillarum*. In order not to bring bacterial pathogens from the laboratory facilities to the sea cages, a collection (n=10) of surviving fish from this group and previously infected fish were sent to a veterinarian lab in August 2008 for bacteriological examinations. These were then screened for the presence of *Y. ruckeri* and *V. anguillarum*.

2.9. Mechanical damage of the musculature

Mechanical punctual damage of the skin and musculature was performed on the left side of 50 fish two weeks prior to transfer to seawater using a constructed homemade device. The device contained 8 x 4 (32) needles (25g, Becton Dickinson) equally distributed on an area of 2.7 x 6.3 cm. The length of the needles, which penetrated the fish skin and musculature, was 0.6 cm. The device was penetrated once through the skin below the dorsal fin with one half of the needles above the lateral line and the other half below.

2.10. Feeding

The fish were fed with commercial trout feed of approximately 1-1.5% of body weight per day throughout the entire experiment. The sizes of the pellets were in accordance with the manufacturer's recommendations in relation to size of the fish. Until at a size of approximately 40 g the fish were fed with a diet of DAN-EX trout feed (Danafeed, Horsens, Denmark). Once transferred to the sea cages fishes were fed with Aqualife / EFICO trout feed and Ecolife Pearl (Biomar, Brande, Denmark).

2.11. Elisa assays for detection of Y. ruckeri and V. anguillarum

An enzyme-linked immunosorbant assay (ELISA) was used to detect the presence of the *Yersinia ruckeri* O1 and *Vibrio anguillarum* O1 in the plasma from slaughtered fish. The ELISAs were run separate for each antigen using the same protocol (Mikkelsen et al., 2006).

In the following, all steps in the protocol were carried out at room temperature.

In order to prepare the antigens (*Yersinia ruckeri* O1 and *Vibrio anguillarum* O1) bacteria were grown for 12 and 24 hours at room temperature in LB media, respectively. The bacteria were then centrifuged for 13 minutes at 1800 rpm and resuspended in coating buffer (pH 9,6).

Initially, the wells of microtiter plates (Immunosorb, Nunc, product no. 269620) were coated using 200 µl of antigen solution diluted in coating buffer (10^6 bacteria / well) and further incubated at 4 °C over night. Unbound bacteria were removed by three washes in PBS-Tween, pH 7.2. The next day, the plates were blocked using 200 µl of 0.5% bovine serum albumin (BSA) in PBS and shaken for 15 minutes. The wells were then further washed three times in PBS-Tween. To each well, 100 µl of rainbow trout

serum diluted 1:10 in PBS with 0.1% BSA was added and the plates were gently shaken for 30 minutes. The wells were then washed in order to remove unbound plasma. Further, 100 µl of Rabbit-anti trout Ig diluted 1:3000 in PBS with 0.1% BSA (Sigma Aldrich) was then added to the wells and the plates were gently shaken for 10 minutes and the wells were thereafter washed three times. Subsequently, 100 µl of HRP conjugated goat-anti rabbit antibody (BioRad, product no. 172-1019) diluted 1:3000 in PBS with 0.1% BSA was added to the wells followed by shaking for 10 minutes. The wells were then washed three times. Lastly, 100 µl of enzyme substrate (TMB; Sigma Aldrich, product no. T5525) was added to the wells and the plates were incubated for 10 minutes. The reaction was stopped using 50 µl of stop solution (1M H_2SO_4). It was added when a strong colour in the wells was obtained or after maximally 15 minutes of incubation. The absorbance was when measured at 450 nm using a Synergy 2 Multi Mode microplate reader (BioTek Instruments).

2.12. Slaughtering of fish for sensory analysis

Prior to slaughtering fish were killed by a blow to the head. They were further gill-cut and bled for five minutes in ice-water. After slaughter and gutting the fish were stored on ice until the next day where after they were filleted. The obtained fillets were then used for the applied sensory analysis. Fillets from the left side of the fish were used for smoking while right side fillets were used for analysis of the fresh fish.

2.13. Smoking of fish fillets

The fish fillets were smoked at Bisserup Havbrug according to standard procedures. In general, the fillets were firstly dry salted for three hours followed by a short dripping of water. Further, the smoke was generated using a mix of peat litter from beech and crowberry heather. The fillets were then smoked for 40 hours at 28 °C.

2.14. Sensory profiling – fresh fillets

The trout filets were evaluated by sensory profiling after 3 and 7 days of ice storage by a sensory panel consisted of 9 assessors that had been selected, tested, and specifically trained in descriptive analysis (ISO 8586-1, 1993; ISO 8589, 1988) of rainbow trout (Hyldig, 2009a). The first three sessions were quantitative, i.e. the assessors were trained to evaluate the descriptors on a linear scale. Each attribute was evaluated using

a 15 cm unstructured linear scale with two anchor points that were "little" and "much" of attribute intensity. The anchor points were placed 1.5 cm and 13.5 cm from zero on the scale (Meilgaard et al., 1999). The sensory analysis was performed in separated booths under normal daylight and at ambient temperature (ISO 8589, 1988). The assessors used water and flat bread to clean the palate between samples. Data were collected using a computer system (FIZZ Network Version 2.0, Biosystems, France). One fillet from each fish was analysed once by one single assessor. The samples were placed in individual porcelain bowls and covered with porcelain lids with three digit codes. The amount of fish prepared for each assessor was approximately 50 g. The fish samples were heated in a pre-warmed convection oven (RATIONAL Combi-Dämpfer CCM) with air circulation for 15 min at 100°C. After heat treatment the samples were immediately served for the panel. The sensory attributes were for odour; sweet, cooked potato, sea, mushroom and warm milk, for the taste; sweet, fish oil, mushroom, sourish, cooked potato and bitter, for the texture; flaky, juicy, firm, toughness, oiliness and fibrousness and brightness, colour, discoloured for appearance (Hyldig, 2009b). The evaluations were performed in separated booths under normal daylight and at ambient temperature.

2.15. Sensory profiling – smoked fillets

The cold smoked trout file was evaluated by sensory profiling by a sensory panel consisted of 8 assessors that had been selected, tested, and specifically trained in descriptive analysis (ISO 11035, 1994) of rainbow trout (Hyldig, 2009a). The assessors were trained to evaluate the descriptors on a linear scale during two sessions and in the following session all the cold smoked samples were evaluated. Each attribute was evaluated as described for fresh fish. The sensory attributes were for odour; smoked, sweet, sourish, wood tar and off-odour, for the taste; smoked, amine, sourish, sweet, rancid, salty and off-flavour, for the texture; elastic (with a fork), elastic, firm, juicy, toughness, crispiness and oiliness and discoloured, colour and iridescence for appearance (Hyldig, 2009b). Each sample was cut in slices (3 mm wide) and three pieces were put into individual Petri dishes for each assessor.

2.16. Statistical analysis

Statistical tests for difference in antibody titre levels were performed using t-test. The statistical analysis of the sensory data was performed using one-tailed Fishers exact test in order to compare the experimental groups to control fish. Only the parameters regarding texture were used in the statistical analysis. The survival curves of the challenged fish were plotted using a Kaplan-Meier plot and significance was tested using a log-rank test. All statistical analysis was performed in the Graphpad Prism version 4.0 software.

3. Results

3.1. Challenge by Yersinia ruckeri

A number of 733 previously *Yersinia ruckeri* vaccinated and 754 non-vaccinated fish were challenged with *Yersinia ruckeri*. Mortality following challenge by *Y. ruckeri* was initiated on day three post challenge and continued until day 17 post challenge (figure 1). Antibiotics was given from day six post challenge and continued until the mortality had ceased according to veterinarian regulations. A final cumulative mortality of 36.2% and 40.5% was obtained in vaccinated and naïve fish challenged by *Y. ruckeri*, respectively and no significant difference was seen between these two groups ($P > 0.05$). No mortality was observed for control fish.

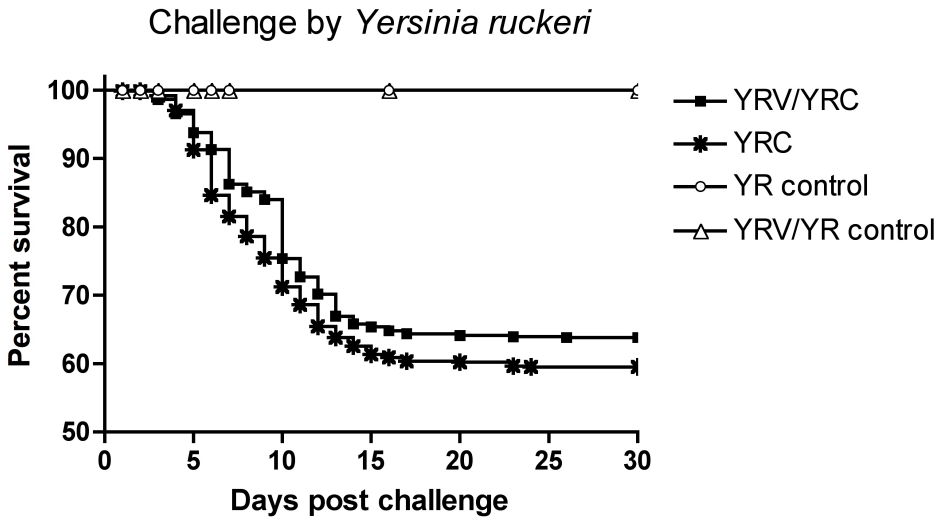

Figure 1. Challenge of rainbow trout by *Yersinia ruckeri*. Vaccinated and non-vaccinated fish showed almost the same survival following intraperitoneal challenge of 36.2 and 40.5%, respectively, showing that the vaccine did not give significant protection relative to vaccinated and non-vaccinated control fish ($P > 0.05$). YRV: *Y. ruckeri* vaccinated; YRC: *Y. ruckeri* challenged. Control fish were not challenged.

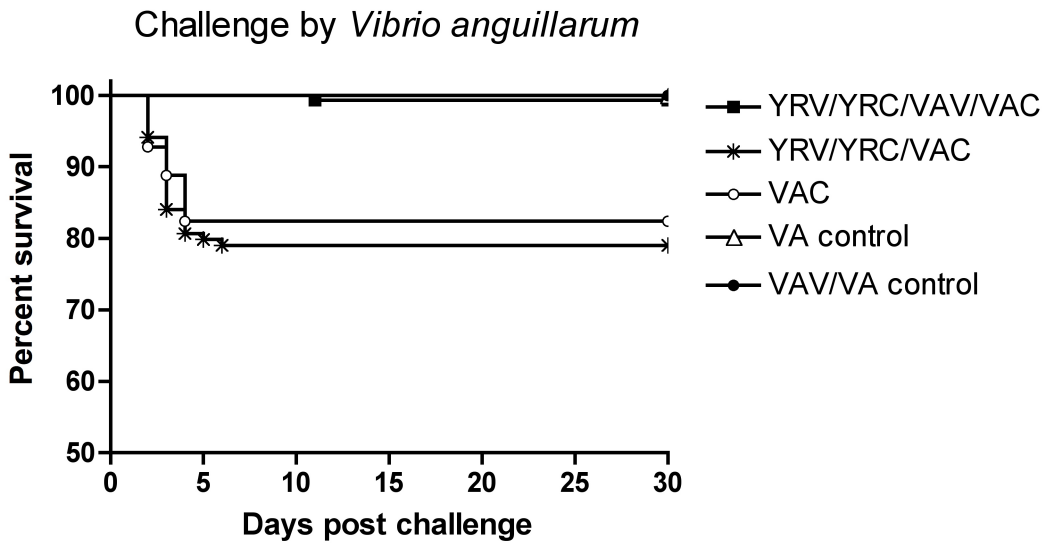

Figure 2. Challenge of rainbow trout by *Vibrio anguillarum*. Non-vaccinated fish and previously *Y. ruckeri* vaccinated + challenged showed a lowered survival following challenge by *V. anguillarum* of 82.4 % and 79.9 %, respectively ($P < 0.05$). No significant different survival was seen between the two groups ($P > 0.05$). YRV: *Y. ruckeri* vaccinated; YRC: *Y. ruckeri* challenged; VAV: *V. anguillarum* vaccinated; VAC: *V. anguillarum* challenged. Control fish were not challenged.

3.2. Challenge by Vibrio anguillarum

A number of 149 previously *Y. ruckeri* vaccinated + *Y. ruckeri* challenged + *V. anguillarum* vaccinated fish were challenged by *V. anguillarum*. Further, 123 previoulsy *Y. ruckeri* vaccinated + *Y. ruckeri* challenged and 124 non-vaccinated fish were challenged by *V. anguillarum*. The survival of non-vaccinated fish following challenge by *V. anguillarum* was higher than for *Y. ruckeri* and vaccinated fish were protected against the bacterium (figure 2). Only 0.7% in total of vaccinated fish died following challenge. Mortality was initiated on day 2 following challenge in naïve fish. A final survival of 82.4 % was obtained in naïve fish following challenge. In the fish that were previously vaccinated against and infected by *Y. ruckeri*, the final survival was 79.0 %. Between these two groups there was no significant difference in survival ($P > 0.05$), but the survival of these two groups was significantly lower than for vaccinated fish ($P < 0.05$).

3.3. Detection of Yersinia ruckeri and Vibrio anguillarum by ELISA

The results of the ELISAs for detection of *Y. ruckeri* and *V. anguillarum* showed presence of antibodies against both bacteria in all groups of fish (figure 3 and 4). The levels of specific antibodies against *Y. ruckeri* and *V. anguillarum* were measured from every

single fish in table 1. For both bacteria, there was no statistical difference between any of the groups in antibody titre (t-test, $P > 0.05$).

Table 1. Sampled fish used for ELISA against *Y. ruckeri* and *V. anguillarum*.

Group	Total no. of fish
Control	39
Y. ruckeri challenged	12
V. anguillarum challenged	10
Y. ruckeri challenged + *V. anguillarum* vaccinated	9
Y. ruckeri vaccinated + challenged + *V. anguillarum* challenged	8
Y. ruckeri vaccinated + challenged + *V. anguillarum* vaccinated + challenged	18
Mechanically damaged	15

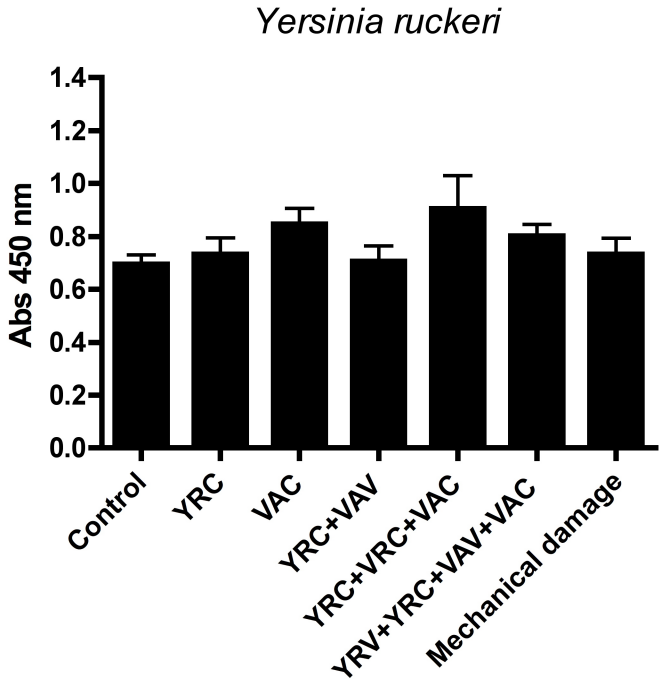

Figure 3. Antibodies against *Y. ruckeri*. The results are shown as absorbance at 450 nm + standard error of mean (S.E.M.). There was no significant difference in antibody titer between any of the groups ($P > 0.05$). YRV: *Y. ruckeri* vaccinated; YRC: *Y. ruckeri* challenged; VAV: *V. anguillarum* vaccinated; VAC: *V. anguillarum* challenged. Control fish were not challenged.

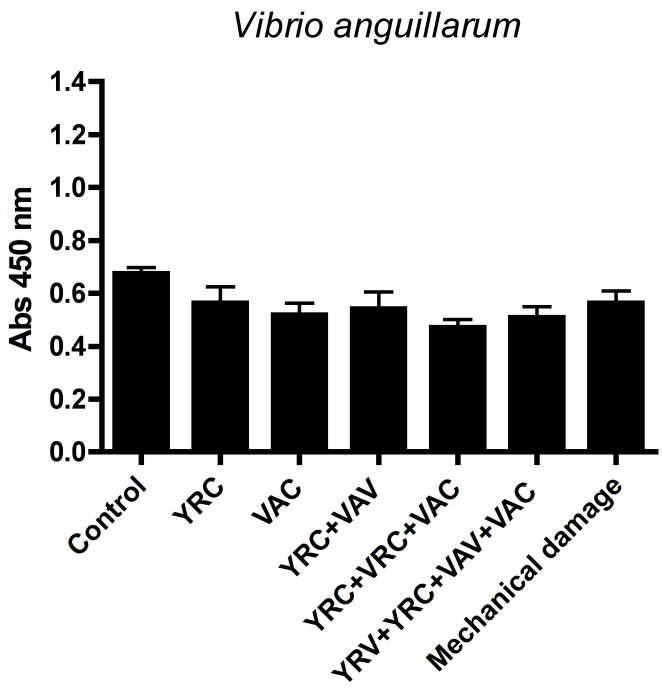

Figure 4. Antibodies against *V. anguillarum*. The results are shown as absorbance at 450 nm + standard error of mean (S.E.M.). There was no significant difference in antibody titer between any of the groups ($P > 0.05$). YRV: *Y. ruckeri* vaccinated; YRC: *Y. ruckeri* challenged; VAV: *V. anguillarum* vaccinated; VAC: *V. anguillarum* challenged. Control fish were not challenged.

3.4. Groups of fish used for sensory analysis

A breakdown of the pump providing the water flow for the land-based fish tanks at Bisserup took place in April 2009. The technical failure resulted in a loss of 757 fish, thus some of the experimental groups were lost. Due to this, the subsequent sensory analysis was reduced to the groups described table 2. If possible, the numbers of fish from each group used for sensory analysis were 8 and 9 for cold-smoked fillets and fresh fillets, respectively. For fresh fish, significant differences were seen between previous diseased fish and control fish after 7 days of storage (table 2). Previously *V. anguillarum* infected fish were significantly less flaky and had a lower oiliness in comparison to control fish ($P < 0.01$). Further, they had a significantly higher toughness in comparison to control fish ($P < 0.01$). Fish previously vaccinated against and challenged with *Y. ruckeri* and later *V. anguillarum* challenged had also a significantly higher toughness and a higher fibrousness in comparison to control fish ($P < 0.01$). No difference between previous diseased fish and mechanical damaged fish in comparison to control fish was seen for smoked fish ($P > 0.05$).

Table 2. Fresh fillets stored on ice for 7 days. All experimental groups are compared to the control group. n.s. = non significant. * = 95 % significant. ** = 99 % significant.

	Flaky	Juicy	Firm	Oiliness	Toughness	Fibrousness
Vac	**	n.s.	n.s	**	**	n.s
Mechanical	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
YRV_YRC_Vac	n.s.	n.s.	n.s.	n.s.	**	**
YRV_YRC_VAV_Vac	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

4. Discussion

The results presented herein are the first published that shows a link between previous infections and sensory quality parameters in rainbow trout. Sensory analysis is a validated and useful method to study different quality parameters of meat regarding odour, taste and texture (Molnar, 1995). In this study, fillets from previously infected and mechanically damaged rainbow trout were examined for sensory analysis as fresh and smoked. The time points of three and seven days of ice-storage of fresh fillets prior to the sensory analysis were chosen in order to make the results comparable to what could be expected within a natural situation in fish farming. Further, the texture is known to undergo changes together with storage time, making it obvious to do measurements at different time points (Faergemand et al., 1995). Only the textural parameters were used for the statistical analysis since changes in this quality parameter could be expected in connection to previous diseases. Both were included in order to examine the usual consumer products industrially processed from that species. Significant results were seen in the study for fresh fillets after seven days of ice-storage regarding textural quality parameters, while no differences between smoked fillets was observed. For fresh fillets, previously challenged rainbow trout by *V. anguillarum* showed changes in most parameters. Fillets from this group were less flaky, a lower oiliness and a higher toughness in comparison to control fish. Further, fish previously vaccinated against and challenged with *Y. ruckeri* and later *V. anguillarum* challenged had also a significantly higher toughness and fibrousness compared to control fish. These results show that previous disease has an impact in the resulting quality of the fish meat. Further, it also shows that the differences were more pronounced after a longer period of ice-storage post slaughter. Both diseases are giving rise to bleedings and damages in the musculature, which further leads to an inflammatory response as the initial step of the tissue regeneration (Høie, 1999; Larsen and Pedersen, 1999). This

is characterised by influx of immune cells in order to combat the pathogen and clear the infected tissue for dying and dead cells (Diegelmann and Evans, 2004). This process together with the later stages of tissue regeneration may typically lead to establishment of scarring in affected regions, as seen for farmed Atlantic salmon following infection by *Moritella viscosa* (Salte et al., 1994). Scarring is a result of deposition of collagen between the live cells and this might account for the textural differences between previously infected and control fish. The textural characteristic 'hardness' has previously been correlated with the amount of collagen in the meat from the shellfish Abelone (*Haliotis discus*) (Hatae et al., 1996). Further, the mechanically damage performed may have had no impact on the fish. A less heavy inflammatory response could be expected following damage in comparison to infected fish since no pathogens were represented in the tissue (Ingerslev et al., 2010). Further, non-vaccinated, *V. anguillarum* challenged fish had the highest textural toughness indicating that vaccination protects against disease and thus tissue damage and scarring. The fish used in the study had been reared under conventional farming conditions and could then be compared to a natural situation. The infections of *Y. ruckeri* and *V. anguillarum* were performed in a way where challenged fish were treated by antibiotics a few days after mortality was observed in order to mimic the situation during a natural outbreak of disease in fish farming. The normal route of entry of bacterial pathogens in the host during a natural infection is through the outer surfaces such as the skin and gills (Lovoll et al., 2009). However, in contrast to a natural infection, the fish were intraperitoneally (i.p.) challenged in order to control the dose of bacteria used for infection. Nevertheless, this was not expected to have an influence of the resulting pathological changes in the musculature compared to a natural route of infection since the bacteria spread systemically through the blood (Høie, 1999; Larsen and Pedersen, 1999). No effect of the *Y. ruckeri* vaccine on the survival following challenge was seen. However, this could be expected in relation to earlier observations (Raida and Buchmann, 2008). Due to the size of the fish at the time of vaccination, the fish were immersion vaccinated against *Y. ruckeri*. This has not shown to be an effective way to vaccinate if the fish are further i.p. challenged. However, the fish were i.p. vaccinated against *V. anguillarum* and for these almost full protection of the vaccine was seen. The ELISA data showed no significant difference any of the different groups either for *V. anguillarum* or *Y. ruckeri*. A survey on the occurrence of typically bacterial pathogens from Danish marine rainbow trout farms

have revealed that both species of bacteria are isolated from fish in these (Pedersen et al., 2008). Hence, the observed antibody titres of the bacteria indicate that the fish have been primed from naturally occurring bacteria in the water and due to this have developed antibodies (Ellis, 1988). However, the presence of *Y. ruckeri* described in the survey by Pedersen *et al*. (2008) could also be due to carriage of the bacteria in the fish from the freshwater phase earlier in life. The time from the last infection and until slaughter was about 16 months. Hence, the long-term effects of disease were examined in this study. It could further be expected that the short-term effects are more prominent, since signs of disease could be expected to be diminished over time. From the results it can be concluded that disease has an impact on product quality-associated parameters and that these are characterised by applying the fresh meat a lowered flakiness and oiliness, but a higher textural toughness and fibrousness. These results may be useful knowledge for the industry, since knowledge about previous history of disease may be used in the choice of strategy regarding the use of the 'raw material' following slaughter. Thus, it might be beneficial to use previously infected fish for smoked products since the impacts of previous disease are greater on the fresh fish. Lastly, the findings underline the importance of vaccination in order to prevent subsequent quality changes.

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Paper II

Fibroblasts express immune relevant genes and are important sentinel cells during tissue damage in rainbow trout (*Oncorhynchus mykiss*)

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Fibroblasts Express Immune Relevant Genes and Are Important Sentinel Cells during Tissue Damage in Rainbow Trout (*Oncorhynchus mykiss*)

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Abstract

Fibroblasts have shown to be an immune competent cell type in mammals. However, little is known about the immunological functions of this cell-type in lower vertebrates. A rainbow trout hypodermal fibroblast cell-line (RTHDF) was shown to be responsive to PAMPs and DAMPs after stimulation with LPS from *E. coli*, supernatant and debris from sonicated RTHDF cells. LPS was overall the strongest inducer of IL-1β, IL-8, IL-10, TLR-3 and TLR-9. IL-1β and IL-8 were already highly up regulated after 1 hour of LPS stimulation. Supernatant stimuli significantly increased the expression of IL-1β, TLR-3 and TLR-9, whereas the debris stimuli only increased expression of IL-1β. Consequently, an *in vivo* experiment was further set up. By mechanically damaging the muscle tissue of rainbow trout, it was shown that fibroblasts in the muscle tissue of rainbow trout contribute to electing a highly local inflammatory response following tissue injury. The damaged muscle tissue showed a strong increase in the expression of the immune genes IL-1β, IL-8 and TGF-β already 4 hours post injury at the site of injury while the expression in non-damaged muscle tissue was not influenced. A weaker, but significant response was also seen for TLR-9 and TLR-22. Rainbow trout fibroblasts were found to be highly immune competent with a significant ability to express cytokines and immune receptors. Thus fish fibroblasts are believed to contribute significantly to local inflammatory reactions in concert with the traditional immune cells.

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Introduction

Throughout the distant evolutionary lineage from the very primitive invertebrates to the phylogenetically more 'modern' vertebrates, an inflammatory reaction is established when tissue is damaged or infection is initiated [1–4]. The types of cells and the signalling molecules taking part in this process are dependent on the evolutionary position of the organism [5]. Among the vertebrates, the inflammatory response is in general characterised by recruitment of cells like polymorphnuclear neutrophilic leucocytes, monocytes and macrophages to the tissue of injury and/or infection [6,7]. Invasion of these cell types mediate enhanced phagocytosis and expression of pro-inflammatory cytokines as IL-1β, TNF-α, and of numerous chemokines like IL-8 and other CXC chemokines [8-10]. The inflammatory immune reaction may not only be elicited by 'traditional' immune cells like macrophages and neutrophils that are recruited or already present in the tissue. Several reports suggests that in humans, the conventional non-immune cell-type fibroblast, also have immune regulating capabilities [11–13] and can thus serve as highly important sentinel cells [14]. Fibroblasts are the least specialised member of the connective tissue family, whose main function is to maintain the structural integrity of connective tissue by secreting precursors of extracellular matrix rich of type I and/or type-III collagen [15]. At the same time, human fibroblasts are known to express a wide range of cytokines. These include, among others, IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, TNF-α, IFN-γ, CCL20 and macrophage colony stimulating factor [12,16]. These cytokines are also known in lower vertebrates like the fish species rainbow trout and zebrafish (*Danio rerio*) [17-26]. In addition, human fibroblasts also respond to the secretion of cytokines like IL-1β, TNF-α e.g. by enhancing the production of CCL20 and hyaluronan [12,27]. Besides expressing various Toll-like receptors (TLRs) [28] these characteristics indicate the presence of several other immune receptors on fibroblasts [12] and subsequent downstream pathways to convey signalling through these [29–31]. Further, some human fibroblasts have also been found to be phagocytic and capable of generating respiratory burst reactions as well as being responsive to lipopolysaccharide (LPS) from *E. coli*. [32–35]. The sensitivity to LPS most likely relates to the presence of CD14/TLR2/TLR4 receptors on the fibroblast surface, which further indicate that fibroblast serve an important sentinel function through pathogen-associated molecular pattern (PAMP) recognition [11,32,36–38]. It then seems reasonable that fibroblasts also respond to damage-associated molecular patterns (DAMPs) like necrotic cells and other hydrophobic portions (hyppos) from 'self' parts exposed through tissue damage [39].

Whether fibroblasts in lower vertebrates also exhibit phagocytic capacity and/or serve as sentinel cells is currently not known, but previous results from carp (*Cyprinus carpio*) could indicate this.

Thirty minutes following injury of the skin, significant up-regulation of CXC receptors, IL-10 and TNF-α was reported [40]. This could indicate the presence of local, responsive cells that were already present in the tissue prior to injury. In lower vertebrates such as fish from the carp family, presence of TLR2 and TLR4 have been shown [41]. However, in evolutionary older fish species like the salmonids, they do not seem to be present although other TLRs have been reported [42–44]. Due to the phylogenetic position of fish, they are by choice considered to be an excellent animal to study immune mechanisms since it is the earliest divergent vertebrate, which have both an innate and adaptive immune system [1,3]. Conversely, the scarce availability of antibodies in teleost research to date often hampers the opportunity for functional experiments. However, the ongoing sequencing of immune genes in fish makes real-time RT-PCR for measuring gene expression a good alternative. As in humans, the adaptive immune system in fish is characterised by the presence of antigen-specific receptors (T cell receptors), immunoglobulin on lymphocytes and MHC class I and II surface molecules [1,45]. However, the immunoglobulin isotype repertoire in teleost fish is more limited than in e.g. mouse and humans and consists of only IgM, IgD and a unique fish isotype termed IgT [46,47]. The specific immune cell-repertoire consists of T- and B-cell subpopulations, whereas the non-specific cells consist of phagocytic cells like monocytes/macrophages, granulocytes and natural killer cells [1,48]. Several different fibroblast cell lines have been characterised from fish. In rainbow trout (*Oncorhynchus mykiss*) at least three fibroblast cell lines termed RTG-2, RTG-P1 and RTHDF exist of where the RTG-2 cell line is expressing the immune genes Mx, iNOS, IL-1β and IL-18 [49-55]. In order to examine the immune capabilities of fibroblasts in fish, an *in vitro* assay was setup using the rainbow trout RTHDF cell-line [53]. The cells were subjected to either LPS from *E. coli*, debris or supernatant from sonicated RTHDF cells in order to introduce fibroblast receptors to both PAMP and DAMP stimuli. Further, in order to examine the role of fibroblasts *in vivo*, a model of sterile, mechanical tissue damage of muscle tissue in rainbow trout was established. From both experiments, the muscle tissue and the fibroblasts were subsequently subject to real-time RT-PCR. Expression of the pro-inflammatory cytokines IL-1β and IL-8 as well as the anti-inflammatory cytokine IL-10 was measured since these are key molecules in the initial, inflammatory reaction [56–59]. The multifunctional gene transforming growth factor-β (TGF-β) was also included in order to show whether regenerative and proliferative responses were activated following tissue damage. Finally, we examined the expression of the four different TLRs; 3, 5, 9 and 22 in order to show if these were sensitive to PAMPs and/or DAMPs [42,60,61]. Together, this study provides new and important information about the role of fibroblasts in lower vertebrates in relation to inflammation, tissue damage and immune competence.

Materials and Methods

Culturing of RTHDF Fibroblasts

Rainbow trout hypodermal fibroblasts (RTHDF [53]) were cultured in Leibovitz L-15, supplemented with 15% (w/v) foetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100 μg/ml) at 21°C and atmospheric air, as described previously [53]. Trypsin solution for cell detachment was made by dissolving 0.1% (w/v) trypsin and 1 mM disodium EDTA in phosphate-buffered saline, PBS [137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4] [62]. All cell culture reagents were used cold from the refrigerator. Cells were subcultured in 25 cm^2 tissue culture flasks 3 days prior to the experiments and were confluent when harvested. The amount of cells per culture flask when confluent was estimated to 4.5×10^5 as earlier described [53]. All cell culture reagents were purchased from Life Technologies Inc. (Naperville, IL, U.S.A.) and cell culture plastic wear were purchased from TPR (Trasadingen, Switzerland). Chemicals were from Sigma Aldrich, unless otherwise stated.

Stimulation of RTHDF Fibroblasts with LPS, Cell-Debris and Supernatant

Confluent 25 cm^2 cell culture flasks of RTHDF cells containing 3 ml of cell-culture medium were either incubated with sonicated RTHDF fibroblasts, the supernatant from the sonicate or *E.coli* 0111:B4 LPS (Sigma-Aldrich). The sonicate was prepared by an initial trypsination of confluent cell culture flasks followed by sonication for 30 s (amplitude 10 microns) using a MSE Soniprep 150 sonicator (Sanyo). The sonicate was then centrifuged for 2 min at 14.000× g and the clear supernatant was transferred to a new tube. The pellet containing necrotic cells and cell debris was thereafter resuspended in L-15 medium and used for stimulation. The RTHDF fibroblasts were then stimulated in triplicate of cell culture flasks per sampling point. The amount of sonicate added per flask of stimulated cells originated from one flask of RTHDF fibroblasts. The LPS stimulation was performed using 20 μg ml^{-1} of LPS and the amount of supernatant used was 100 μl per cell culture flask. Non-stimulated cells were used as controls. Harvesting of cells for isolation of total RNA was then performed 1 hour, 4 hours and 24 hours post stimulation. This was accomplished by removal of the cell culture medium from the flasks followed by addition of 500 μl of lysis buffer plus 5 μl of β-mercaptoethanol from the GenElute Mammalian™ Total RNA Miniprep Kit (Sigma-Aldrich). The cells were then scraped off the bottom of the flasks with a cell scraper (Greiner Bio-one) and RNA was isolated according to the manufacturers instructions.

Phagocytic Assay

Phagocytosis of the RTHDF fibroblasts was studied using carboxylate-modified polystyrene fluorescent latex beads with a diameter of 1.0 μm (Sigma-Aldrich) as described by Ganassin *et al.* [63]. A suspension of beads was prepared by adding 2 μl of the commercial latex suspension (2.5% solids latex) to 5 ml of growth medium, which replaced the regular growth medium. The cell-cultures were then observed at 1 hour, 4 hours and 24 hours after incubation and observations were made with a Nikon fluorescence microscope. Prior to observation, the cells were washed four times with PBS in order to wash away adhered, non-ingested beads.

Rearing Conditions of Mechanically Damaged Fish

Unvaccinated, healthy rainbow trout (*Oncorhynchus mykiss*) reared at Agerskov Dambrug (Bording, Jutland), where delivered to the experimental facilities at the National Institute of Aquatic Resources, Technical University of Denmark at 21 March 2007. The fish were then acclimatised one month prior to mechanical injury and maintained in 200 l plastic tanks with aerated local tap water at 14°C and exposed to a light regime of 16 hours of light followed by 8 hours of dark. Average weight (g ± SD) and length (cm ± SD) at the samplings were 9.5±2.6 and 9.5±0.9, respectively ($n = 40$). At each sampling point five injured fish and five control fish were collected. All procedures were conducted in accordance with the regulations set forward by the Danish Ministry of Justice and animal protection committees by Danish Animal Experiments Inspectorate permit 2007/561-1302 and in compliance with European Community Directive 86/609.

Procedures for Mechanical Injury and Tissue Sampling

Thirty fish were mechanically injured just below the caudal end of the dorsal fin at April 26 using a home made device containing twenty five sterile needles made from 19G syringes (Becton Dickinson) with a thickness of 1.1 mm equally distributed on an area of 6 mm×6 mm. The needles had a depth of 6 mm to ensure penetration of both the skin and underlying muscle tissue of the fish. Prior to mechanical injury, fish were anaesthetised in MS-222 (50 mg/l) (Sigma-Aldrich). The injury was performed posterior to the dorsal fin above the lateral line and the device was penetrated twice through the skin, giving rise to fifty holes per fish. Prior to sampling of muscle tissue, fish were killed in an overdose of MS-222. Sampling occurred at 4, 8 and 24 hours post injury using a sterile disposable scalpel. The tissue collected was 1) muscle tissue from the site of injury and 2) non-injured muscle tissue from the opposite side of the fish relative to the injury tissue and 3) muscle tissue from non-injured control fish. By sampling the internal control it was possible to show whether the responses in the injured fish were local or systemic. Tissue was collected from five injured and five control fish per sampling point and was transferred to cryo tubes containing RNA *later*® (Sigma-Aldrich) and stored at −20°C until isolation of RNA. For this, fifty mg of the sampled tissues were homogenised by sonication for 30 s (amplitude 10 microns) using a MSE Soniprep 150 sonicator (Sanyo) and RNA was further isolated using a GenElute Mammalian^{TM} Total RNA Miniprep Kit (Sigma).

CDNA Synthesis

The RNA quality and quantity from the RTHDF cells and the muscle tissue was checked by $OD_{260/280}$ measurements on a GeneQuant II Spectrophotometer (Pharmacia Biotech) and the RNA was finally treated with DNase-I (Sigma-Aldrich) to remove any genomic DNA. Random hexamer primed reverse transcription reactions were performed from 400 ng of total RNA in a 20 µl setup using TaqMan® Reverse Transcription reaction (Applied Biosystems). The synthesised cDNA samples were diluted 1:10 in MilliQ H_2O and stored at −20°C.

Quantitative RT-PCR

Quantitative RT-PCR was performed using a Stratagene MX3000P^{TM} real-time PCR system, dual-labelled TaqMan® probes conjugated with either a 5' HEX or a 5' FAM fluorophor,

Table 1. Sequences of primers and probes used for the real-time PCR analysis.

Gene	Primer	Sequence (5′-3′)	GenBank acc. no.	Amplicon (bp)
	Forward	ACCCTCCTCTTGGTCGTTTC		
EF-1α	Reverse	TGATGACACCAACAGCAACA	**AF498320**	63
	Probe	GCTGTGCGTGACATGAGGCA		
	Forward	AGCCGCAACGTCAAGTCT		
RPS20	Reverse	GTCTTGGTGGGCATACGG	**NM_001124364**	104
	Probe	TGTGCAGACCTTATCCGTGGAGCT		
	Forward	AGGACAAGGACCTGCTCAACT		
IL-1β	Reverse	CCGACTCCAACTCCAACACTA	**AJ278242**	72
	Probe	TTGCTGGAGAGTGCTGTGGAAGAA		
	Forward	GAGCGGTCAGGAGATTTGTC		
IL-8	Reverse	TTGGCCAGCATCTTCTCAAT	**AJ310565**	72
	Probe	ATGTCAGCGCTCCGTGGGT		
	Forward	GGGTGTCACGCTATGGACAG		
IL-10	Reverse	TGTTTCCGATGGAGTCGATG	**AB118099**	121
	Probe	ATCTCGACACGGTGCTGCCCAC		
	Forward	ACGCCACAGCCAGCTTAG		
TGF-β	Reverse	CGCACACAGCAACTCTCC	**X99303**	87
	Probe	TCTCGGAAGAAACGACAAACCA		
	Forward	ACGGCTCAACCTGAATATGG		
TLR-3	Reverse	GCTCTCCAGTGCCCTTAGTG	**DQ459470**	97
	Probe	----------		
	Forward	GGCATCAGCCTGTTGAATTT		
TLR-5m	Reverse	ATGAAGAGCGAGAGCCTCAG	**AB091105**	89
	Probe	GCTCAGTCATATCGTGTGAGGAGGA		
	Forward	GCAACCAGTCCTTCCACATT		
TLR-9	Reverse	AAACCCAGGGTAAGGGTTTG	**NM_001129991**	73
	Probe	----------		
	Forward	AAGGCGCTTCGAGAGTTGAAT		
TLR-22	Reverse	TGGAGAGAGGCTGAAATGATGAG	**AJ628348**	148
	Probe	----------		

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a 3' BHQ_1 quencher and desalted primers (Sigma-Genosys). The assays for TLR-3, TLR-9 and TLR-22 examined were run using SYBR® Green (Sigma-Aldrich) instead of a probe. The genes chosen for investigation were IL-1β, IL-8, IL-10, TGF-β, TLR-3, TLR-5m (membrane bound form), TLR-9 and TLR-22. For use as an internal control and for normalisation of the results the reference gene ribosomal protein S20 (RPS20) and elongation factor-1α (ELF-1α) were validated for their transcriptional stability in muscle tissue and the RTHDF cells (data not shown). RPS20 was used within muscle tissue since it was more stably expressed between the different individuals and injured versus non-injured fish compared to ELF-1α. For the RTHDF cells, the elongation factor-1α gene was found more suitable [64]. The sequence for the primers and probes, amplicon length and GenBank accession numbers are shown in Table 1. The primers were optimised according to $MgCl_2$ and primer concentrations. The cycling conditions for the TaqMan® assay were 94°C for 2 min followed by 40 cycles of 94°C for 15 s and 60°C for 1 min. The cycling conditions for the SYBR® Green assays were the same but the run was terminated by a melting curve analysis where the fluorescence was continually measured during a temperature increase from 60°C to 95°C. Wells for the TaqMan® assays contained 12.5 µl of JumpStart™ *Taq* ReadyMix™ (Sigma–Aldrich), 0.25 µl ROX (Sigma–Aldrich; diluted 10x), 2.5–5.5 mM $MgCl_2$ (Sigma–Aldrich), 0.75–1.25 µl forward and reverse primer (10 mM), 1 µl TaqMan® probe (200 nM), 5 µl of diluted cDNA and autoclaved MilliQ water to a volume of 25 µl. Wells for the SYBR® Green assays contained 12.5 µl of SYBR® Green JumpStart™ *Taq* ReadyMix™ (Sigma-Aldrich), 0.25 µl ROX (Sigma–Aldrich; diluted 10x), 3.5–5.5 mM $MgCl_2$ (Sigma-Aldrich), 1 µl forward and reverse primer (10 mM), 5 µl of diluted cDNA and autoclaved MilliQ water to a volume of 25 µl. The expression results were analysed using the $2^{-\Delta\Delta Ct}$ method after verification that the primers amplified with an efficiency of approximately 100% (doubling of the product between every cycle in the log-linear phase) and data were shown as $-\Delta\Delta C_t$-values and fold expression relative to non-injured control fish or non-stimulated RTHDF cells [65]. The threshold cycle (*Ct*) was determined manually and set to 0.01 in the lower level of the log-linear area. The statistical analysis was performed on the ΔC_t values by a two-tailed T-test in cases of normally distributed data and Mann-Whitney U-test in cases when they were not normally distributed. The statistical software GraphPad Prism version 4.03 was used to calculate the statistics and create graphs.

Results

Constitutive Expression of Selected Genes

All genes except for TLR-22 in the RTHDF cells were expressed within 40 cycles of PCR in non-injured tissue and non-stimulated cells (Figure 1). Except for TLR-9, the constitutive level of expression of the immune genes was higher in the RTHDF cells than in muscle tissue. The highest difference was seen for IL-1β and IL-8 that were approximately 20 and 330 folds higher expressed in the RTHDF cells compared to muscle tissue, respectively. The constitutive expression level of the housekeeping genes was lower for the RPS20 in the muscle tissue compared to the ELF-1α in the RTHDF cells and both genes showed a lower transcriptional variance between individuals/cell replicates than the immune genes.

Expression in RTHDF Cells following Stimulation with LPS, Debris and Supernatant

Figure 2 A-E shows the effect of *E. coli* LPS (0111:B4), debris or supernatant from sonicated RTHDF cells on the expression of IL-1β, IL-8, IL-10, TLR-3 and TLR-9 in the RTHDF cells after

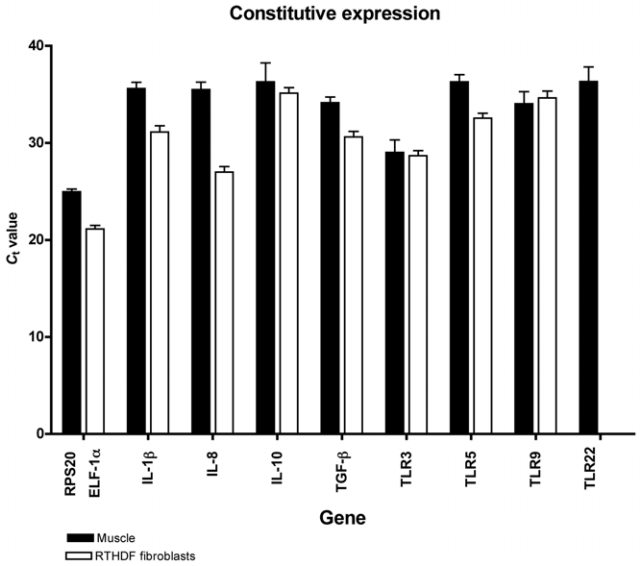

Figure 1. Constitutive expression of the examined genes. The C_t-values for RPS20, ELF-1α, IL-1β, IL-8, IL-10, TGF-β, TLR-3, TLR-5m, TLR-9 and TLR-22 are shown for muscle tissue (black bars) and for RTHDF cells (white bars). The data are presented as mean expression of the control fish from all samples points and mean expression of control RTHDF cells from all sample points. The C_t value is defined as the threshold cycle number of PCR at which the sample fluorescent signal passes a fixed threshold above the baseline.
doi:10.1371/journal.pone.0009304.g001

1, 4 and 24 hours of stimulation. No influence on the expression of TGF-β and TLR5m was seen and TLR-22 was not expressed in the RTHDF cells after 40 cycles of PCR (data not shown). Overall the variation in expression between cell replicates was much lower compared to individual fish. The highest effect on all genes was seen for IL-1β and IL-8 after stimulation with LPS. The expression increased significantly after 1 hour of stimulation for both genes peaked after 4 hours to 42.5 and 22 folds, respectively, followed by a decrease in expression to 9.4 and 9.9 folds after 24 hours, respectively ($P<0.05$). The pattern for IL-10 was different and the response was slower than for IL-1β and IL-8. IL-10 was only significantly expressed at 24 hours after stimulation by 2.7 folds relative to non-stimulated cells ($P<0.05$). Stimulation by debris revealed significant impact on only IL-1β, but the response was weak and only significantly elevated at 4 hours post stimulation by 2.2 folds ($P<0.05$). Supernatant from the sonicated RTHDF cells increased the expression of both IL-1β and IL-10, but weakly compared to the effects of LPS. The IL-1β expression increased to 1.9 folds after 4 hours of stimulation, while for IL-10 it was 2 folds after 24 hours relative to non-stimulated cells ($P<0.05$). TLR-3 and TLR-9 showed similar expression patterns. Both LPS and supernatant increased the expression significantly after 24 hours; LPS to approximately 4 fold for both genes and supernatant to 1.6 and 2.5 folds for TLR-3 and TLR-9, respectively ($P<0.05$).

Phagocytic Assay

The addition of latex beads to the RTHDF cells did not indicate that the cells were able to phagocytose (data not shown). After incubation with beads and washing, a few beads were still attached to the glass slide surrounding the cells, and there was no indication that any beads were taken up and positioned inside the RTHDF cells.

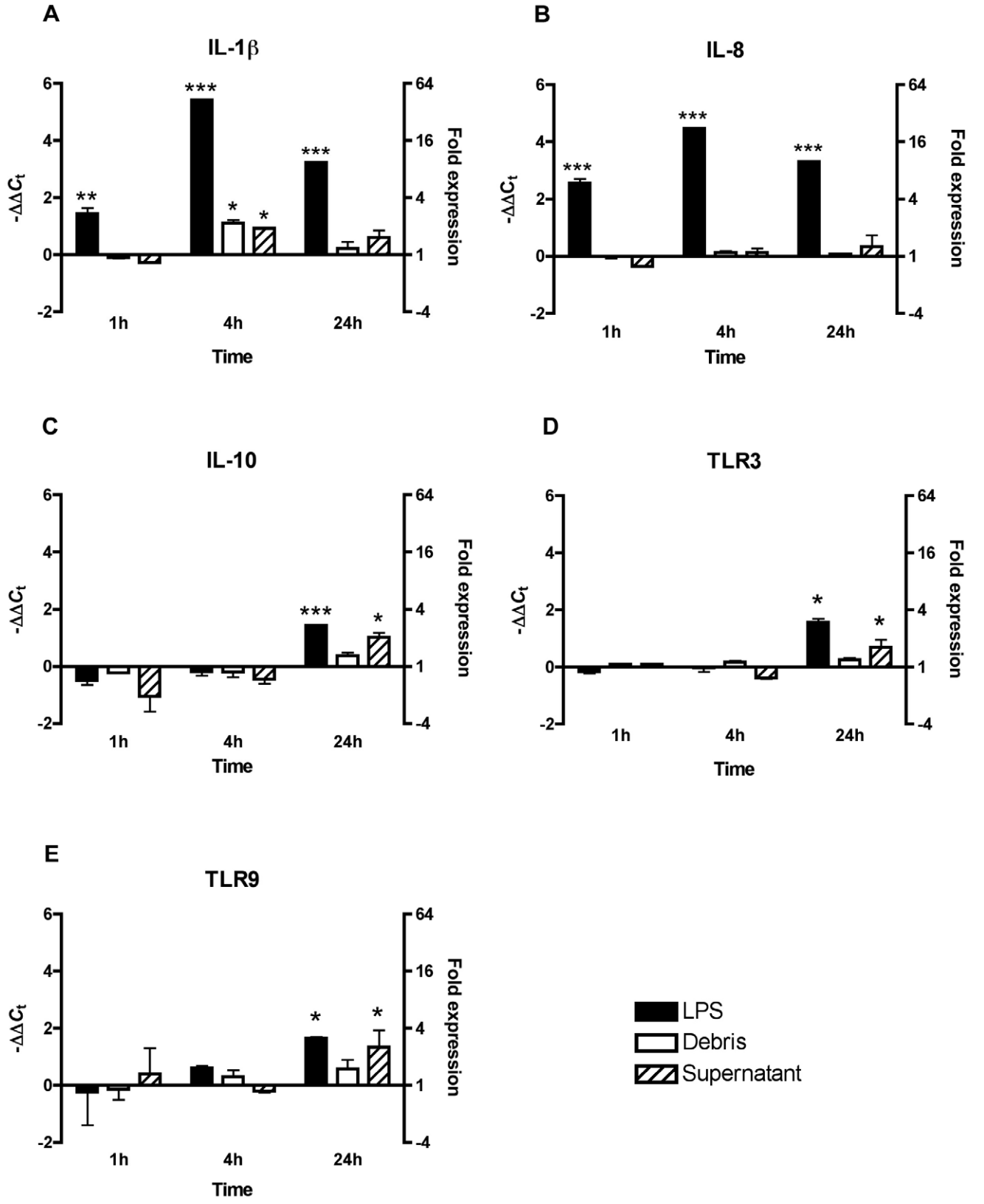

Figure 2. Quantitative real-time PCR for the RTHDF cells. Expression is shown for the genes (A) IL-1β, (B) IL-8, (C) IL-10, (D) TLR-3 and (E) TLR-9. Black bars represent expression in *E.* coli LPS stimulated cells relative to control cells; white bars indicate expression in cells stimulated with debris from sonicated RTHDF cells relative to control cells and striped bars denotes expression in cells stimulated with supernatant from sonicated RTHDF cells relative to control cells. The data are normalised relative to the expression of elongation factor-1α and analysed using the $\Delta\Delta C_t$ method. Data are shown as $-\Delta\Delta C_t$ values and fold expression. Bars represent mean values of $-\Delta\Delta C_t$ + SD values from three cell replicates. * Depicts statistical significance between stimulated cells and control cells (*$P<0.05$; **$P<0.01$; ***$P<0.001$). A $-\Delta\Delta C_t$ value of 0 means no regulation relative to control cells.
doi:10.1371/journal.pone.0009304.g002

Mechanical Injury of Muscle Tissue and the Effects on Gene Expression

The device used for mechanical tissue damage is shown in figure 3 A–B. The following effects of mechanical injury of the muscle tissue on the expression of the genes IL-1β, IL-8, TGF-β and TLR-3, 5 m, 9 and 22 at the time points 4, 8 and 24 hours post injury (p.i.) is shown in figure 4 A-F. No significant changes were seen for IL-10 ($P>0.05$) (not illustrated). The study showed a strong induction of immune related genes, especially of the cytokines IL-1β, IL-8 and TGF-β in the injured muscle tissue at all samplings. The average up-regulation in the injured fish for the three sampling points was between 5.2 to 21.8 folds for IL-1β; 20.0 to 59.3 folds for IL-8 and 4.9 to 13.9 folds for TGF- β. For all three genes the mean expression level was increasing from 4 hours to 24 hours post injury and the expression at the site of injury was significantly higher at all sampling points compared to the non-injured samples from the same fish ($P<0.05$).

The expression of the TLRs in muscle tissue showed significant changes in the expression for TLR-3, 9 and 22, but not for TLR-5m. TLR-3 was significantly up-regulated at the site of injury 24 hours post damage relative to the control fish corresponding to 4.6 folds while no changes was seen for the internal control samples ($P<0.05$). The expression of TLR-9, however, was significantly up-regulated at the injury site 4 hours and 24 hours by 2.5 and 7.2 folds, respectively ($P<0.05$). TLR-22 showed significant differences between injured and non-injured site 4 hours and 24 hours p.i. indicated by small down-regulations at the non-injured site of 3.2 and 2.8 folds, respectively ($P<0.05$). Twenty-four hours post injury, the expression of TLR-22 was significantly up-regulated by 5 folds at the site of injury relative to control fish ($P<0.05$).

Discussion

Previous studies have shown that mammalian fibroblasts have immune regulatory capabilities due to their ability to express cytokines and immune receptors on their surface and their reactivity to pathogen associated molecular patterns (PAMPs) [12,13]. In the present study it is demonstrated, by using the rainbow trout as a model, that these characteristics of the fibroblasts are evolutionary old and that Toll-like receptor and cytokine gene orthologoues to the mammalian counterparts are induced following stimulation in fibroblasts from this fish species. Further, it was shown that rainbow trout fibroblasts are reactive to damage associated molecular patterns (DAMPs). *Seong* and *Matzinger* (2004) proposed the DAMP model and according to this, any molecule that is normally not exposed can be a DAMP if it is revealed during, after or because of injury or damage [39]. Hence, in order to show if 'foreign' molecules were more potent inducers than 'self' in the fibroblasts, the sonicated fibroblasts were separated into debris containing cell-surface fractions and supernatant containing endogenous molecules like mitochondrial DNA and intracellular proteins [39,66]. Interestingly, both debris and supernatant induced the expression of IL-1β, whereas only the supernatant stimulated IL-10, but IL-8 was not induced at all. However, debris might also contain 'foreign' parts of molecules and structures since the interior side of the cell wall is normally not exposed and could explain why debris stimulated the IL-1β expression. Together, this shows that fibroblasts potentially are able to take part in the inflammation in the body and react to signals released from injured cells. Worthy of note, the results also showed that only LPS induced IL-8 expression in fibroblasts *in vitro*, whereas neither fraction of the injured cells or supernatant could do so. Hence, subsequent neutrophil recruitment via IL-8 chemoattraction most likely relies on stimulation by PAMPs rather than by DAMPs. Alternatively, other cell types in the injured tissue could account for the increase in IL-8 expression observed *in vivo*. However, the constitutive expression of IL-8 was higher in the RTHDF cells than in muscle tissue, indicating that the contribution of IL-8 from fibroblasts is not without significance. In this study, the idea of looking into the immune regulating capabilities of fibroblasts *in vivo* was derived from the results that were obtained from the *in vitro* study using the RTHDF cell-line. From this, we hypothesized that local cells in the musculature of the rainbow trout were able to express cytokine genes following tissue damage and hence were able to react to DAMPs. Previously, it has been shown that skeletal muscle tissue in humans produce IL-6, IL-8 and IL-15, and it could be suggested that local

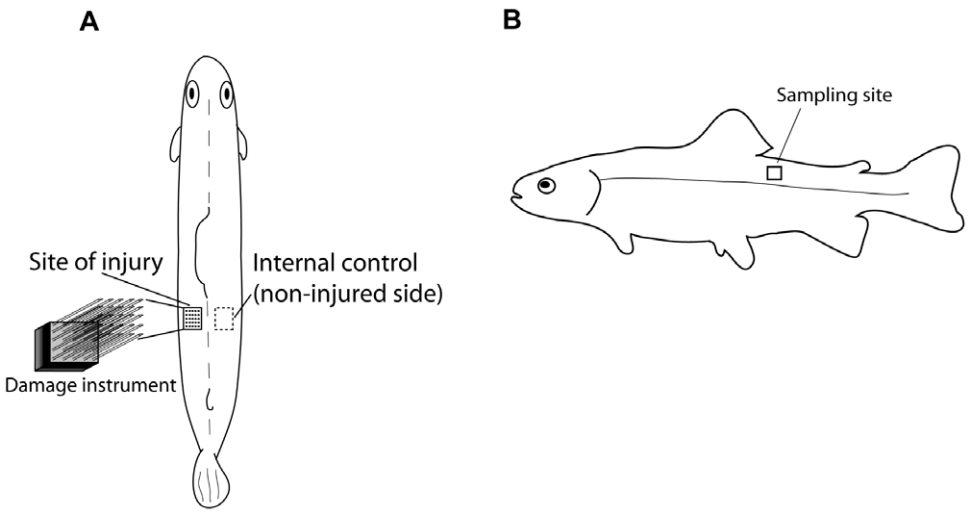

Figure 3. Damage procedures and sampling from mechanically injured rainbow trout. The fish were injured on the left side behind the dorsal fin using the damage instrument. Muscle tissue samples were taken in the injured area while the internal control samples were taken from the same place relative to the dorsal fin on the right side of the fish (A). The vertical position of the site of injury/sampling site is shown in (B). Sampling of muscle tissue from non-injured control fish was performed in the same area as shown for injured fish (not shown).
doi:10.1371/journal.pone.0009304.g003

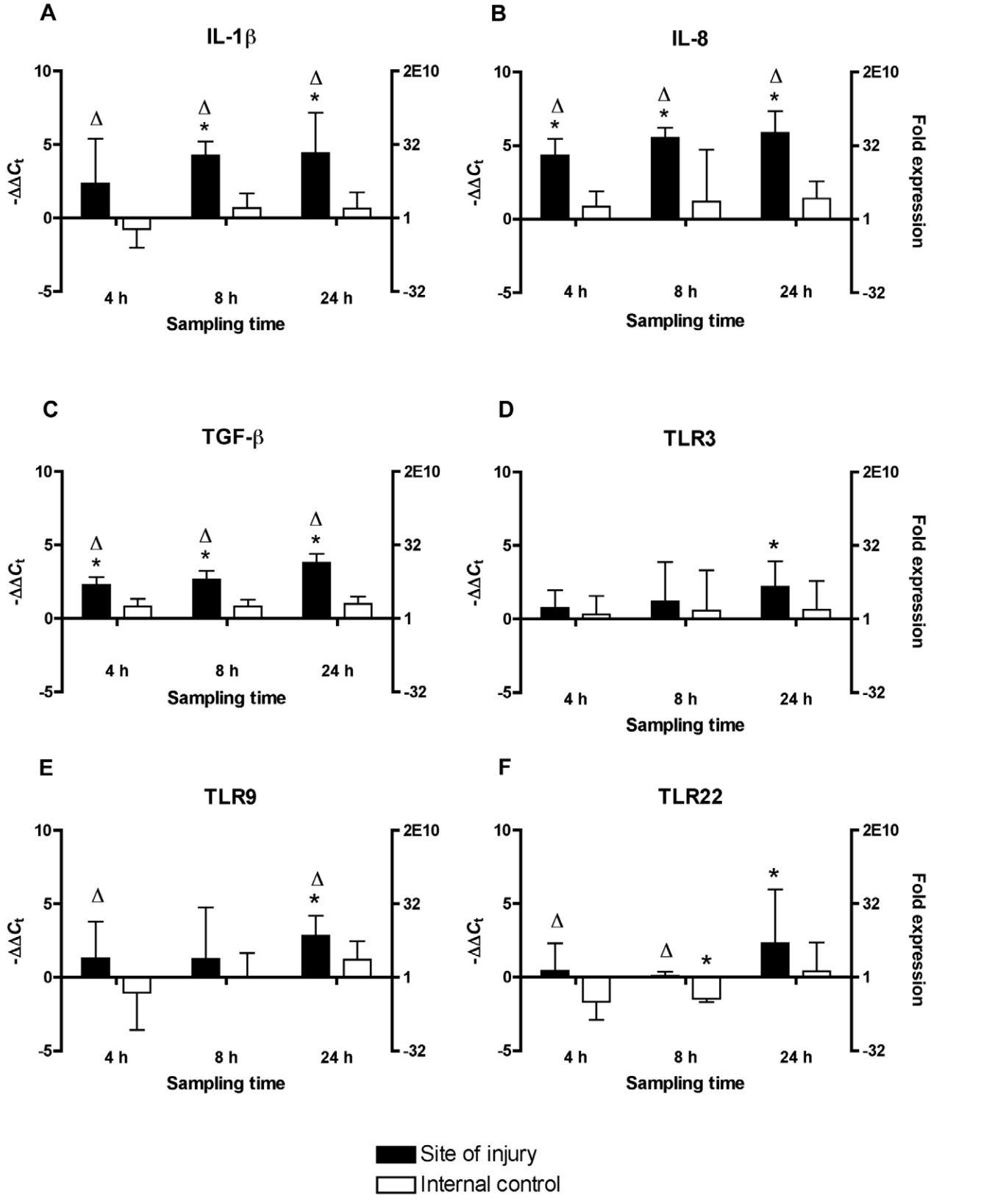

Figure 4. Quantitative real-time PCR for mechanically damaged fish. Expression is shown for the genes (A) IL-1β, (B) IL-8, (C) TGF-β, (D) TLR-3, (E) TLR-9 and (F) TLR-22. Black bars represent expression at the site of injury relative to control fish and white bars indicate expression at the non-damaged internal control site relative to control fish. The data are normalised relative to the expression of ribosomal protein S20 and analysed using the $\Delta\Delta C_t$ method. Data are shown as $-\Delta\Delta C_t$ values and fold expression. Bars represent mean values of $-\Delta\Delta C_t$ + SD values from five individuals. * Depicts statistical significance between injured fish and control fish ($P<0.05$); Δ denotes statistical significant difference between site of injury and internal control site ($P<0.05$). A $-\Delta\Delta C_t$ value of 0 means no regulation relative to control fish.
doi:10.1371/journal.pone.0009304.g004

fibroblasts contributes to this [67]. By applying the damage model in the rainbow trout, internal controls in muscle tissue from the injured fish opposite to the damaged side could be sampled. This gave a unique opportunity to show whether the responses were local or systemic. An activation of local cells was initiated at the injury site due to a rapid induction four hours post sampling of IL-1β, IL-8 and TGF-β. This did not occur within the internal control site. It seems realistic that an up-regulation earlier than four hours took place since the level of especially IL-8 was high four hours post injury. The *in vitro* experiment supports this idea, since LPS stimulated RTHDF cells expressed IL-1β and IL-8 significantly higher than control cells already one hour after stimulation. Further, in zebrafish an induction of H_2O_2 following tail cutting has been observed in the wound a few minutes after cutting, indicating a very rapid activation of local cells [68]. During the evolution from the early vertebrates (fish) to mammals a number of different TLRs have evolved. Fishes comprise a large heterogeneous group with a high degree of evolutionary distance between species, which is also reflected in the numbers and specific types of TLRs present. In the rainbow trout, five different TLRs named 3, 5, 9 and 22 are known, where TLR-22 is only found in fish [61]. In the evolutionary more 'advanced' pufferfish *Fugu rubripes* and the zebrafish *Danio rerio*, a complete set of TLRs orthologous to the ten mammalian TLRs is described [69]. In addition, they also express TLR-21 and 22, where TLR-21 is unique to fish [61]. Both the damage model and the fibroblast *in vitro* experiment showed that the TLR-3 and 9 genes were inducible by DAMPs at the site of injury and by the supernatant from the sonicated fibroblasts, but not by the debris. Hence, this was in accordance with the DAMP model proposed by *Seong* and *Matzinger* since endogenous proteins and molecules released following tissue damage were able to trigger the expression of immune genes [39]. Further, LPS induced TLR-3 and 9 expression in the fibroblasts showing that PAMPs could also stimulate these cells. In contrast, TLR-5 was not affected either in mechanically damaged tissue or in stimulated fibroblasts, indicating that this receptor is triggered by other ligands. TLR-22 was induced by mechanical damage in the rainbow trouts, but not expressed in the non-stimulated RTHDF cells. In the mammals, TLR-3 binds dsRNA, but is also a sensor of tissue necrosis during acute inflammation [70]. Our findings in the rainbow trout fibroblasts suggest that this characteristic is evolutionary old. It is not known whether TLR-3 and 9 are endogenous positioned in cells from rainbow trout, as generally seen in mammalian cells. However, although mostly expressed endosomally, TLR-3 have been shown to be surface expressed in human skin and lung fibroblasts, in certain human fibroblast cell lines and on epithelial cells. Whatever might be the case in fish; our results show that these receptors are inducible in the rainbow trout fibroblasts without the cells being able to phagocytise latex beads. This is in contrast to e.g. macrophages and granulocytes in the rainbow trout, which are phagocytic cell-types [48,71]. Future studies using nano particles could reveal if the RTHDF cells are able to take up smaller particles [72]. Further, the ability of supernatant to induce TLR-9 expression in the fibroblasts showed that this receptor also functions as a sensor of necrosis. In the closely related species Atlantic salmon (*Salmo salar*), an increase in TLR-9 expression after CpG stimulation have been shown in lymphoid tissue indicating a homologue function of this receptor to the mammalian TLR-9 [73]. Since the salmonids lack TLR-2, it might therefore be suggested that TLR-3 and 9 in these species could have a similar role as TLR-2 in the mammals, where this receptor is also a sensor of necrotic cells, while TLR-9 in the mammals recognises bacterial and viral DNA, unmethylated CpG and DNA without CpG motifs [39,74]. Together, these results indicate that evolutionary fibroblasts are primitive cells, which have maintained the ability to express genes of immune functions, when stimulated. Their function as being positioned in the tissue, as 'already there' before the more potent immune cells are recruited, seems advantageous when damage, injury or infection occurs. However, due to their lack of phagocytosing capability it seems that their immune capacity is not fully developed like traditional immune cells such as macrophages and granulocytes.

Author Contributions

Conceived and designed the experiments: HCI TL MEN. Performed the experiments: HCI CGO. Analyzed the data: HCI MEN. Contributed reagents/materials/analysis tools: HCI CGO MEN. Wrote the paper: HCI MEN.

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Paper III

Inflammatory and regenerative responses in salmonids following mechanical tissue damage and natural infection

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Inflammatory and regenerative responses in salmonids following mechanical tissue damage and natural infection

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ABSTRACT

Locale responses in muscle tissue against either a sterile tissue damage or infection were compared in salmonid fish in order to examine the inflammatory responses and regeneration of tissue. From higher vertebrates both damage and infection are known to cause inflammation since DAMPs released from injured cells as well as PAMPs from the surface of pathogens are immunogenic. To examine this in salmonid fishes, Atlantic salmon (*Salmo salar*) were infected with *Moritella viscosus*, the causative agent of winter ulcer. Muscle tissue was sampled from infected fish at 4, 7 and 14 days post infection. Samples were obtained from site of lesions and from locations without clinical signs of disease and lesions. The tissue damage was performed in rainbow trout (*Oncorhynchus mykiss*) by applying sterile needles to skin and muscle tissue to one side of the fish. Samples were taken 7, 14, 21, 28 and 42 days post injury from the injured side and non-injured site (internal control). From both infected and damaged fish, samples were subject to real-time RT-PCR for measuring the expression of IL-1β, IL-8, IL-10, Hsp70, iNOS, TGF-β, TLR-5m, TLR-9, TLR-22, TGF-β, MMP-2, CTGF, myostatin-1αβ and collagen-1α which are coding for immunological factors and tissue regeneration. Locale, inflammatory responses were seen as strong up-regulation of IL-1β and IL-8 in both groups of fish, but it was more pronounced in infected fish. Expression of the toll-like receptors showed induction of TLR-5m following infection, but TLR-9 and TLR-22 following damage. Further, in both studies the regenerative genes TGF-β, MMP-2, CTGF, myostatin-1αβ were induced, but showed different kinetics. Collagen-1α was only induced in infected fish, probably due to heavier tissue damage in these.

1. Introduction

Tissue damage in fish occurs due to many events like handling in aquaculture, physical trauma and infectious diseases. Healing and regeneration of tissue damage imply complex processes involving both physiological factors and immune components [1]. The immune apparatus is strongly activated during the healing process more or less immediately after wounding and has a great impact on tissue repair [2]. Roughly, in mammals the wound healing response due to tissue damage can be divided into several overlapping phases; haemostasis, inflammation, proliferation and remodelling [3]. In lower vertebrates like the salmonids, the responses towards tissue injury and the following regeneration of tissue on molecular basis are poorly described. However, some studies have been done in the cyprinid species zebrafish (*Danio rerio*) and common carp (*Cyprinus carpio*) [4–10]. In higher vertebrates, the initial phase of the immune response is generally dominated by innate immune factors rather than adaptive. In order to remove foreign material like bacteria, non-functional host cells and damaged matrix components, neutrophils are attracted to the site of injury shortly after the haemostasis and later followed by influx of macrophages. Inflammatory cytokines like IL-1β, -6, -8, TNF-α and a number of chemokines play a crucial role and are up-regulated in order to attract leucocytes (chemotaxis) and regulate cell proliferation, differentiation and control synthesis of extracellular matrix [11]. The inflammatory response in fish shows many similarities to mammals by expression of pro-inflammatory cytokines, vasodilation, chemotactic influx of leucocytes from the blood into the inflammation site [12]. However, fish lack histamine and this may

Abbreviations: DAMP, Damage Associated Molecular Patterns; PAMP, Pathogen Associated Molecular Patterns; IL-, interleukin; Hsp70, heat-shock protein-70; iNOS, inducible nitric oxide synthase; TGF-β, transforming growth factor-β; TLR-, toll-like receptor; MMP-2, matrix metalloproteinase-2; CTGF, connective tissue growth factor; NLR, NOD-like receptor; RLR, RIG-like receptor; TNF-α, tumor necrosis factor.

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have an influence on the inflammatory response [13]. If the inflammatory response does not get chronic due to ongoing infection like e.g. with *Heliobacter pylori* in the intestine of some mammalian species, it will decline and be followed by a proliferative phase [3]. This phase is initiated by expression and production of matrix proteins for reestablishment of tissue structure and formation of new tissue. In this phase TGF-β does also play an important role [14]. The fibroblasts are activated in order to produce collagen, which is essential to restore structure and function of the tissue [15]. In addition to synthesis of matrix proteins, a new epithelial layer is produced and angiogenesis is initiated to establishment of new blood vessels. Unlike mammals, the epithelium is composed of live cells in fish [13]. The remodelling of the tissue comprises the last and final phase of a normal wound healing process where the repaired tissue is organised into the correct structure by cross linking of fibrillar collagen and the formation of a scar takes place [3]. Scarring is due to excessive accumulation of collagen and can among others be mediated by a disbalanced immune/inflammatory response during the healing cascade. This process is termed fibrosis, which is also known to occur in fish following infections of the skin and the musculature by the bacterium *Moritella viscosus* [16]. Dependent on the way tissue damage occurs, it might be reflected in the way the immune system is activated and reacts towards the stimuli. The immune system is capable of reacting on signals delivered from PAMPs through pathogen recognition receptors such as TLRs, NLRs and RLRs leading to downstream activation of pro-inflammatory genes [17,18]. Further, DAMPs released from necrotic, apoptotic and injured cells such as exposed mitochondria or heat-shock proteins may also bind to these receptors and activate the same signalling events [19]. In order to better understand the initial immune reactions towards PAMP (infection) and DAMP (sterile tissue damage) stimulations and the following phase of tissue regeneration, we performed a comparative study on salmonids. For the infection study, Atlantic salmon (*Salmo salar*) were bath challenged by the gram-negative bacterium *M. viscosus*. This bacterium is considered as the main causative agent of winter ulcer in sea during cold periods and gives rise to localised swelling of the skin followed by development of lesions [20]. For examination of the local response in fish following tissue damage without pathogens, rainbow trout (*Oncorhynchus mykiss*) were mechanically damaged through the skin and into the musculature using sterile needles. From both experiments, muscle tissue was sampled and were further subject to real-time RT-PCR and transcription of genes related the immune system were measured; interleukin-1β, -8, -10, inducible nitric oxide synthase, heat-shock protein-70, transforming growth factor-β, toll-like receptor-3, 5m (membrane bound form), 9 and 22. The regenerative responses were characterised by measurement of the genes matrix metalloproteinase-2, myostatin-1αβ, collagen-1α and connective tissue growth factor.

2. Materials and methods

2.1. Fish and rearing conditions from M. viscosus experiment

Unvaccinated Atlantic salmon (*S. salar* L.) (80–110 g), were obtained from Sørsmolt AS (Sannidal, Norway). The fish were transported to the Norwegian Institute for Water Research's aquaculture station (Solbergstrand, Norway) in a transportation tank containing 800 l freshwater. The fish were adapted to seawater in two separate 1400 l tanks prior to the challenge. Tank 1 contained 60 control fish and tank 2 contained 159 fish. The fish were kept in seawater (salinity 31–35‰) at 8.9 °C, with an oxygen content maintained stable at 8.2 mg l^{-1}. Water quality was monitored during the experiment using WTW type pH/Cond 340i and oxi 340i (Christian Berner, Oslo, Norway). The National Committee of Ethics as required by Norwegian law approved the experiment.

2.2. Bacteria and challenge procedures of Atlantic salmon

M. viscosus (NVI 96/09/1016, recently passaged through Atlantic salmon) was cultured in Brain Heart Infusion Broth (BHIB) containing 2% NaCl at 9 °C for 48 h with shaking. Following reduction of the water volume in the challenge tank to approximately 500 l, bacteria were added to a final concentration of 7×10^5 cfu ml^{-1}. The final bacterial suspension was controlled by colony counting following serial dilution in 2% saline and plating of 100 ml aliquots (in duplicate) on blood agar containing 2% NaCl (BA 2%) and incubation for 48 h at 15 °C. During the challenge the water flow was stopped, and the oxygen saturation level was maintained at 8.2 mg l^{-1}. The rapid mortality rate was decelerated by increasing the water temperature from 8.9 to 13.4 °C on day 4 and reducing the salinity from 35 to 10‰ on day 8. Control fish (untreated) were maintained under identical conditions. All fish were fed to appetite for the duration of the trial.

2.3. Sampling and culturing of M. viscosus infected Atlantic salmon

Samples from live fish were taken 4, 7 and 14 days post-challenge and dead fish were removed from the tanks daily. Due to ethical reasons, no samples later than 14 days post-challenge were taken. Parallel samples of control fish were taken at the same time as the challenged fish. All fish were anaesthetized by immersion in 0.005% benzocaine/water before handling. Blood samples for serology were centrifuged at 3000×*g* for 15 min at 4 °C and plasma was stored at −20 °C. Sampled fish were examined bacteriologically for the presence of *M. viscosus*, and other bacterial species. Blood, kidney and ulcer inocula were plated onto BA 2%. The plates were initially examined following incubation at 15 °C for 48 h, and after further 5 days of incubation. *M. viscosus* colonies were presumptively identified by colony viscosity, colony pigmentation and typical hemolysis. Muscle samples from the centre of ulcer and muscle tissue from regions in the fish where the skin appeared non-infected (internal control) was dissected using a clean scalpel and transferred to cryo tubes containing RNA *later*® (Sigma) and stored at 4 °C for 24 h, then −20 °C until isolation of RNA.

2.4. Fish and rearing conditions of mechanically damaged rainbow trout

Unvaccinated, healthy rainbow trout reared at Agerskov Dambrug (Bording, Jutland), where delivered to the experimental facilities at the National Institute of Aquatic Resources, Technical University of Denmark at March 2007. The fish were then kept at the facilities until the mechanical injury and maintained in 200 l plastic tanks with aerated local tap water at 14 °C and exposed to a light regime of 16 h of light followed by 8 h of dark. The mechanical tissue injury was performed November 2007. The fish were fed with a standard commercial trout feed (DAN-EX, DANA FEED A/S) by 1.5% bodyweight/day. Average weight (g ± SD) and length (cm ± SD) at the day of mechanical injury were 42.8 ± 7.9 and 15.0 ± 1.0, respectively ($n = 80$). At the last sampling 42 days post damage the average weight (g ± SD) and length (cm ± SD) were 84.0 ± 12.5 and 18.9 ± 0.9, respectively ($n = 10$). At each sampling point five injured fish and five control fish were collected. All procedures were conducted in accordance with the regulations set forward by the Danish Ministry of Justice and animal protection committees by Danish Animal Experiments Inspectorate permit 2007/561-1302 and in compliance with European Community Directive 86/609.

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2.5. Procedures for mechanical injury and tissue sampling

Forty fish were mechanically injured using a home made apparatus containing twenty five sterile needles made from 19G syringes (Becton Dickinson) with a thickness of 1.1 mm equally distributed on an area of 6 mm × 6 mm. The needles had a depth of 6 mm to ensure penetration of both the skin and underlying muscle tissue of the fish. Prior to mechanical injury, fish were anaesthetised in MS-222 (50 mg/l) (Sigma–Aldrich). The injury was performed posterior to the dorsal fin above the lateral line and the apparatus was penetrated twice through the skin, giving rise to fifty holes per fish. Prior to sampling of muscle tissue fish were killed in an overdose of MS-222. Sampling occurred at 7, 14, 21, 28 and 42 days post injury using a sterile disposable scalpel. The tissue collected was 1) muscle tissue from the site of injury and 2) non-injured muscle tissue from the opposite side of the fish relative to the injury tissue and 3) muscle tissue from non-injured control fish. By sampling the internal control it was possible to show whether the responses in the injured fish were local or systemic. Tissue was collected from five injured and five control fish per sampling point and was transferred to cryo tubes containing RNA *later*® (Sigma–Aldrich) and stored at −20 °C until isolation of RNA.

2.6. RNA isolation and cDNA synthesis

Fifty mg of the sampled tissues were homogenised by sonication for 30 s (amplitude 10 microns) using a MSE Soniprep 150 sonicator (Sanyo) and RNA was further isolated using a GenElute Mammalian™ Total RNA Miniprep Kit (Sigma–Aldrich). RNA quality and quantity was checked by $OD_{260/280}$ measurements on a NanoDrop™ 2000 spectrophotometer (Thermo) and the RNA was finally treated with DNase-I (Sigma–Aldrich) to remove any genomic DNA. Random hexamer primed reverse transcription reactions were performed from 400 ng of total RNA in a 20 μl setup using TaqMan® Reverse Transcription reaction (Applied Biosystems). The synthesised cDNA samples were diluted 1:10 in MilliQ H_2O and stored at −20 °C.

2.7. Quantitative RT-PCR

Quantitative RT-PCR was performed using a Stratagene MX3000P™ real-time PCR system, dual-labelled TaqMan® probes conjugated with either a 5′ HEX or a 5′ FAM fluorophor, a 3′ BHQ_1 quencher and desalted primers (Sigma–Genosys). The genes chosen for investigation were interleukin-1β (IL-1β), interleukin-8 (IL-8), interleukin-10 (IL-10), inducible nitric oxide synthase (iNOS), transforming growth factor-β (TGF-β), collagen-1α (Coll-1α), matrix metalloproteinase-2 (MMP-2), myostatin-1αβ (Myo-1αβ; co-amplification of the α and β isotypes), connective tissue growth factor (CTGF), heat-shock protein-70 (Hsp70), toll-like receptor-5m (membrane bound form), 9 and 22 (TLR-5m, TLR-9 and TLR-22). The assays for Hsp70, TLR-9 and TLR-22 were run using SYBR® Green (Sigma–Aldrich) instead of a probe. For use as an internal control and for normalisation of the results the reference gene ribosomal protein S20 (RPS20) was used since it was stably expressed between the different individuals and infected versus non-infected fish. The sequence for the primers and probes, amplicon length and GenBank accession numbers are shown in Table 1 (rainbow trout primers) and Table 2 (Atlantic salmon primers). The primers were optimised according to $MgCl_2$ and primer concentrations. The cycling conditions for the TaqMan® assay were 94 °C for 2 min followed by 40 cycles of 94 °C for 15 s and 60 °C for 1 min. The cycling conditions for the SYBR® Green assays were the same but the run was terminated by a melting curve analysis were the fluorescence was continually measured during a temperature increase from 60 °C to 95 °C. Wells for the TaqMan® assays contained 12.5 μl of JumpStart™ *Taq* ReadyMix™ (Sigma–Aldrich), 0.25 μl ROX (Sigma–Aldrich; diluted 10×), 2.5–5.5 mM $MgCl_2$ (Sigma–Aldrich), 0.75–1.25 μl forward and reverse primer (10 mM), 1 μl TaqMan® probe (200 nM), 5 μl of diluted cDNA and autoclaved MilliQ water to a volume of 25 μl. Wells for the SYBR® Green assays contained 12.5 μl of SYBR® Green JumpStart™ *Taq* ReadyMix™ (Sigma–Aldrich), 0.25 μl ROX (Sigma–Aldrich; diluted 10×), 3.5–5.5 mM $MgCl_2$ (Sigma–Aldrich), 1 μl forward and reverse primer (10 mM), 5 μl of diluted cDNA and autoclaved MilliQ water to a volume of 25 μl. The expression results were analysed using the $2^{-\Delta\Delta Ct}$ method after verification that the primers amplified with an efficiency of approximately 100% (doubling of the product between every cycle in the log-linear phase) and data were shown as $-\Delta\Delta C_t$-values and fold expression relative to non-injured control fish [21]. The threshold cycle (*C*t) was determined manually and set to 0.01 in the lower level of the log-linear area. A two-tailed *T*-test in cases of normally distributed data and Mann–Whitney *U*-test in cases where the data were not normally distributed performed the statistical analysis on the Δ*C*t values. The *T*-tests were paired when comparing data from site of damage with internal controls since the samples were taken from the same fish. Unpaired *T*-tests were used when comparing data from site of damage with control fish or when comparing data from internal controls to control fish. The statistical software GraphPad Prism version 4.03 was used to calculate the statistics and create graphs.

3. Results

3.1. Mortality after challenge with M. viscosus

Mortality was initiated two days after challenge and the cumulative mortality increased rapidly (Fig. 1). The first clinical signs of infection was localised swelling of the skin three to four days after challenge, and a few fish had open ulcers. At day 7 and 14 post infection the infected fish had all open ulcers. The water temperature was increased from 8.9 to 13.4 °C on day 4 and this lead to a rapid decline of the mortality on day 5, followed by an increase again on day 6. The salinity was reduced from 35 to 10‰ on day 8 and the cumulative mortality ceased to 33% on day 13. No fish died from the control group.

3.2. Bleedings and inflammation in mechanically damaged tissue

The device used for mechanical damage is shown in Fig. 2. Pictures of the musculature at the site of needle injection after removal of the skin from representative fish are shown in Fig. 3. Bleedings in the musculature were observed at day 7, 14 and 21 post damage, while these had disappeared at day 28 post damage. At day 7 post damage, the damaged area showed signs of inflammation by swelling of the skin. However, these signs were almost gone at day 14 post damage.

3.3. Gene expression following mechanical damage and infection by M. viscosus

3.3.1. Interleukin-1β (IL-1β) and interleukin-8 (IL-8)

The expression of the examined genes in mechanically damaged fish is shown in Fig. 4. The expression patterns of the pro-inflammatory cytokine genes IL-1β and IL-8 were very similar and the responses were localised to the site of injury since the expression in the internal control was not significantly different from the control fish ($P > 0.05$). Both genes showed the strongest up-regulation at the site of injury 7 days post damage corresponding to 26.5 and 30.0 folds relative to control fish for IL-1β and IL-8, respectively ($P < 0.05$). This up-regulation was decreasing at day 14 and 21, but

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Table 1
Sequences of rainbow trout primers and probes used for the real-time PCR analysis in mechanically damaged fish.

Gene	Primer	Sequence (5′–3′)	GenBank accession no.	Amplicon length (bp)
RPS20	Forward	AGCCGCAACGTCAAGTCT	NM_001124364	104
	Reverse	GTCTTGGTGGGCATACGG		
	Probe	TGTGCAGACCTTATCCGTGGAGCT		
IL-1β	Forward	AGGACAAGGACCTGCTCAACT	AJ278242	72
	Reverse	CCGACTCCAACTCCAACACTA		
	Probe	TTGCTGGAGAGTGCTGTGGAAGAA		
IL-8	Forward	GAGCGGTCAGGAGATTTGTC	AJ310565	72
	Reverse	TTGGCCAGCATCTTCTCAAT		
	Probe	ATGTCAGCGCTCCGTGGGT		
IL-10	Forward	GGGTGTCACGCTATGGACAG	AB118099	121
	Reverse	TGTTTCCGATGGAGTCGATG		
	Probe	ATCTCGACACGGTGCTGCCCAC		
TGF-β	Forward	ACGCCACAGCCAGCTTAG	X99303	87
	Reverse	CGCACACAGCAACTCTCC		
	Probe	TCTCGGAAGAAACGACAAACCA		
iNOS	Forward	AACGAGAGCCAACAGGTGTC	AJ300555	80
	Reverse	GGTGCAGCATGTCTTTGAGA		
	Probe	CCCGTGCGTAACGTGAAGGA		
Coll-1α	Forward	TGAGGGAACTCCTGGTAACG	AB052835	74
	Reverse	ACTCACCACGTTCTCCCTTG		
	Probe	CTTCTGGTCGCGATGGTGCT		
MMP-2	Forward	GGCCCAGATCAGAGGAGAG	AB021698	94
	Reverse	TACCAGCATGGGACCTGAA		
	Probe	ATTCAGGTCAACCAACTTCCGG		
Myo-1αβ	Forward	CTTTGGCTGGGACTGGATTA	DQ138300 + DQ136028	65
	Reverse	CGCACTCACCAGAGCAGTAG		
	Probe	CCCAAGCGCTACAAGGCCAA		
CTGF	Forward	CGCGTGCCACTACAACTG	CX247885	78
	Reverse	GCCATGTCTCCCAGCATC		
	Probe	CCCCGGCGAGAATGACATATT		
Hsp-70	Forward	AGATCAGCGAGGAGGACAAA	NM_001124228	148
	Reverse	AGGAAATGGTCTGGTTGCAC		
TLR-5m	Forward	GGCATCAGCCTGTTGAATTT	AB091105	89
	Reverse	ATGAAGAGCGAGAGCCTCAG		
	Probe	GCTCAGTCATATCGTGTGAGGAGGA		
TLR-9	Forward	GCAACCAGTCCTTCCACATT	NM_001129991	73
	Reverse	AAACCCAGGGTAAGGGTTTG		
TLR-22	Forward	AAGGCGCTTCGAGAGTTGAAT	AJ628348	148
	Reverse	TGGAGAGAGGCTGAAATGATGAG		

was still significant ($P < 0.05$). On day 28 and 42 post damage the expression had declined and were similar to non-damaged control fish ($P > 0.05$).

The expression of the examined genes in *M. viscosus* infected fish is shown in Fig. 5. The expression pattern of IL-1β and IL-8 showed similar trends as what was seen in mechanically damaged fish, but the expression was higher up-regulated. The expression level of both genes was not significantly changed at day 4 post infection, but highly elevated at day 7 post infection in muscle tissue with ulcers (2090 and 5792 folds for IL-1β and IL-8, respectively) compared to control fish and non-ulcerated areas on infected fish ($P < 0.05$). Further, the expression of IL-1β and IL-8 at day 7 post infection was not completely limited to the visible ulcers, since IL-1β and IL-8 compared to control fish were also significantly elevated in muscle tissue of areas on the fish where ulcers did not seem to appear (32 and 87 folds for IL-1β and IL-8, respectively) ($P < 0.05$). At day 14 the expression of both genes in the ulcers had decreased relative to control fish (102 and 256 folds for IL-1β and IL-8, respectively) ($P < 0.05$), and no difference was seen between control fish and visibly non-ulcerated sites on infected fish ($P > 0.05$).

3.3.2. Interleukin-10 (IL-10)

The individual variation of the anti-inflammatory cytokine IL-10 in the control fish, but also in the injured fish, was very high in the mechanical damage study. Therefore, the expression was not significantly different at any of the sampling points (data not shown) ($P > 0.05$).

In fish infected by *M. viscosus*, a significant elevation was seen in the ulcers at day 4 (11 folds) and 14 post infection (5 folds) relative to control fish ($P < 0.05$). No change was seen in the ulcers at day 7 post infection ($P > 0.05$).

3.3.3. Toll-like receptor-5m (TLR-5m)

The toll-like receptor TLR-5m (m = membrane bound) known to bind flagellin was not significantly regulated at the first three samplings in mechanically damaged fish ($P > 0.05$). At day 28 post damage, a small increase was seen in the site of damage (2.6 folds, $P < 0.05$), which was followed by a significant down-regulation at the internal control site 42 days post damage (2.5 folds, $P < 0.05$). Expression of TLR-5m in *M. viscosus* infected fish showed a high up-regulation 7 (9.8 folds) and 14 days (4.9 folds) post infection in ulcers ($P < 0.05$), but no changed expression in areas of the fish, where ulcers did not seem to appear ($P > 0.05$).

3.3.4. Toll-like receptor-9 and -22 (TLR-9 + TLR-22)

The two toll-like receptors TLR-9 and TLR-22 showed very similar expression patterns in mechanically damaged fish. TLR-9 was significantly up-regulated at the site of damage throughout the entire experimental period relative to the internal control site and control fish (3.9–11.2 folds, $P < 0.05$). TLR-22 was strongly induced at day 7,14 and 21 (10.1–21.9 folds) relative to the internal control site and control fish ($P < 0.05$) followed by a decrease at 42 days post damage ($P > 0.05$).

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Table 2
Sequences of Atlantic salmon primers and probes used for the real-time PCR analysis in *Moritella viscosus* infected fish.

Gene	Primer	Sequence (5′–3′)	GenBank accession no.	Amplicon length (bp)
RPS20	Forward	AGCCGCAACGTCAAGTCT	AY953432	104
	Reverse	GTCTTGGTGGGCATACGG		
	Probe	TGTGCAGACCTTATCCGTGGAGCT		
IL-1β	Forward	AGGACAAGGACCTGCTCAACT	AY617117	72
	Reverse	CCGACTCCAACTCCAACACTA		
	Probe	TTGCTGGAGAGTGCTGTGGAAGAA		
IL-8	Forward	GAGCGGTCAGGAGATTTGTC	NM_001140710	72
	Reverse	TTGGCCAGCATCTTCTCAAT		
	Probe	ATGTCAGCGCTCCGTGGGT		
IL-10	Forward	GGGTGTCACGCTATGGACAG	EF165029	118
	Reverse	TGTTTCCGATGGAGTCGATG		
	Probe	ACCTCGACACGGTGTTGCCCAC		
TGF-β	Forward	ACGCCACAGCCAGCTTAG	EU082211	87
	Reverse	CGCACACAGCAACTCTCC		
	Probe	TCTCGGAAGAAACGACAAACCA		
iNOS	Forward	AACGAGAGCCAACAGGTGTC	AJ300555	80
	Reverse	GGTGCAGCATGTCTTTGAGA		
	Probe	CCCGTGCGTAACGTGAAGGA		
Coll-1α	Forward	TGAGGGAACTCCTGGTAACG	CK897549	74
	Reverse	ACTCACCACGTTCTCCCTTG		
	Probe	CTTCTGGTCGCGATGGTGCT		
MMP-2	Forward	GGCCCAGATCAGAGGAGAG	NM_001139932	94
	Reverse	TACCAGCATGGGACCTGAA		
	Probe	ATTCAGGTCAACCAACTTCCGG		
Myo-1αβ	Forward	CTTTGGCTGGGACTGGATTA	NM_001123549 + NM_001123634	65
	Reverse	CGCACTCACCAGAGCAGTAG		
	Probe	CCCAAGCGCTACAAGGCCAA		
CTGF	Forward	CGCGTGCCACTACAACTG	NM_001139999	78
	Reverse	GCCATGTCTCCCAGCATC		
	Probe	CCCCGGCGAGAATGACATATT		
Hsp-70	Forward	AGATCAGCGAGGAGGACAAA	BT059340	57
	Reverse	AGGAAATGGTCTGGTTGCAC		
TLR-5m	Forward	GAAGATTTGCTTGGCTTTCG	AY628755	106
	Reverse	TTCCACATCGTTTCCAGTCA		
TLR-9	Forward	CTCAGCCTCTGTCCTTTCACTT	EF672331	71
	Reverse	CAGAGAATGCATCCTTCCCTAC		
TLR-22	Forward	GCCAACCTCTCCCTGCTAC	AM233509	63
	Reverse	AAAGGATGAGGACCCGATG		

In *M. viscosus* infected fish, the expression patterns of both genes were very different. The expression of TLR-9 was not changed in the ulcers at any of the samplings, but a small decrease (5.9 folds) was seen at day 7 post infection at sites on infected fish, where ulcers did not seem to appear ($P < 0.05$). For TLR-22, a significantly decreased expression relative to control fish (12.5 folds) was seen in the ulcers 7 days post infection ($P < 0.05$). At day 14 post infection, this was followed by an increase in TLR-22 expression (4.6 folds) in the ulcers relatively to sites on infected fish, where ulcers did not seem to appear ($P < 0.05$).

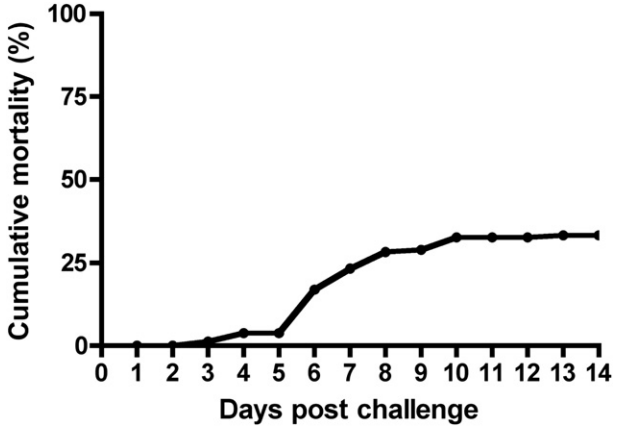

Fig. 1. The cumulative mortality (%) of the *Moritella viscosus* infected fish. The fish started dying on day three following challenge. From between day 5 and 10 the highest mortality rate was seen, while the mortality thereafter declined. No fish died on day 13 and 14 post-challenge.

3.3.5. Heat-shock protein-70 (Hsp70)

The heat-shock protein-70 gene (Hsp70) was only weakly regulated in the mechanical damage study. The expression at the site of damage was up-regulated 2.8 fold 7 days post injury and thereafter down-regulated again to constitutive levels ($P > 0.05$). At day 14 post damage, a significant down-regulation was seen at the internal control site (3.5 folds, $P < 0.05$). No regulation was seen at the last three samplings ($P > 0.05$).

In *M. viscosus* infected fish no significant change in expression of Hsp70 was seen ($P > 0.05$). This was due to a high individual variance between single fish and a few high responders (not shown).

3.3.6. Inducible nitric oxide synthase (iNOS)

The expression of the inducible nitric oxide synthase gene iNOS, which catalyses the production of nitric oxide (NO) from L-arginine was not significantly regulated at any of the sampling point in mechanically damaged fish ($P > 0.05$) (not shown).

The same was seen for *M. viscosus* infected fish, where no change in expression was observed at any of the sampling points ($P > 0.05$).

3.3.7. Transforming growth factor-β (TGF-β)

The transforming growth factor-β gene (TGF-β) functions both as a cytokine but also as a molecule controlling cellular proliferation

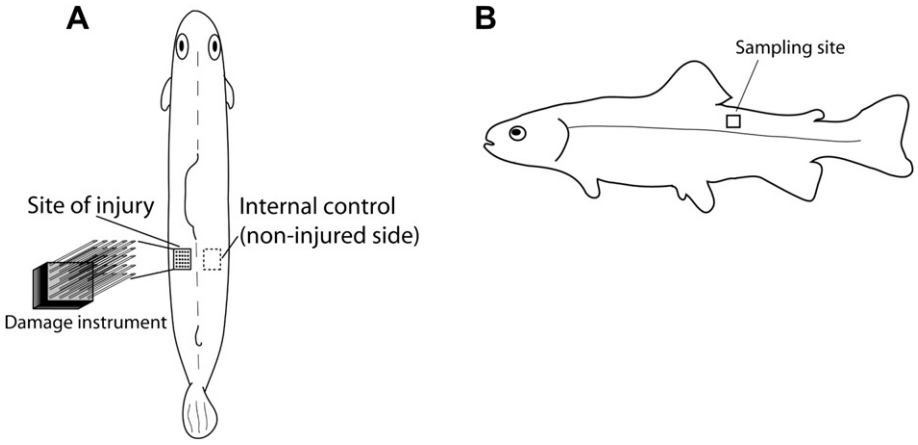

Fig. 2. Illustration of the tissue damage apparatus, the site of damage on the fish (A) and the sampling method from mechanically damaged fish (A and B). The fish were damaged on the left side and one muscle tissue sample was further taken at the damaged site while another was sampled from non-damaged muscle tissue on the right side of the fish. Samples from *Moritella viscosus* infected fish were taken by the same principle from ulcers and visibly non-ulcerated muscle tissue, respectively.

and differentiation. In the mechanically damaged fish, TGF-β was significantly up-regulated at day 7, 14 and 21 at the site of injury (4.2–12.9 folds) relative to non-injured control fish and the internal control site ($P < 0.05$). The pattern was different in *M. viscosus* infected fish. At day 7 post infection, a small increase of TGF-β (1.6 folds) in ulcers was seen relative to sites on the fish where ulcers did not seem to appear ($P < 0.05$). This was followed by a high induction (35.2 folds) in the ulcers 14 days post infection ($P < 0.05$), while no change in expression was seen at any of the samplings at sites of the infected fish where ulcers did not seem to appear ($P > 0.05$).

3.3.8. Matrix metalloproteinase-2 (MMP-2)

The matrix metalloproteinase-2 gene (MMP-2) is a protease degrading collagen and extracellular matrix. In mechanically damaged fish, the MMP-2 gene showed like TGF-β also a local up-regulation at the site of injury compared to the internal control site and control fish ($P < 0.05$). The expression was significantly elevated at day 7, 14, 21 and 28 (1.9–4.4 folds) and peaked 14 days post damage ($P < 0.05$). In fish infected by *M. viscosus*, the expression of MMP-2 was changed at one sampling point only. In ulcers, a significant up-regulation was seen 14 days post infection

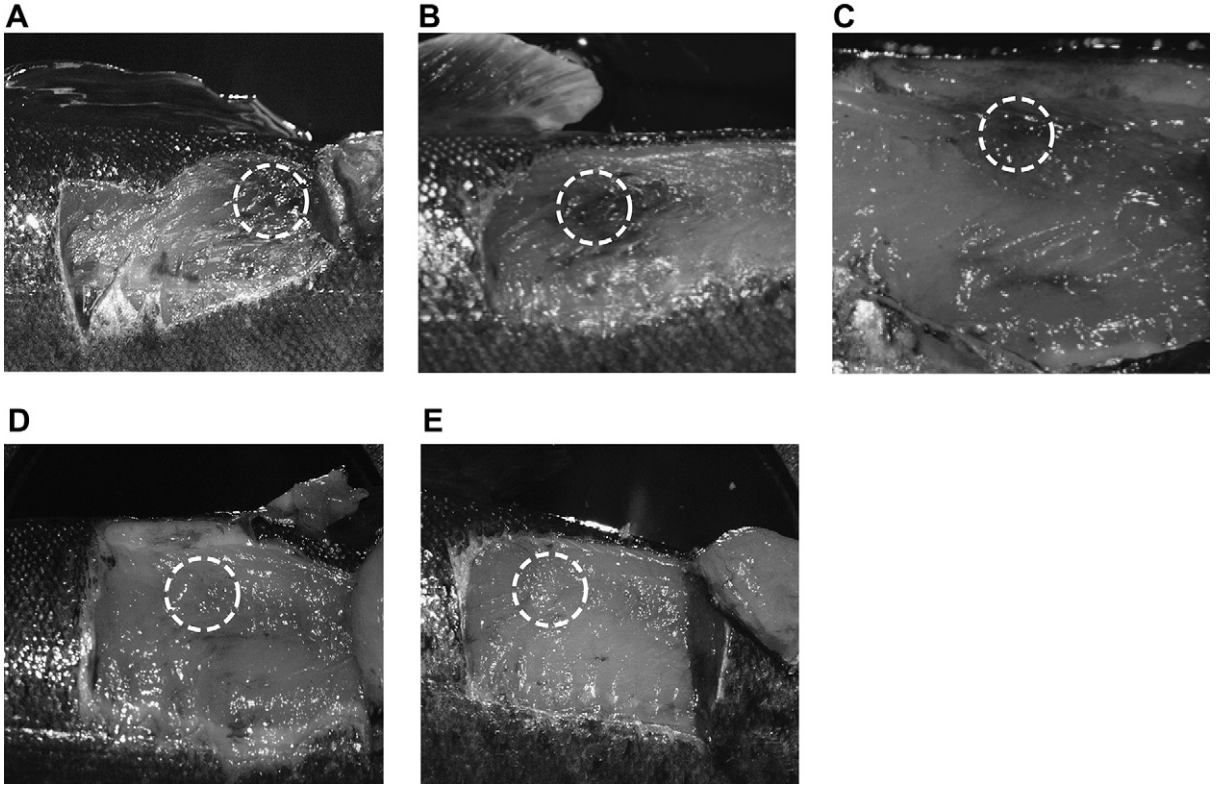

Fig. 3. Pictures of the musculature at the site of damage (white, dashed circles) in mechanically damaged fish at each sampling point day 7 (A); 14 (B); 21 (C); 28 (D) and 42 (E) post damage. At the first three samplings visible bleedings were seen, while they had disappeared 21 days post damage.

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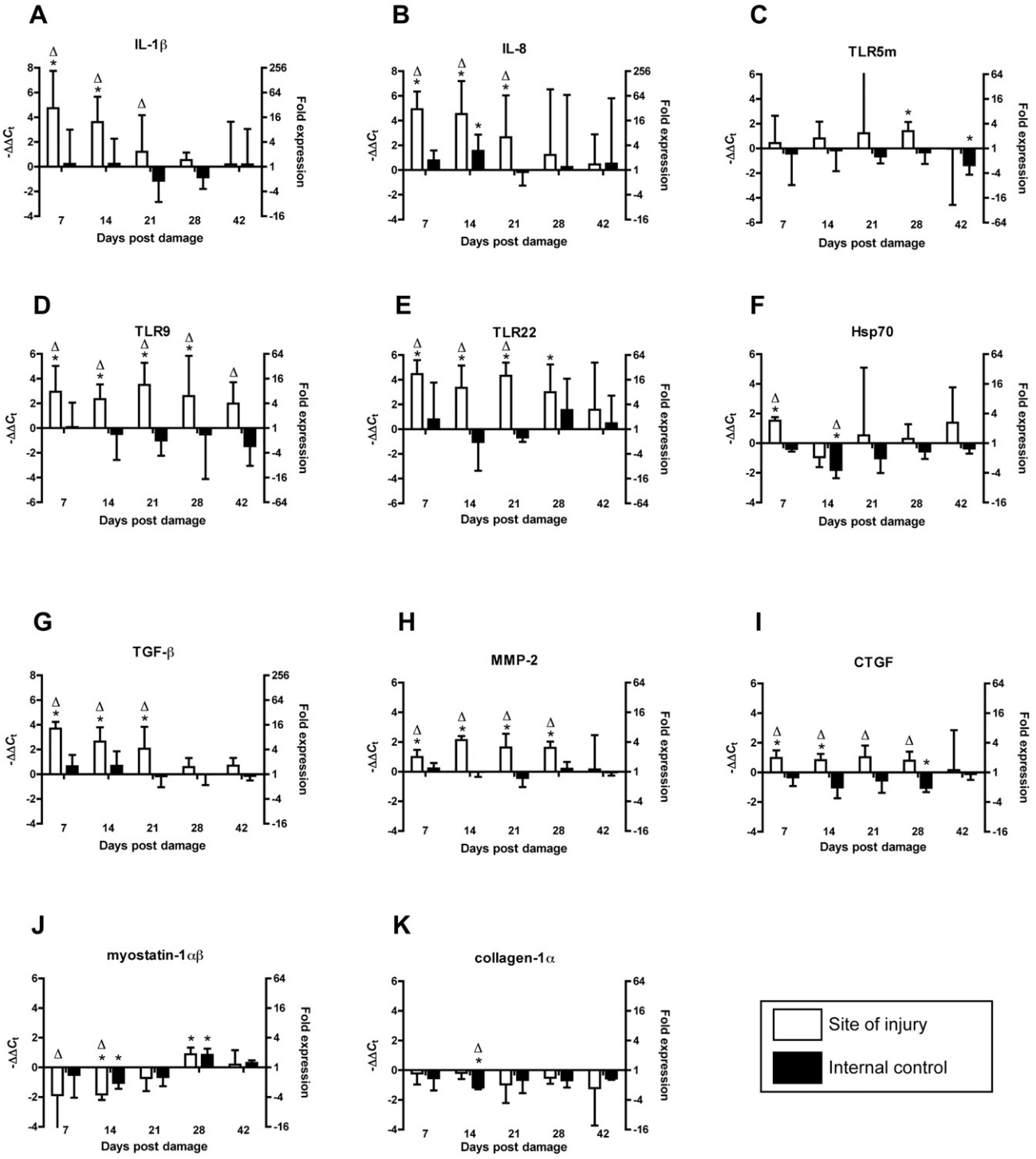

Fig. 4. Quantitative real-time PCR of the genes IL-1β, IL-8, TGF-β, TLR-5, TLR-9, TLR-22, Hsp70, TGF-β, MMP-2, CTGF, myostatin-1αβ and collagen-1α in mechanically damaged fish (A–K). White bars represent expression at the site of injury relative to control fish and black bars indicate expression at the non-damaged internal control site relative to control fish. The data are normalised relative to the expression of ribosomal protein S20 and analysed using the $\Delta\Delta C_t$ method. Data are shown as $-\Delta\Delta C_t$ values and fold expression. Bars represent mean values of $-\Delta\Delta C_t$ + SD values from five individuals. * Depicts statistical significance between injured fish and control fish ($P < 0.05$); Δ denotes statistical significant difference between site of injury and internal control site ($P < 0.05$). A $-\Delta\Delta C_t$ value of 0 means no regulation relative to control fish.

relative to control fish and sites on infected fish, where ulcers did not seem to appear (3 fold, $P < 0.05$).

3.3.9. Connective tissue growth factor (CTGF)

The connective tissue growth factor gene (CTGF) takes part in wound healing, modelling of extracellular matrix and fibrosis showed similar expression pattern. CTGF was significantly up-regulated at all samplings except at 42 days post injury relative to the internal control (1.7–2.0 folds) and significantly up-regulated compared to control fish 7 days and 14 days post injury (1.8–1.9 folds) ($P < 0.05$). In *M. viscosus* infected fish, a significant induction of CTGF was seen in ulcers at day 7 and 14 post infection relative to

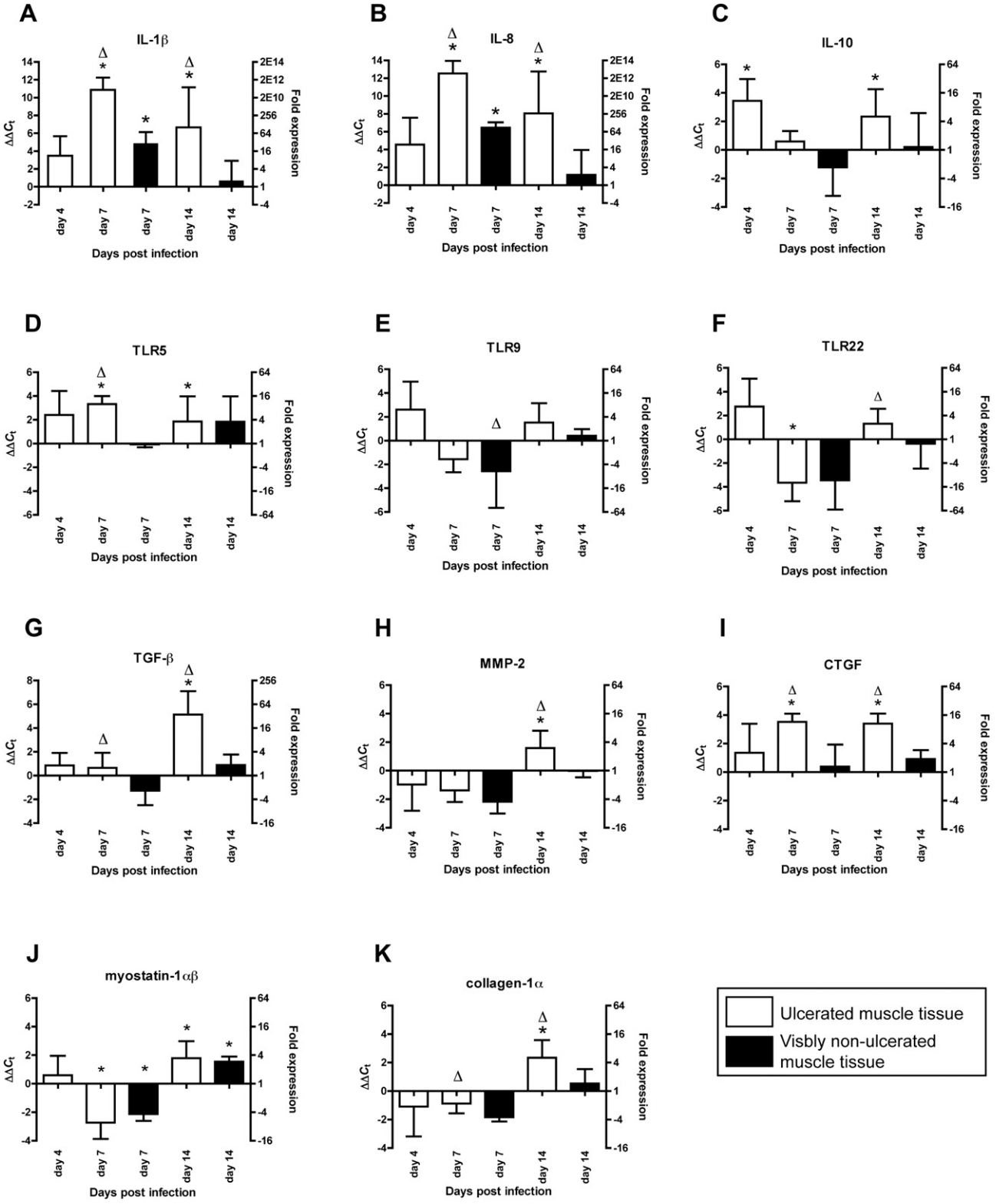

Fig. 5. Quantitative real-time PCR of the genes IL-1β, IL-8, IL-10, TGF-β, TLR-5, TLR-9, TLR-22, TGF-β, MMP-2, CTGF, myostatin-1αβ and collagen-1α in *Moritella viscosus* infected fish (A–K). White bars represent expression in ulcerated muscle tissue relative to control fish and black bars indicate expression in visibly non-ulcerated muscle tissue from the same fish relative to control fish. No samples from visibly non-ulcerated muscle tissue were taken at day 4 post-challenge. The data are normalised relative to the expression of ribosomal protein S20 and analysed using the $\Delta\Delta C_t$ method. Data are shown as $-\Delta\Delta C_t$ values and fold expression. Bars represent mean values of $-\Delta\Delta C_t$ + SD values from five individuals. * Depicts statistical significance between injured fish and control fish ($P < 0.05$); Δ denotes statistical significant difference between site of injury and internal control site ($P < 0.05$). A $-\Delta\Delta C_t$ value of 0 means no regulation relative to control fish.

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control fish and to sites on infected fish, where ulcers did not seem to appear (10.5–11.5 folds, $P < 0.05$). Only local reactions was also seen for this gene, since no change in transcription level was seen in infected fish at sites where ulcers did not seem to appear ($P > 0.05$).

3.3.10. Myostatin-1αβ

The myostatin-1 gene controls muscle differentiation and growth. Myostatin-1 has two different isoforms in rainbow trout termed α and β. The co-expression of these two isoforms in mechanically damaged fish showed a significant down-regulation at the site of injury 7 and 14 days post injury relative to the internal control (−3.5 to −3.7 folds) and a significant down-regulation compared to control fish 14 days post injury (−3.5 folds, $P < 0.05$). The down-regulation was followed by a small up-regulation 28 days post injury at the site of injury but also at the internal control site relative to control fish (1.8–1.9 folds, $P < 0.05$). In *M. viscosus* infected fish, the responses showed similar trends as seen in the mechanically damaged fish. Seven days post infection a significant systemic decrease was seen, since the transcriptional level compared to control fish was lower in ulcers and at sites where ulcers did not seem to appear ($P < 0.05$). A decrease in myosstain-1αβ expression corresponding to 6.6 folds in ulcers and 4.4 folds in sites where ulcers did not seem to appear was seen. Fourteen days post infection this was changed to a systemic transcriptional increase (2.9–3.5 folds, $P < 0.05$).

3.3.11. Collagen-1α

Collagen-1α is one the most abundant type of collagen and is present in e.g. tendon, skin and in high amounts in scars. The expression of collagen-1α in mechanically damaged fish was not changed following tissue injury throughout the experiment. However, a small down-regulation was seen at the internal control site (2.2 folds) relative to injured tissue and control fish ($P < 0.05$). In *M. viscosus* infected fish, a local down-regulation in ulcers 7 days post infection was seen (2.1 folds), which was followed by an up-regulation at day 14 post infection (5.0 folds, $P < 0.05$). No transcriptional change was seen in infected fish at sites, where no ulcers seemed to appear ($P > 0.05$).

4. Discussion

In the current study, immunological and regenerative responses following damage of muscle tissue in rainbow trout and infection of Atlantic salmon by *M. viscosus* were studied. Overall, the results showed many similar expression patterns for the examined immune and regenerative genes between the two experiments. Due to ethical reasons, unfortunately no samples were taken no later than 14 days post infection so comparisons at later time points between the two studies was not possible.

In both experiments a strong inflammatory response was seen. The inflammatory response is initiated in order to combat invading pathogen(s) and to clean the damage- and/or infection site for cell debris and foreign material [1]. The inflammatory cytokines IL-1β and IL-8 were heavily up-regulated following damage and infection. However, the up-regulation of IL-1β and IL-8 in *M. viscosus* infected fish was much higher than in mechanically damaged fish. Probably as a consequence of this, the anti-inflammatory cytokine IL-10 was highly up-regulated in the infected fish in order to control the inflammatory response, whereas no significance was seen in the mechanically damaged fish [22]. Removal of the skin from mechanically damaged fish in the needle injection area showed a high correlation between the up-regulation of IL-1β and IL-8 in relation to bleedings in the underlying muscle tissue at the different samplings. Bleedings were seen in the musculature until at least day 21 post damage, while they had ceased at day 28. The sampling at day 28 post damage was the first where expression of IL-1β and IL-8 in the damaged site had declined to similar levels as control fish and the internal control. This clearly demonstrates that IL-1β and IL-8 are useful as indicators of inflammation in fish. Further indications of cellular stress was initiated in the mechanically damaged fish, since Hsp70 was up-regulated 7 days post damage. Hsp70 is known as a marker of cellular stress and its ability to trigger and amplify inflammation [23,24]. During infection in fish, increased expression of inducible nitric oxide syntethase (iNOS) has previously been shown in different immunologically active organs such as head kidney, spleen, liver, skin and gills [25,26]. IL-1β has been shown to induce iNOS expression, but surprisingly no significant transcriptional changes was seen for iNOS in either of the models [27]. A high individual variation in expression in all groups of fish could account for this.

Activation of the immune system following physical tissue damage (non-pathogenic mediated) and infection, respectively, is initiated and triggered by a diverse array of inducers in mammals [1]. Roughly, non-pathogenic inducers that are released during necrosis, apoptosis and damage are categorised as DAMPs while pathogenic surfaces are termed PAMPs [19]. The DAMPs are mostly intracellular molecules like nuclear and cytosolic proteins that due to a change in redox potential when outside the cell become immunogenic, but they can also be extracellular [28].

The results clearly showed that these immune mechanisms also exist in lower vertebrates since inflammatory reactions and up-regulation of pro-inflammatory cytokines were seen in both studies. Hence, DAMPs trigger the innate immune system in fish. The initial induction of these genes occurs via activation of receptors on the surface of the cells, which further downstream leads to activation of NFκ-B that induce the transcription of cytokines [17]. This, in turn means that receptors on the cell-surface like TLRs or scavenger receptors were triggered, which the expression patterns indicated [29–32]. In *M. viscosus* infected fish, TLR-5m was highly up-regulated on day 7 and less on day 14 post infection, while not following damage. However, a minor increase in TLR-5m expression was seen at day 28 post damage. This difference between the two models could be expected since TLR-5 is recognising flagellin from bacterial flagella and hence *M. viscosus* might have induced its expression [33]. On the contrary, the results for TLR-9 were surprising. TLR-9 is in higher vertebrates known to be induced by unmethylated CpG DNA motifs, which is present in all bacteria [34]. However, the TLR-9 was not induced in *M. viscosus* infected fish while it was significantly up-regulated in mechanically damaged fish at all samplings. Expression of TLR-9 in salmonid fish have not yet been described following a bacterial infection, but it has been found to be inducible following stimulation with synthetic CpG ODNs and a plasmid vector encoding the VHS virus glycoprotein G in gilthead seabream (*Sparus aurata*) and rainbow trout, respectively [35,36]. On the other hand, TLR-9 has been shown to recognise other patterns of DNA, which are not methylated or even non-DNA compounds [37,38]. This could explain why it was induced following mechanical damage. Hence, TLR-9 seem to have a broad number of ligands, which could explain why DAMPs triggered the expression of TLR-9.

Similar findings were seen for TLR-22. However, the results for TLR-22 in *M. viscosus* infected fish were not clear since both down- and up-regulations were seen. The TLR-22 receptor is only found in aquatic animals and it has been coupled to virus recognition and is called a functional substitute of human cell-surface TLR3 [39]. Mammalian TLR3 is sensible to RNA from necrotic cells and therefore a sensor of tissue necrosis [40]. Consequently, according to the up-regulation of TLR-22 in the mechanically damaged fish, a further similarity to the mammalian TLR3 is then sensibility of fish TLR-22 to DAMPs. Together, the functions of the fish TLR-9 and TLR-22 as

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sensors of tissue necrosis might substitute for the TLR2 receptor in higher vertebrates, which do not seem to exist in salmonid fish [19].

A comparison of PAMPs and DAMPs in connection to the damage model and infection may, however, be taken with caution. A pure PAMP stimulation of the immune system cannot be performed with live pathogens because DAMPs are released due to tissue damage caused by the pathogen. Hence, studying responses in infected fish is a result of a mixed PAMP and DAMP reaction. On the other hand, inactivated bacteria would not generate an immune response of the same strength and character as live bacteria and therefore a challenge model using live bacteria must be preferred [41]. Further, it is potentially difficult to compare quantitative differences in responses between two species, since they could react differently against the same stimuli. On the other hand, rainbow trout and Atlantic salmon are very closely resembled species, but some observations from fish farming indicate that Atlantic salmon show a stronger inflammatory response in the peritoneal cavity following administration of oil adjuvants compared to rainbow trout (unpublished).

In both experiments a new sampling model was introduced since an internal control based on sampling of non-damaged muscle tissue in damaged fish or non-ulcerated muscle tissue in infected fish was taken. This gave a unique opportunity to study local tissue reactions in single fish, since this internal control sample could demonstrate if responses in damaged or ulcerated areas of the fish were systemic or rather limited to the affected area. By using this model it was shown that the responses towards both mechanical damage and infection were localised and largely limited to either the site of damage or visible ulcers. *M. viscosus* produces skin ulcers with different degree of pathology. It can vary from light damaging of the skin with superficial lesions extending down only to the stratum compactum of the skin, but in chronic cases open lesions extending beyond the skin and down into the musculature can appear [16,20]. Sampling from ulcers in this study was performed from open ulcers at day 7 and 14 and, if possible, at day 4 since the ulcers at this sampling were mostly sub acute and not visible in all infected fish. Despite most of the examined genes were regulated mainly in the ulcerated musculature of the *M. viscosus* infected fish, Løvoll et al. (2009) reported from the same experiment that some examined immune genes were systemically regulated [20]. Expression of IL-1β, C3, ISG15 and CD83 mRNA was seen in head kidney from about day 2 post infection and the expression correlated with the amount of *M. viscosus* DNA in the head kidney. Further, the amount of bacterial DNA was higher in head kidney compared to 'visibly' non-infected muscle tissue [20]. Together, those results are in line with the findings reported here, where the up-regulation was strongest in ulcers, but at the same time a significant up-regulation of IL-1β, IL-8, TLR-9 and myostatin-1αβ was seen in visibly non-ulcerated muscle tissue in challenged fish. Hence, some genes showed a systemic response. Despite clear locale reactions in ulcers of infected fish, the regulations seen in mechanically damaged fish seemed to be more limited to the site of damage compared to infected fish. This was seen for IL-1β and IL-8 in the *M. viscosus* infected wish, where a systemic up-regulation was seen on day 7. This seems likely, since the bacterium as mentioned above is spreading around the in the body through the bloodstream [20]. Again, this showed that by including the internal control sample unique information about locale versus systemic responses was obtained.

Together with activation of the immune system following damage and infection, both models showed that tissue remodelling is initiated in concert with the immune system. Molecules coding for cellular proliferation and differentiation (TGF-β), degrading collagen and extracellular matrix (MMP-2) and fibrotic responses (CTGF) were up-regulated in both studies. In contrast to the mechanically damaged fish, it appeared to be more evident with a two-phase reaction in the *M. viscosus* infected fish. The kinetics for MMP-2 and TGF-β showed that the expression of these two genes peaked after peaking of pro-inflammatory cytokine expression in *M. viscosus* infected fish, whereas they were co-expressed together in the mechanically damaged fish. It seems reasonable that the immune system 'pauses' regeneration as long as infection is going on, and at that moment clearance of the pathogen has the highest priority. Further, it appears to be advantageous starting regeneration simultaneously with cleaning-up of dead cells and destroyed tissue after a sterile damage, when no PAMPs are present. In other words, it would be physiological advantageous to 'win the war before rebuilding of new houses', while 'presence of ruins does not prevent rebuilding of new houses'. In mammals, the outer skin is keratinised and is not composed of live cells as in fish [42,43]. Tissue regeneration in terrestrial mammals has been described as overlapping phases, but which are still split-up into more clearly divided phases. From a rational point of view, it seems reasonable that it probably is more important for an aquatic animal to exhibit a faster regeneration compared to non-aquatic animals in order to be protected from waterborne pathogens and maintain osmotic balance. This theory is supported by results from zebrafish (*D. rerio*), where a very fast regeneration of tissue compared to mammals was shown [44]. On the other hand, the expression of the myostatin-1αβ gene and CTGF was initiated early in both studies indicating that some parts of tissue regeneration were started while the infection is going on. Myostatin-1αβ was down-regulated at day 7 in both groups of fish and at day 14 post damage. It has been reported, that this gene controls muscle growth and that expression of myostatin inhibits muscle growth [45,46]. Hence, due to these down-regulations, muscle growth may have been primed in the early phase following infection or damage. The more heavy inflammation seen in the infected fish relative to damaged fish, measured as a much higher transcription of IL-1β and IL-8, could probably explain the differences seen for expression of collagen-1α. In fish infected by *M. viscosus*, a strong induction of collagen-1α was seen 14 days post infection, whereas no transcriptional change was observed at the site of damage in the damaged fish. This specific type of collagen is present in high amounts in scars of mammalian species. Hence, since the pathogen-induced tissue damage was heavier than the sterile, it seems obvious that it consequently would lead to a higher amount of collagen deposition and scarring during the tissue repair. To support this further, from farmed fish it is very common that fish previously infected by *M. viscosus* are downgraded in quality due to a high deposition of scarring in the filets [16].

In summary, we have described a new model to show locale tissue reactions following damage and infection in the salmonid fish species rainbow trout and Atlantic salmon by introducing an internal control sample. Hereby, unique information about locale versus systemic reactions was obtained. Further, we showed the immunological and regenerative responses following stimulation by PAMPs and DAMPs, respectively, in order to examine the differences between these in salmonid fish. Both ways of stimulation resulted in an inflammatory response by activating IL-1β and IL-8 and a number of toll-like receptors. Activation of the toll-like receptors examined showed clear differences in expression pattern dependent on stimulation by either PAMPs or DAMPs. A number of genes coding for tissue regeneration were also induced following infection and tissue damage, and the activation seems overall to occur as parallel processes together with the immune system.

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