Mucosal immune response in common carp (*Cyprinus carpio* L.) Host pathogen interactions in relation to β-glucan stimulation

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Technical University of Denmark

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

Contents

“Jeśli nie możesz ich przekonać, zamąć im w głowach”

“If you can't convince them, confuse them”

Harry Truman

Summary

Control of fish diseases is a great concern in aquaculture because of losses in the production. Drug choices for the treatment of common infectious diseases are becoming increasingly limited and expensive and, in some cases, unavailable due to the emergence of drug resistance in bacteria and fungi. This is why number of biological compounds, as an alternative to the drugs, has been used to reduce the risk of diseases and improve fish welfare by enhancement of non-specific defence system. Among them, β-glucans, naturally occurring polysaccharides found in the cell wall of plants, bacteria and fungi, are some of the most powerful and extensively investigated immune modulants. β-glucans have been proven to enhance the immune system and pharmacologically they are classified as biological response modifiers (BRM).

The focus of the present thesis was on: 1. creation of a model for the examination of the biological impact of two commercially available β-glucan enriched products on the wound healing process in common carp (*Cyprinus carpio* L.) in sterile, controlled conditions; 2. investigation of potential impact of intravenously injected β-glucan on mucosal immune response and immunoglobulin switch-like process in common carp. In order to reach these objectives, different methods were used such as real-time quantitative PCR (RT-PCR) in order to measure the expression of immune-related genes involved in wound healing process, ELISA for specific antibody detection, cortisol assay for measurement of stress level, respiratory burst assay for radical production measurement and image analysis.

The results of this study showed that previous infections gave rise to changes regarding texture quality parameters in fresh fish meat, and were a starting point for use of immune modulators such as β-glucans. Further work showed that bath in two commercially available β-glucan enriched products, specifically MacroGard and 6.3 kDa oat fiber, had a direct positive effects on the wound closure in common carp and promoted faster wound healing compared to non-treated fish. We showed the immunological and regenerative response following stimulation with PAMPs and DAMPs in controlled conditions, without the exposure to pathogens, which resulted in an inflammatory response by activating IL-1b, IL-6 and IL-8. Local differences in expression pattern dependent on stimulation by DAMPs alone or DAMPs/PAMPs combination. In addition, the absence of marked differences on the respiratory burst activity in head-kidney cells supports the idea of a localized immune response to the site of injury. Due to direct and constant contact between skin and β-glucan, bath treatment was an obvious choice to investigate. However, intravenous injection of β-glucan showed it has a biological effect on skin as well. Once again we observed immunological changes taking place in skin of common carp, with IgM/IgZ immunoglobulin switch-like process. Finally, β-glucan seems to work in dose-dependent manner, with elevating cortisol level when injected at high doses.

Sammendrag

For at sikre en sund og rentabel i akvakultur produktion er kontrol af fiskesygdomme af stor betydning. Muligheden for brug af medicin til behandling af almindelige infektiøse sygdomme bliver mere og mere begrænset og kostbare, og er i nogle tilfælde ikke mulig på grund af resistens i bakterier og svampe. Derfor er der stor fokus på alternativer til medicinering, som kan reducere risikoen for sygdomme og forbedre fiskevelfærden gennem stimulering af det ikke-specifikke forsvarssystem. β-glucaner er naturligt forekommende polysaccharider, der findes i cellevæggen hos planter, bakterier og svampe. β-glucaner er nogle af de mest effektive og bedst undersøgte stoffer med immunmodulerende egenskaber i fisk. β-glucaner har vist sig at forbedre immunsystemet og farmakologisk klassificeres de som biologiske respons-modifikatiorer. Fokus i denne afhandling er: 1. Udvikling af en model til undersøgelse af den biologiske virkning af to kommercielt tilgængelige β-glucan berigede produkter på sårhelingsprocessen i almindelig karpe (*Cyprinus carpio* L.) under sterile, kontrollerede betingelser; 2. Undersøgelse af betydningen af intravenøst injiceret β-glucan på det mukosale-immunrespons og en antistof switch-lignende proces i karper. For at nå disse mål, blev der anvendt forskellige metoder, såsom kvantitativ realtime-PCR (RT-PCR) til måling af ekspressionen af immun-relaterede gener involveret i sårhelingsprocessen, ELISA for bestemmelse af specifikke antistoftitere samt cortisol-måling af stressniveau, real-time respiratorisk burst assay til bestemmelse af produktionen af frie-radikaler og billedanalyse af sårhelingsprocessen. Det er blevet vist at behandlede sygdomsudbrud giver anledning til ændringer i kvalitetsparametre som tekstur i frisk fiskekød. Hvilket har været udgangspunktet for yderligere at undersøge anvendelses af immunmodulatoren β-glucaner. Den videre undersøgelse af effekter af β-glucan har vist, at to kommercielt tilgængelige β-glucan berigede produkter, MacroGard og 6,3 kDa havrefibre, har en direkte positiv effekt på sårlukning i karper ved badbehandling med disse. Således var sårheling hurtigere i β-glucan behandlede fisk i sammenligning med ikke-behandlede fisk. Det immunologiske og regenerative respons efter stimulering med PAMPs (Pathogen-associated molecular patterns) og DAMPs (danger-associated molecular patterns molecules) under kontrollerede forhold, uden at eksponeringen for patogener er ligeledes blevet undersøgt, og en inflammatorisk reaktion ved aktivering af IL-1b, IL-6 og IL-8 blev fundet. Herudover blev der set lokale forskelle i ekspressionsmønsteret afhængige af om stimuleringen var med DAMPS alene eller DAMPS / PAMPs kombination. Desuden understøtter fraværet af væsentlige forskelle i den respiratorisk burst aktivitet i hoved-nyreceller ideen om at det lokale respons på et vævsbeskadigelsen er det primære innate respons. Yderligere blev der fundet immunologiske effekter af intravenøs injektion af β-glucan i huden. Således blev der observeret immunologiske forandringer i huden som tyder på en immunoglobulin switch-lignende proces med IgM / IgZ.

Endvidere blev det fundet at iv. injektion med β-glucan har en dosisafhængig effekt på stress niveauet i karper udtrykt som forøgelse af plasma cortisol niveau.

1. General introduction

The common carp (*Cyprinus carpio* L.) can be considered the first fish specie that was widely distributed by humans (Balon, 1995). Nowdays it is the third most frequently introduced specie worldwide (Welcomme, 1992). The common carp also accounts for the world's second highest farmed fish production, mainly from polyculture in Asia (Milstein, 1992), and the production of ornamental varieties is even more important in monetary value (Balon, 1995). A change in the main objective of common carp production can be observed in Europe (FAO report 2004-2012; FAO. © 2004-2012. Cultured Aquatic Species Information Programme. Cyprinus carpio. Cultured Aquatic Species Information Programme). Furthermore, due to its outstanding importance in freshwater aquaculture many aspects of its physiology, nutrition, genetics, and diseases have been studied during past decades. Adverse changes in the natural environment, the increasing intensity of carp production in many areas, extensive inter-regional transport of common carp and other cyprinids, and the ban on using several traditional medicaments (fungicides, antibiotics and insecticides) call for the intensification of research on carp diseases. The development of vaccines and immune modulators, to increase the natural resistance of fish to pathogens, seems to be the most promising solution for avoiding the application of antibiotics (Rodgers and Furones, 2009). Nowadays, number of biological compounds has been used to reduce the risk of diseases and improve fish welfare by enhancement of non-specific defence system. Among them, β-glucans are some of the most powerful and extensively investigated immune modulants. They are naturally occurring polysaccharides from cereals (e.g. barley, oat), algae, mushrooms and yeast cell walls. Glucan exerts a beneficial effect on a variety of experimental disease states of bacterial, viral, fungal and parasitic origin (DiLuzio and Williams, 1978; Williams *et al*., 1983, Williams and DiLuzio, 1980, 1985, Williams *et al*., 1978, Cook *et al*., 1980; Yano *et al*., 1991, Raa *et al*., 1992, Duncan and Klesius 1996, Rodriguez *et al*. 2009, Zhang *et al*. 2009). Many studies investigated the effect of beta glucans on fish immunity (Dalmo *et al*., 1997; Palic *et al*., 2006; 2007; Dalmo and Bøgwald, 2008; Yano *et al*., 1991; Raa *et al*., 1992; Duncan and Klesius 1996; Rodríguez *et al*., 2009; Zhang *et al*., 2009).

The overallobjective of the PhD project is to determinate and understand the impact of the β-glucan as immune modulator in relation to the mucosal immune response in common carp (*Cyprinus carpio* L.). It was achieved by introducing β-glucans from "outside" and "inside" the fish and subsequently monitoring changes in parameters of innate immune response of common carp, such as pro-inflammatory cytokines expression, respiratory burst activity, antibody ratio and cortisol level. For this purpose two models were applied: 1. bath model where biological effect of β-glucan enriched products on wound healing process in carp was examined, and 2. intravenous injection

model, where natural and specific antibody ratio and dose-related side effects of β-glucan were investigated.

1.1 Immunity in fish

Immunity system exists in animals (vertebrates and invertebrates) and plants (Ausubel 2005) to protect themselves against variety of microorganisms (Janeway and Medzitov 2002). Immunity is divided into two: innate immunity, non-specific and fast reacting defense mechanism, which includes physical parameters, cellular and humoral factors (Magnadóttir, 2004), and adaptive immunity with a highly specificy and diverse system characterized by memory occurrence.

Fish are the earliest vertebrates that have developed both arms of the immune system, reflected by the innate and the adaptive immune response (Figure 1). Mucosal immune system in skin, gill and gut forms the first line of defense and the connective tissues associated are densely populated with immune cells. Fish lack bone marrow as a primary immune organ, and their hematopoietic cells are produced in so-called head kidney (HK) where myeloid and lymphoid immune cells raise (van Muiswinkel, 1995). The capacity of the head-kidney to trap antigens and produce antibodies has been demonstrated in a few species (Ellis *et al*., 1989; Zapata and Cooper, 1990). Moreover head kidney combines immune functions with corticosteroid production and is an intriguing link between the endocrine and the immune system (Manning, 1994). The HK together with the thymus, centre of T-lymphocytes maturation, are sites for the interaction of immune system with antigens (Tort *et al*., 2003). Fish lack lymph nodes as secondary immune organs (Tort *et al*., 2003). In consequence blood filtration and erythrocytic destruction is performed by the melanomacrophagic centres, which are accumulation of macrophages associated to elipsoid capillaries. These centers may retain antigens as immune complexes for long periods (Espenes *et al*., 1995).

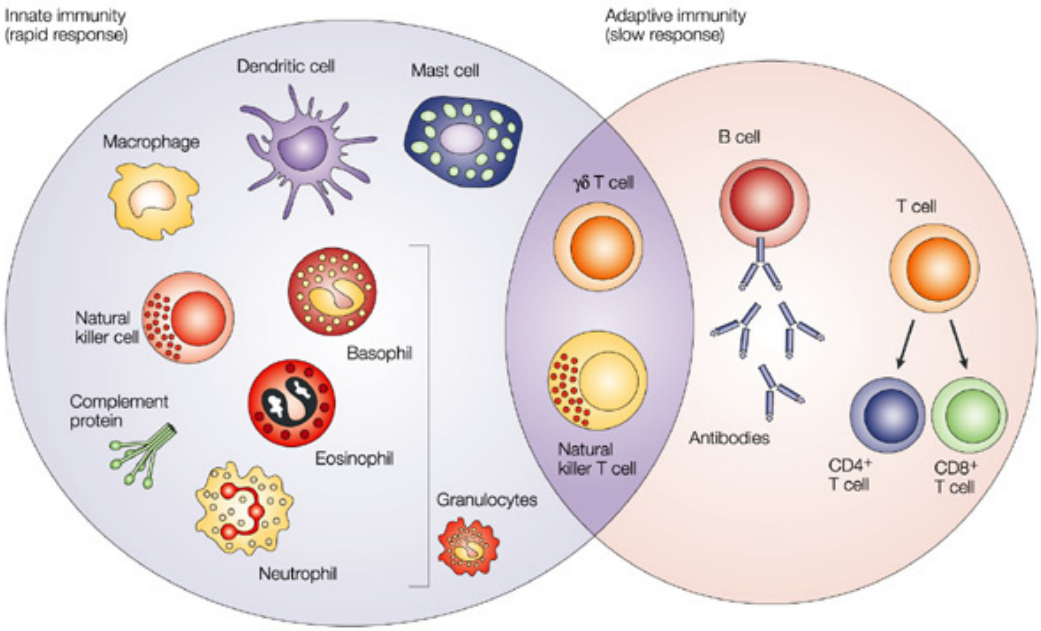


Figure 1. Mammalian model: illustration of cells involved in innate and adaptive immune response (Dranoff, 2004). The innate immune response functions as the first line of defense against infection. It consists of soluble factors, such as complement proteins, and diverse cellular components including granulocytes (basophils, eosinophils and neutrophils), mast cells, macrophages, dendritic cells and natural killer cells. The adaptive immune response is slower to develop, but manifests as increased antigenic specificity and memory. It consists of antibodies, B cells, and CD4+ and CD8+ T lymphocytes. Natural killer T cells and T cells are cytotoxic lymphocytes that straddle the interface of innate and adaptive immunity. Most of the cells involved in mammalian immunity are present in fish, and they are myeloid cells, neutrophilic granulocytes and macrophages. Pathogens are killed by natural cytotoxic cells, the fish equivalent of mammalian natural cells. Further, fish possess mast cells, however they do not have histamine granules (Mulero et al., 2007). In the present PhD thesis, both, innate (**PAPER 2**) and adaptive immunity (**PAPER 3**) have been examinated.

Although the immune system of teleost fish is different from that of mammals, there are some common features. Immune cells communicate via cytokines and associated receptors, which is important for a fast and effective attack on pathogens (Bird *et al*., 2006). Many genes encoding molecules associated with defense mechanism of fish have counter parts in mammals, suggesting a common ancestor of key mediators of immune system (Plouffe *et al*., 2005). In vertebrates, cytokines are the key regulators of both, innate and adaptive immunity, and elicit broad range of actions on cell growth and differentiation (Bird *et al*., 2006). In general, the cytokines repertoire in fish is alike in mammals, however function of each fish cytokine has not been described to the same extent as it has mammals. However, in fish many genes are duplicated and it results in the creation of numerous novel or semi-novel genes and functions in fish, known as the "more genes in fish than mammals" concept (Ohno 1970). It is, therefore, not surprising that there are already 5 genomic databases sequenced thus far for this taxon; the zebrafish, medaka, stickleback, tiger pufferfish and the green spotted pufferfish (www.ensembl.org). Large scale of teleost fish genome

analysis has revealed numerous gene duplications that are thought to originate from the whole-genome duplication - WGD (Stein 2007).

Tremendous progress has been made in isolating these molecules from fish in recent years, (ILs) such as IL-1β (Zou *et al*., 1999; Bird *et al*., 2002; Seppola *et al*., 2007), IL-6 (Bird *et al*., 2005b; Costa *et al*., 2011), IL-8 (Seppola *et al*., 2007; Oehlers *et al*., 2009; Qiu *et al*., 2009.); tumor necrosis factor alpha (TNF-α) (Hirono *et al*., 2000; Secombs *et al*., 2001) and interferons e.g. interferon gamma (IFN-γ) (Zou *et al*., 2004), and many more are predicted to be found. Genetic diversity translates to protein diversity, and as such, it is therefore very possible that in teleost fish, there will be a lot of unique and differing functionalities amidst the background of conserved functions. In fact, many of these fish-specific features are now starting to be unraveled (Aoki *et al*., 2008).

The adaptive immune response provides the vertebrate immune system with the ability to recognize and remember specific pathogens, and to mount stronger and faster response to pathogens. To mount such an immune response, a specific pathogen or antigen needs to be recognized (Aoki *et al.*, 2008). In teleostean fishes, as in mammals, a first antigen encounter will induce some long-lived memory cells that retain the capacity to be stimulated by the antigen, which results in faster and more vigorous secondary response (dos Santos *et al*., 2001; Kaattari *et al*., 2002). The essential features of adaptive immunity are immunoglobulins and the major histocompatibility complex (MHC). The first evidence of for MHC genes in teleost fish was published by Hashimoto *et al*., in 1990. Today, MHC genes have been isolated and described for all major vertebrates, including cartilaginous fish (Barl, 1998; Ohta *et al*., 2000) and bony fish (Kruiswijk *et al*., 2002; Stet *et al*., 1998). The teleost fish MHC is equivalent to its mammalian counterpart, but the class I and class II genes appear in different linkage group and are not clustered (Stet *et al*., 2003).
Mammals have five types of immunoglobulin (IgA, IgD, IgG, IgM and IgE; Elgert KD 1998; from Immunology: Understanding the immune system, Chapter 4). The main immunoglobulin studied in in teleost is tetrameric IgM (Håvarstein *et al*., 1998; Kaattari *et al*., 1998) and additionally, IgD and IgT/IgZ have been recently described, but functional implications have not yet been established (Flajnik, 2002; Danilova *et al*., 2005; Savan *et al*., 2005a; Savan *et al*., 2005b; Hansen *et al*., 2005; Zhang *et al*. 2010). Moreover, IgZ or IgT were not sequenced in all teleost species (Bengten *et al*., 2006). IgG is not found in fish and no class switch seems to be possible (Magor *et al*., 1999). However, Przybylska and Nielsen (**PAPER 3**) have showed, that dose-related injection with β-glucan stimulates IgZ1 gene expression in skin of common carp, while expression of main fish immunoglobulin IgM stays unchanged.

Common carp (*Cyprinus carpio* L.) is a close relative to zebrafish (*Danio rerio*), and they are representatives of the most abundant and widespread primary freshwater fish family, Cyprinidae (FishBase, http://www.fishbase.org/). In this manuscript, some references will be made to zebrafish due to ample genomic resources including a nearly fully sequenced genome and over a million expressed sequence tags, while genomic data for the rest of the cyprinids are quite scarce (Orbam and Wu, 2006).

1.2 Mucosal immune system

The mucosal immune system in vertebrates is the part of the immune system that responds to and protects against pathogens that enter the body through mucosal surfaces. The mucosal membranes are endowed with powerful mechanical and chemical cleansing mechanisms that degrade and repel most foreign matter and, in addition, a large and highly specialised innate and adaptive mucosal immune system protects these surfaces (Holmgren and Czerkinsky, 2005). The mammalian mucosal immune system is composed of inductive sites, where antigens sampled from mucosal surfaces stimulate cognate naïve T and B lymphocytes, and effector sites, where the effector cells after extravasation and differentiation get to work (Brandtzaeg and Pabst, 2004). Inductive sites for mucosal immunity consists of organized mucosa-associated lymphoid tissue (MALT) as well as local and regional draining lymph nodes (LN), whereas the effector sites consists of distinctly different histological compartments, including the lamina propria (LP) of various mucosae, the stroma of exocrine glands and the surface epithelia (Brandtzaeg and Pabst, 2004). Depending on the anatomical regions, MALT is subdivided into: bronchus-associated lymphoid tissue (BALT), gut-associated lymphoid tissue (GALT) and skin-associated lymphoid tissue (SALT) (Brandtzaeg and Pabst, 2004). In vertebrates, mucosal immune system provides three main functions: (i) mucus membrane protection against infections, (ii) prevention of the uptake of antigens, microorganisms and foreign materials, (iii) and avoidance of the of harmful immune response development against these pathogens (Holmgren and Czerkinsky, 2005).

1.2.1. Mucosal immunity in fish

Skin, gill and intestine are the first barrier between the fish and surrounded environment. MALT is present in fish, however it is less organised than in mammals: fish do not possess organized lymphoid tissue such as Peyer's patches and M cells or secretory IgA (SIgA) (Rombout *et al*., 2011). However, 2nd segment of the gut (hindgut) seems to play important function in antigen uptake (Rombout *et al*., 1993). Uptake of macromolecules by intestinal epithelial cells and many diffusely distributed leucocytes including intraperitoneal lymphocytes (IELs) are described in numerous fish species, such as common carp (Rombout *et al*., 1993), goldfish (Temkin and

McMillan, 1986), Atlantic salmon (Uran *et al*., 2008). Moreover, as recently showed by Przybylska *et al*. (**PAPER 2**), carp skin responses to β-glucan stimulation when bathing, resulting in an inflammatory response by activating IL-1β, IL-6 and IL-8 and local differences in expression pattern dependent on major stimuli: DAMPs or DAMPs/PAMPs combination.

1.2.1.1. Mucus

Fish and other aquatic vertebrates are covered with mucus blanket, which is synthesized and secreted by epidermal goblet cells. Mucus present in vertebrates consists of many components, two of which are predominantly important in gel formation: water and large filamentous, highly glycosylated glycoproteins called mucins (Verdugo 1990; Strous and Dekker, 1992; Bansil *et al*., 1995; Kesimer *et al*., 2010).

Fish mucus has wide range of functions: (i) protects underlying epithelium against various types of pathogens, (ii) respiration, (iii) ionic and osmotic regulation (Shephard, 1994). In vertebrates, in order to protect epithelial surface against damage, foreign particles and microorganisms are entrapped in mucus: mucus blanket is also a reservoir of numerous innate immune factors such as lysozyme, immunoglobulins, complement proteins, lectins, C-reactive proteins, proteolytic enzymes and various antibacterial proteins and peptides, e.g. defensins (Shepherd, 1994; Cole *et al*., 1997; Subramanian *et al*., 2007; Narvaez *et al*., 2010). Vertebrates' mucins and mucus have rheological properties, foe example viscosity and elasticity, which are important for their physiological function (Roussel and Delmotte, 2004). Based on biochemical characterisation, 19 mucin genes have been identified in humans (Dekker *et al*., 2002), and their gene structure and role in the infection process has been proved (McAuley *et al*., 2007; Thornton *et al*., 2008). Up to date, only two mucin genes, Muc2c and Muc5b were cloned and sequenced in fish (van der Marel *et al*., 2012) cause accurate assembly of the mucin genes is difficult due to the large size of the centraltandem repeats (Cox *et al*., 2010). However, the mucin genes were highly conserved and mucins described in fish showed a high similarity to mammalian Muc2 and Muc5B. Analysis of tissues and organs for mucin expression in carp revealed a clear separation in the site of expression between the two mucins. Carp Muc5b appears to be homologous to mammalian Muc5B. Human Muc5B has been found to be mainly expressed in the mucous glands of the respiratory mucosa and salivatory glands, as well as in the gall bladder, pancreas and cervix, whereas carp Muc5b was expressed with high copy numbers in skin and gills (Roussel and Delmotte, 2004; van der Marel *et al*., 2012). Carp Muc2c was only, but highly, expressed in the first and second intestinal segment (van der Marel *et al*., 2012). Moreover, differences Muc5b and Muc2c expression were observed in carp with different β-glucan feeding regimens. Consistent, but not significant, down-regulation of

Muc2c in the intestine and gills was seen in the β-glucan fed fish, while Muc5b was significantly increased in skin, with slight up-regulation in gills. Additionally, Muc5b expression is affected by β-glucan enriched bath, as it was recently reported (Przybylska *et al*., **PAPER 2**). Although, in vertebrates, Muc5B is one of the largest gel-forming glycoprotein in the body, cells may differ in the density of mucin granules, suggesting a diversity of the mucins synthesized even by an individual cell (Roussel and Delmotte, 2004; Kesimer *et al*., 2010; Sheehan *et al*., 2000).

1.2.1.2. Skin

Fish skin is a complex structure composed of two layers, the outer stratified epithelium (epidermis) and the inner layer (dermis), which are separated by a basal membrane that is composed of undifferentiated cells (Figure 2.) (Buchmann, 1999). In contrast to terrestrial vertebrates, fish skin comprises living cells and hence is metabolically active (Martin, 1997; Press CM and Evensen, 1999). Pluripotent cells in fish skin, actively participate in wound healing, tissue remodelling, and are able to differentiate into epithelial cells, mucous cells, club cells or sensory cells (Rakers *et al*., 2010). The thickness and cells composition of epidermis varies among fish species. In common carp, epithelial cells, goblet cells, club cells and keratynocytes are mainly located in the epidermis, while chromatophores, melanophores and xanthopores are found deeper in the tissue (Iger and Bonga, 1994). Fish epidermis forms not only the first barrier against environment, but represents the initial site for complex immune responses against microorganisms (Kearn, 1999). Immunological components, such as lymphocytes, macrophages and granulocytes were found in skin of teleost species (Iger *et al*., 1988; Peleteiro and Richards, 1990; Herbomel *et al*., 2001). Moreover, the presence of IgM was detected in the goblet cells of trout skin (Peleteiro and Richards, 1988). In addition, presence and role of specific antibodies in skin and mucus against e.g. infection of skin by *I. multifiliis* was previously showed (Clark *et al*., 1996; Dickerson and Clark, 1998). Recent work presented by Przybylska *et al*. **(PAPER 2)** and Przybylska and Nielsen **(PAPER 3)** showed that skin together with mucus responds to stimulation with β-glucan.

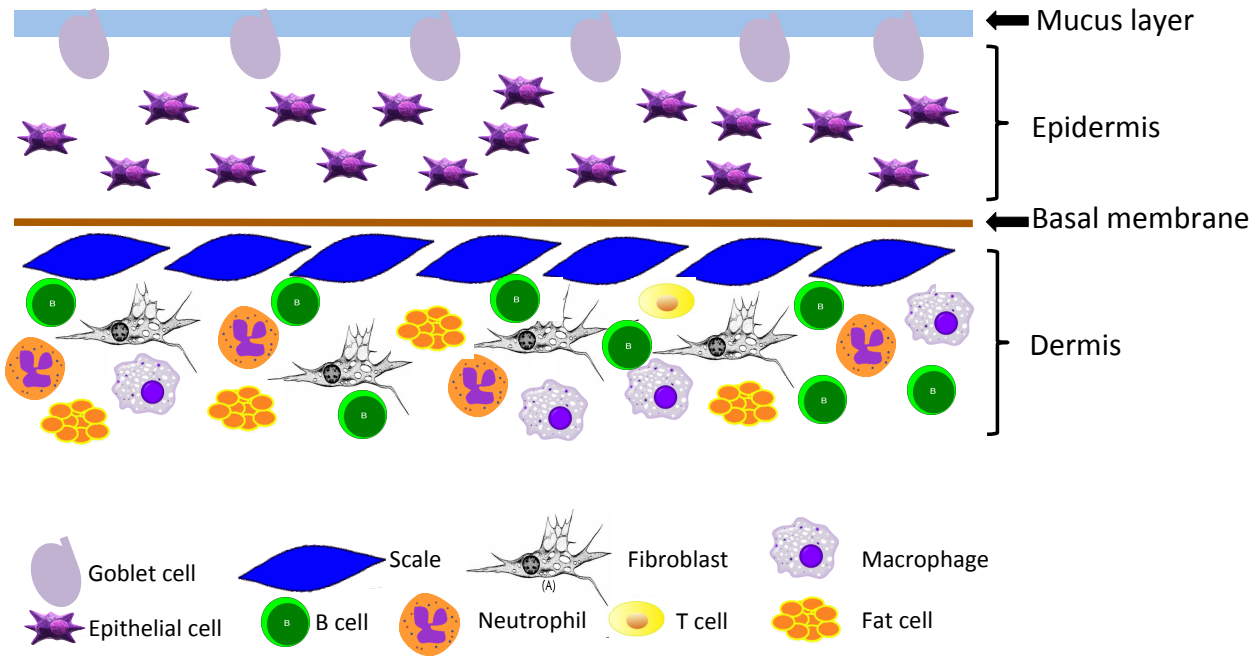


Figure 2. Schematic model of common carp skin. Fish skin is divided in to layers, the epidermis and dermis, which are separated by basal membrane. The outer surface of fish skin is protected by the mucosal blanket, formed by goblet cells (known also as mucous cells). In a healthy skin, many leucocytes can be found. Scales are originated from the dermal layer. Based on recent studies, IgZ(T)$^{+}$ B cells might be the major B cells present in skin.

1.2.1.3. Gill

Gills consist of lamellae, which comprise the main respiratory surfaces of the fish (Wilson and Laurent, 2002). Recent morphological studies on Nile tilapia (*Oreochromis niloticus*) have described the branchial filament as a multilayered epithelium, such as in other teleostean species (Monteiro *et al*., 2010). Lymphocytes (Grove *et al*., 2006a; Lin *et al*., 1998), macrophages (Lin *et al*., 1998; Mulero *et al*., 2008), neutrophils (Lin *et al*., 1998), eosinophilic granulocytes (Barnett *et al*., 1996; Lin *et al*., 1998; Mulero *et al*., 2007) and antibody-secreting cells (ASC) (Davidson *et al*., 1997; dos Santos *et al*., 2001a) have been observed in the gill associated lymphoid tissue (GIALT) of different fish species. Presence of the B cells and T preponderance cells was shown as well (Lin *et al*., 1999). Mucus production has been proven to be higher in the area surrounding the gill cover than in any other skin sites (Shephard 1994).

1.2.1.4. Gut

Fish do not possess organized GALT, and thus, have no lymphoid tissue such as (i) Peyer's patches, an organized lymphoid nodules and mesenteric lymph nodes, (ii) M cells, which sample antigen directly from the lumen of gut and deliver it to antigen presenting cells, (iii) secretory IgA,

produced by activated B-cells in the mucosa that forms immune complexes with pathogens and allergens, preventing them from binding to and penetrating the intestinal mucosa (SIgA; Rombout *et al*., 2005; 2010). Lamina propria of teleost gut harbors a variety of immune cells including, but is not limited to macrophages, granulocytes, lymphocytes and plasma cells, whereas the surface epithelium compartment is mainly composed of T cells and few B cells. Most of our knowledge regarding gut immunity relates to the differential uptake of particles in the anterior gut (foregut or first segment). In cyprinids like carp (*Cyprinus carpio* L.) (Rombout *et al*., 1985) and goldfish (*Carassius auratus auratus*) (Temkin and McMillan, 1986), large intraepithelial macrophages containing phagocytosed material have been observed and therefore these are thought to be the main antigen presenting cells. However, no macrophages were found in the gut of seabream (*Sparus aurata*); (Mulero *et al*., 2008). Presence of IgM positive cells in gut of sea bass, turbot, carp and cod has been shown (Abelli *et al*., 1997; Fournier-Betz *et al*., 2000; Rombout *et al*., 1993, 1998; Schrøder *et al*., 1998).

1.3 Wound healing process

Wounds in fish can be caused by pathogens, such as ectoparasites, for example *Ichthyophthirius multifillis* and lice, Gram-negative bacteria as *Moritella viscosus*, as well as mechanical trauma (Gonzalez *et al*., 2007; Ingerslev *et al*., 2010; Krkosek *et al*., 2011). In vertebrates reduced skin integrity or mucus production facilitates entry of pathogens into the underlying tissue and vascular system, and therefore rapid recruitment of immune cells to the wound site is essential (Singer and Clark, 1999). In all animals, wound healing is a complex, well-organized process which can be roughly divided into three overlapping phases: inflammation, tissue formation and remodelling, in which blood cells, soluble mediators, resident cells (fibroblasts, endothelial cells, goblet cells) and extracellular matrix components are involved (Figure 3) (Singer and Clark, 1999; Gillitzer and Goebeler, 2001; Kanter and Akpolat, 2008). In fish, so far, the inflammatory phase is the best described.

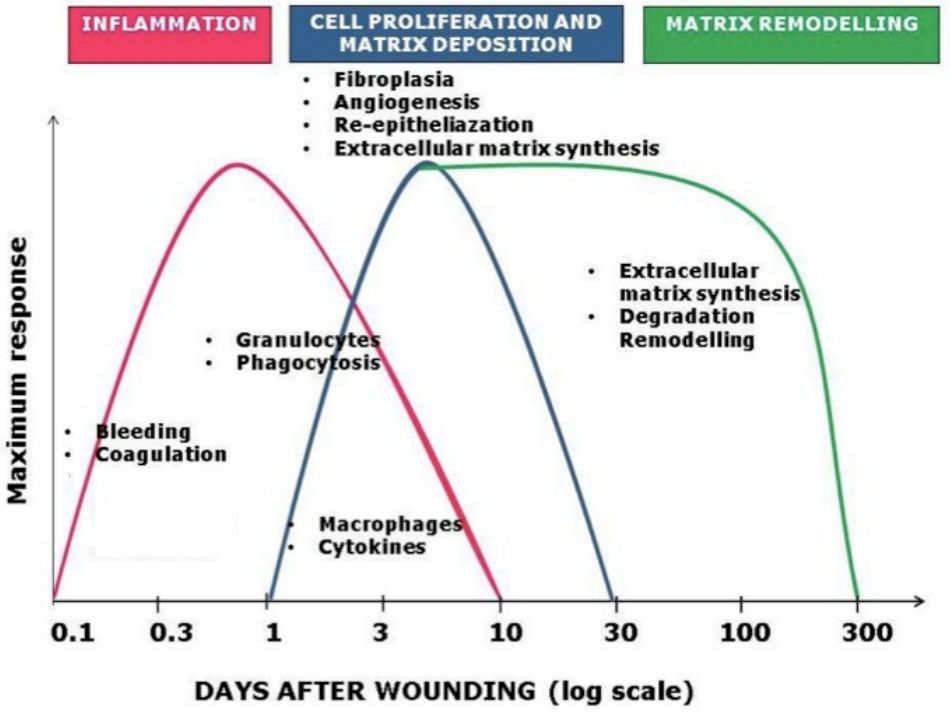


Figure 3. Mamalian model showing time line for three phases of wound healing process (Enoch and Price, 2004; modified). The same three phases are distinguished in wounds occurred in fish. Up to date, the inflammatory (inflammation) phase is the best described. In present work, wound closere in skin and muscle of common carp was investigated, and similar time-wise pattern was obserwed: inflammation phase with bleeding step (day 1), epithelium cells overgrowth (day 3) and complete wound closure at day 14 (**PAPER 2**).

1.3.1 The inflammatory response following the tissue damage

Wound healing process in vertebrates is accompanied by inflammatory reaction (Singer and Clark, 1999). In vertebrates, during tissue injury, blood vessels break and host homeostasis is disrupted. In order to re-establish the homeostasis and provide provisional matrix for cell migration red blood cells together with platelets form a clot (Singer and Clark, 1999). At the same time, inflammatory reaction starts to clean up the site of injury and fight pathogens: numerous vasoactive mediators and chemokines such as interleukins, lymphokines and several related signalling molecules, for example tumor necrosis factor alpha (TNF-α) and interferons are released to recruit neutrophils and macrophages to the site of injury (Singer and Clark, 1999; Gillitzer and Goebeler, 2001). All mention above play important role in recruitment of inflammatory cells to the wounded site as well as contribution to the regulation of re-epithelization, tissue remodelling and angiogenesis (Gillitzer and Goebeler, 2001). In mammals, tissue injury causes release of danger associated molecular patterns (DAMPs): remains of damaged cells, which can trigger immune response in non-damaged cells (Gillitzer and Goebeler, 2001; Metzinger 1994, 2002). In fish, immediately after injury, erythrocytes and thrombocytes become abundant in the affected area (Jiang and Doolittle, 2003).

Alike in mammalian mmodel, fish neutrophiles and macrophages are of particular importance due to their phagocytic activity and cytokines and chemokines production (Gonzalez *et al*., 2007a, b; Martin and Leibovich, 2005). Fish neutrophils appear at the injured site very fast and have been observed in skin and musculature tissue two hours after mechanical damage (Gonzalez *et al*., 2007a).

In vertebrates, chemokines poses unique potential for selective activation of various leukocyte subsets (Belperio *et al*., 2000). Immune cells appeare at the injured site by following increasing gradient of cytokines and chemokines, as well as DAMPs release. First, neutrophils infiltration takes place, in order to clean damaged site of foreign particles and bacteria, and secondly macrophages take over (Singer and Clark, 1999). Mammalian macrophages circulate in blood as monocytes although they mature and differentiate in specific tissue types (Geissmann *et al*., 2010). In response to specific chemoattractants (e.g interleukin-8, IL-8), monocytes are directed to the wound area and become active macrophages. They are considered as a key cells in wound repair due to their capacity to produce inflammatory cytokines as well as growth factors (Riches, 1996). Recently, process of wound closure in skin of common carp was investigated (Przybylska *et al*., **PAPER 2**) where bathing in β-glucans, specifically MacroGard and 6.3 kDa oat fiber, have a direct positive effects on the wound closure in common carp. Immunological and regenerative response following stimulation with PAMPs and DAMPs in controlled conditions was showed. PAMPs/DAMPs stimulation resulted in an inflammatory response by activating IL-1β, IL-6 and IL-8, and local differences in expression pattern dependent on major stimuli: DAMPs or DAMPs/PAMPs combination. Expression of main pro-inflammatory cytokines in skin confirms presence of immune related cells, such as neutrophils and macrophages and their active response to stimulation. In addition, the absence of marked differences on the respiratory burst activity in head-kidney cells supports the idea of localized immune response to the site of injury (Przybylska *et al*., **PAPER 2**).

Wound healing is a complex process, and it has been proven, that not only "typical" immune cells of haematopoietic origin, such as neutrophils or macrophages are involved in wound closure and cytokines production. Fibroblasts have been described in humans as the non-conventional cell type having immune regulation capabilities (Larsen *et al*., 1989; Chen *et al*., 2005). Recent studies in human have shown that fibroblasts can not only respond to cytokines, but also generate these signals (IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, TNF-α and IFN-γ) and influence the behaviour of immune competent cells trafficking to the injury site (Larsen *et al*., 1989; Chen *et al*., 2005; Ruiz *et al*., 2007). That hypothesis has been strengthen by results obtained from research on mammalian fibroblasts. Wei *et al*. (2002) have presented first evidences that glucan can directly stimulate

human fibroblasts. Moreover, *in vitro* studies in mammals presented by Iocono *et al*. (2000) showed that IL-8/CXCL8 had an inflammatory effect on keratinocyte proliferation and collagen lattice contraction. Rennekampff *et al*. (2000) demonstrated results where IL-8/CXCL8 was shown to have stimulatory effect on murine keratinocytes proliferation *in vitro*. Injury could potentiate immune-like performance of fibroblasts, as well as having a direct effect on the resident cells present in skin, such as epithelial cells, resident macrophages and lymphocytes. Several fibroblast cell lines have been established and characterised in fish (Collet and Secombes, 2001; Ossum *et al*., 2004). Ingerslev *et al.,* (2010) reported that fish fibroblasts are susceptible to DAMPs.We believe, wound closure process in fish is alike the one described in mammals. It is supported by results presented by Ingerslev *et al*. (2010) and recently by Przybylska *et al*. **(PAPER 2**).

1.3.2 Cells involved in the inflammatory response

Cells of vertebrates' immune system are active participants in the repair of injured skin (Singer and Clark, 1999). Immune cells can greatly impact the repair process at each of these stages: hemostasis, inflammation, proliferation, and scar formation/remodeling (Enoch and Price, 2004). In vertebrates, immediately after skin injury platelets enter the wounded area, where they aggregate and release mediators that initiate the coagulation cascade, as well as growth factors and cytokines involved in the recruitment of inflammatory and immune cells (Martin, 1997; Singerand and Clark, 1999). At the same time, neutrophils start fighting and destroying any invading microorganisms, and subsequently the damaged site is overtaken by macrophages, which clear the wound of debris and foreign particles (Smith and Lumsden, 1983). Both neutrophils and macrophages release an assortment of growth factors and cytokines (Riches, 1996). Eventually, T cells and other members of the adaptive immune system may become involved in wound closure in mammals (Havran and Jameson, 2010). During re-epithelialization, angiogenesis, the scar formation and remodeling phase, fibroblasts increase in number and produce a permanent scar in the repaired skin (Chen *et al*., 2005; Ruiz *et al*., 2007).

1.3.2.1 Neutrophils

In all animals, neutrophils are the most numerous and important components of host defence against bacteria, viruses and fungal infections (Smith and Lumsden, 1983). In fish, the anterior kidney is the central immune organ where neutrophils are being produced (Bols *et al*., 2001), however they can be found in spleen and blood, and are commonly increased in inflammatory lesions (Afonso *et al*., 1998). In response to inflammation, fish neutrophils migrate from blood stream to site of infection, where they bind, engulf and kill pathogens by proteolytic enzymes, antimicrobial peptides and reactive oxygen radicals production (Lamas and Ellis, 1994; Dalmo *et*

al., 1997; Palic *et al*., 2005). In vertebrates, by degranulation, neutrophils release antimicrobial factors as well as neutrophil extracellular traps (NETs) (Brinkmann *et al*., 2004; Cho *et al*., 2005). NETs can be produced by stimulated neutrophils or in response to cytokine stimulation, and immobilize and kill pathogens (Brinkmann *et al*., 2004; Cho *et al*., 2005; Palic *et al*., 2007). Fish and mammalian neutrophils share morphological and functional similarities in respect to phagocytosis, chemotaxis, respiratory burst intensity, degranulation and NETs release (Lamas and Ellis, 1994; Berman *et al*., 2003; Hermann *et al*., 2004; Rodrigues *et al*., 2004; Palic *et al*., 2005a, b). NETs have been described in mammalian and fish neutrophils, but not in other cell types.

In fish, immune and endocrine systems are linked due to double role of head kidney, therefore neutrophils are affected by high cortisol level (Weyts *et al*., 1998a, b; Flik *et al*., 2006; Palic *et al*., 2007). It was showed that fish exposed to handling and crowding stress for 48 hours showed the decrease in neutrophil activity measured as NETs release (Palic *et al*., 2007). Degranulation of neutrophils and release of myeloperoxidase (MPO) is an important step in oxygen mediated killing of pathogens, and it appears to be essential step in exocytosis in neutrophil extracellular traps (Brinkmann *et al*., 2004). Recently, particulate β-1,3-1,6-glucan from baker's yeast was found to be a potent stimulator of fish neutrophil degranulation and an optimal neutrophil immune modulator during stress conditions in fathead minnow (Ainsworth, 1994; Palic *et al*., 2006; 2007). Previous reports have showed strong respiratory burst response of fish phagocytes to glucans in doses from 50 to 1000 μg/ ml (Castro *et al*., 1999; Jørgensen and Robertsen, 1995). In contrary to that, Przybylska *et al*, (**PAPER 2**) have reported that bath supplemented with β-glucan enriched products had no effect on respiratory burst activity in neutrophils collected from head kidney of common carp. Nevertheless, during injury, neutrophils are the first leukocytes that migrate to into inflamed tissue (Chadzinska *et al*., 1999).

1.3.2.2 Macrophages

Macrophages in vertebrates arise from hematopoietic progenitors, which differentiate directly, or via circulating monocytes, into subpopulation of tissue macrophages (Geissmann *et al*., 2010). In vertebrates, macrophages are multifunctional: they can ingeste microbes, secrete wide range of biologically active molecules (hydrolases, complement components, enzyme inhibitors, cytokines, eikosanoides) and reactive oxygen and nitrogen species (Auger *et al*., 1992; Secombs and Fletcher, 1992; Neumann *et al*., 1995; Jang *et al*., 1995). Depending on the organ, resident macrophages of higher vertebrates posses morphological and phenotypic differences (Gordon and Taylor, 2005). Fish macrophages were shown to have a long life compared to neutrophils and play a crucial role in both, the innate and the adaptive immune system (Joerink *et al*., 2006). In the innate immune

system, they act as phagocytic cells, and producing oxygen and nitrogen radicals, in the adaptive immune system, act as professional antigen presenting cells (APCs). Hanington *et al*., (2009) have summarized the recent findings of macrophage development in teleost. Joerink *et al*. presented (2006) have showed that carp head kidney macrophages are heterogeneous and, upon *in vitro* stimulation, can give rise to functionally different polarization states. First evidences for innate and alternative macrophage polarization *in vivo* in common carp were presented by Chadzinska *et al*. (2008). Moreover, teleost macrophages become activated following stimulation with bacterial products (e.g. lipopolysaccharide) and/or soluble host-derived factors (Graham and Secombes, 1988; Jang *et al*., 1995; Neumann *et al*., 1995). This was strengthen with results presented by Przybylska *et al*., (**PAPER 2**) where during wound healing process, PAMPs/DAMPs stimulation resulted in an inflammatory response by activating IL-1β, IL-6 and IL-8 which might be indirect evidence of macrophage activation.

1.3.2.3 Fibroblasts

In mammals, the fibroblast are the last specialised member of the connective tissue, 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, are responsible for the synthesis, deposition, and remodeling of the extracellular matrix (Alberts, 2008). Conversely, the extracellular matrix can have a positive or negative effect on the ability of fibroblasts to synthesize, deposit, remodel, and generally interact with the extracellular matrix in mammals (Xu J and Clark, 1996; Clark *et al*., 1995). In mammalian model, after migrating into wounds, fibroblasts commence the synthesis of extracellular matrix, and subsequently the provisional extracellular matrix is gradually replaced with a collagenous matrix (Clark *et al*., 1995). In humans, fibroblasts have been described as the non-conventional cell type having immune regulation capabilities: they can not only respond to cytokines, but also generate these signals (IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, TNF-α and IFN-γ) and influence the behaviour of immune competent cells trafficking to the injury site (Larsen *et al*., 1989; Nirodi *et al*., 2000; Chen *et al*., 2005; Ruiz *et al*., 2007). Once an abundant collagen matrix has been deposited in the wound, the fibroblasts stop producing collagen, and the fibroblast-rich granulation tissue is replaced by a relatively acellular scar.

In contrast to higher vertebrates, fish skin is not keratinised and hence, metabolically active (Martin, 1997; Press CM and Evensen, 1999). Several fibroblast cell lines have been established and characterised in fish (Collet and Secombes, 2001; Ossum *et al*., 2004). Ingerslev *et al.,* (2010) reported that fish fibroblasts are susceptible to DAMPs. Moreover, Przybylska *et al*., (**PAPER 2**) have showed that fish bath in water with β-glucan enriched products were healing faster when

compared to non-treated fish. High up-regulation of IL-1β and IL-6 in skin samples from treated carp three days post wounding could be explained by the ability of fibroblasts to express genes of immune functions before the more potent immune cells have been recruited or before new amount of such cytokines has been produced. Presence of DAMPs (wounding) and PAMPs (β-glucans) could potentiate immune-like performance of fibroblasts, as well as having a direct effect on the resident cells present in skin, such as epithelial cells, resident macrophages and lymphocytes.

1.3.2.4 Mucous cells

Fish and other aquatic vertebrates are covered with mucus layer over their entire body surface. Skin, together with mucosa forms the first line of defence against pathogens and is an essential protective barrier in aquatic organisms. Mucus is produced by goblet and mucous cells which are placed in epidermis (Kanter and Akpolat, 2008). In mucus, foreign particles and microorganisms are entrapped, before imping epithelial surface and cause damage (Rogers, 1994). Few studies have emphasized the role of goblet cells and mucus in wound healing process in vertebrates (Geggel *et al*., 1984, Kanter and Akpolat, 2008). Goblet cells differentiation and functioning is affected by mucosal immunity in mammals (Ciacci *et al*., 2002), and gel-forming mucins can be regulated by inflammatory cytokines such as interleukins (IL-1β, -6, -17) or exogenous factors (Roussel and Delmotte, 2004). In fact, Jin-Jang *et al*., (2003) have found that in rat model, that goblet cells are always lost in the presence of intense inflammation and regulation by mucogenesis in goblet cells was associated with the cessation of inflammation. In fish, mucous cells usually originate from the pluripotent cells placed in the middle layer of epidermis, where they develop and mature (Bullock and Roberts, 1974). Mucous cells of different types are found in fish skin and the density of mucous cells varies greatly among fish species, body regions and growth stage (Bullock and Roberts, 1974; Mittal *et al*., 2000; Pinky *et al*., 2008; Yamamoto *et al*., 2011).

1.3.3 Molecules involved in the inflammatory response

In general, the extent of the immune response in vertebrates can vary depends on the major stimuli (Enoch and Price, 2004). It is important that occurred immune response is powerful enough to clean up debris and combat pathogens subsequently. During inflammation phase in mammals, secreated cytokines work in autocrine and paracrine manner and more seldom in an endocrine way (Gillitzer and Goebeler, 2001). In general, fish posses a similar repertoire of cytokines as mammals, however the function of each fish cytokine is not so well established as for mammalian cytokines (Secombes et al., 1996). Some of the cytokines are in high importance during wound healing process and interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8), the best described cytokines in fish (Scapigliati *et al*., 2000, Secombes *et al*., 1996) are among them.

1.3.3.1 Interleukin 1β (IL-1β)

In mammals two isoforms are present: IL-1α and IL-1β, but only IL-1β was characterized in fish. IL-1β is probably the the best described cytokine in fish. It is main pro-inflammatory cytokine with multiple functions such as stimulating cell division, cells activation, expression of other cytokines and induction of fever. Main IL-1 β source in vertebrates are polymorphonuclear laukocytes (constitute nearly 50% of all cells at the wound site; Gillitzer and Goebeler, 2001) and macrophages, as well as some resident cells (Feiken *et al*., 1995; Hübner *et al*., 1996). In vertebrates, IL-1β was shown to be strongly up-regulated during the inflammation (Hübner *et al*., 1996, Grellner *et al*., 2000; Chadzinska *et al*., 2007). In injured sites, IL-1β activates the lining endothelial cells and cause enhancement of leukocyte migration. Cortisol together with LPS synergistically stimulates the expression of IL-1β in fish HK phagocytes (Engelsma *et al*., 2003), however *in vitro* studies in common carp showed inhibition of inflammatory cytokine due to acute stress (Saeij *et al*., 2003). Interleukin 1 is one of the cytokines produced by macrophages in response to an immunogenic stimulus in fish. Previous studies revealed that glucan treatment in fish enhanced the expression of interleukin 1 (Fujiki *et al*., 2000). Injection with *Astragalus* polysaccharide increased the IL-1β mRNA level in a dose-dependent manner in the head kidney, while no significant changes were found in the gill and spleen (Galina *et al*., 2009). Chadzinska *et al*. (2008) reported migration of inflammatory leukocytes from HK into peritoneum during intraperitoneal injection of zymosan particles. Constitutive expression of IL-1β in peritoneal leukocytes (PTL) was two-fold higher than in HK, however during zymosan-induced peritonitis, IL-1β expression showed twice higher expression in peritoneal leukocytes (PTL) than in HK. In addition, Przybylska *et al*. (**PAPER 2**) have showed changes in IL-1β expression following wound healing process and stimulation with β-glucans. Furthermore, Przybylska and Nielsen (**PAPER 3**) reported changes in IL-1β expression followed intravenous injection with β-glucan, suggesting response to PAMPs stimulation.

1.3.3.2 Interleukin 8 (IL-8)

Interleukin 8 belongs to CXC chemokine family and its secretion activates and tracks neutrophil granulocytes to site of inflammation in vertebrates (Jimenez *et al*., 2006). As is well established in mammals, CXCL8 is among the first chemokines expressed during inflammation. The pro-inflammatory cytokines like IL-1β and TNF-a induce CXCL8 expression in endothelial cells, fibroblasts and local immune cells in mammals (Larsen *et al*., 1989; Strieter *et al*., 1989). IL-8 regulates is known regulator of neutrophil trafficking (Rennekampff *et al*., 2000; Werner and Grose, 2003; Grellner *et al*., 2000; Gillitzer and Goebeler, 2001) and augments angiogenesis in

mammals (Li *et al*., 2003; Yao *et al*. 2002). It was recently reported that in two fish species that belong to the cyprinid lineage, common carp and zebrafish, two CXCL8-like lineages evolved: CXCa_L1 (lineage 1) and CXCL8_L2 (lineage 2) (Abdelkhalek *et al*., 2009; van der Aa *et al*., 2010). Presence of two CXCL8 lineages in carp appears to be functional homologs to mammalian IL-8, and has crucial biological role in chemotaxis (van der Aa *et al*., 2010). Results presented by Sangrador-Vegas *et al*., (2002) reported that stimulation rainbow trout fibroblasts induce IL-8 expression. Recently, Przybylska *et al*. (**PAPER 2**) have showed that IL-8 is inducible in skin and muscle of common carp, when stimulated with PAMPs and DAMPs.

1.3.3.3 Interleukin 6 (IL-6)

Expression of IL-8 in mammals is correlated with interleukin 6 (IL-6); (Nielsen *et al*., 2007). IL-6 is a multifunctional cytokine that is produced by a variety of hematopoietic and non-hematopoietic cell types. Vertebrates IL-6 is produced and secreted by macrophage, neutrophils, T cells, as well as resident cells (Hübner *et al*., 1996; Gillitzer and Goebeler, 2001; Lin *et al*., 2003). IL-6 seemd to be one of the key cytokine involved in wound healing process in mammals: experiments on diabetic or IL-6 knockout mice have shown decrease in neutrophil and macrophage number, as well as a reduction in collagen production and deposition (Lin *et al*., 2003, Berdal *et al*., 2007). On the other hand administration of IL-6 at the wound site induces massive leukocyte infiltration in mice (Saba *et al*., 1996) and collagen production (Duncan and Berman, 1991; Greenwel *et al*., 1993). Orthologous of the mammalian IL-6 members have been identified in fish (Bird *et al*., 2005; Iliev *et al*., 2007; Castellana *et al*., 2008) and recently, Varela *et al*., 2011 have confirmed that zebrafish IL-6 shares structural characteristic with IL-6 from other vertebrates. IL-6 was found to follow β-glucans stimulation during bath treatment in skin and muscle of common carp (Przybylska *et al*., **PAPER 2**).

1.3.3.4 Heat shock protein 70 (Hsp70)

Heat shock proteins (Hsps) are highly conserved stress proteins, found in all prokaryotic and eukaryotic cells and are classified according to their molecular weight or to the way they are expressed. Hsps function as “danger signals” – damage associated molecular patterns (DAMPs), released endogenous structures from damaged or dead cells in all vertebrates (Matzinger 2002, Seong and Matzinger, 2004).

Hsp families have been reported in fish cell lines, primary cell cultures, as well as in various tissues from whole animals (Iwama *et al.,* 2004; Toa *et al*., 2004). Up-regulation of the Hsps in fish was observed in various stress conditions e.g. exposure to bacterial pathogens (Jia *et al*., 2000), environmental contamination such as heavy metals (Duffy *et al*., 1999), pesticides (Hassanein *et*

al., 1999). The Hsp response can vary according to tissue (Rabergh *et al*., 2000), Hsp families (Wagstaff *et al*., 1998), stressor (Airaksinen *et al*., 2003), species sensitivity (Basu *et al*., 2002) and season (Fader *et al*., 1994). 70kDa Hsps are the most conserved and the most studied class of Hsps. In mammals, Hsp70 is highly inducible during inflammatory reaction caused by stress (Li *et al*., 1992; Trautinger, 2001; Jacquier-Sarlin *et al*., 1995; Guo *et al*., 2007; Novoselova *et al*., 2006). In fish, Hsp70 responds 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). Stolte et al., (2009) showed that an immune stimulus modulates expression of Hsp70 *in vitro* and *in vivo*. As showed by Przybylska and Nielsen (**PAPER 3**), Hsp70 expression increases during common carp intravenous injection with high dose of β-glucan.

1.3.4 Pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs)

1.3.4.1 PAMP recognition

The immune system has to be effective in distinguishing "self" from "non-self" and combating pathogens (Figure 4). However that recognition relay on a limited and invariant repertoire of receptors. The pathogen-recognition receptors of macrophages, neutrophils, and dendritic cells recognize simple molecules and regular patterns of molecular structure known as pathogen-associated molecular patterns (PAMPs), exogenous molecules (antigens) produced only by potential pathogens (Medzhitov and Janeway, 2000). Janeway Medzhitov (2002) proposed that infection would be detected by innate receptors for microbiological biomolecules that are either absent or different in mammals. These receptors are known as pattern recognition receptors (PRRs) and they recognize structures such as mannose-rich oligosaccharides, peptidoglycans and lipopolysaccharides in the bacterial cell wall, and unmethylated CpG DNA, which are common to pathogens and have been conserved during evolution (Roach *et al*., 2005). PRRs have several functions: stimulate ingestion of the pathogens they recognized, guide cells to the site of infection and induce production of effector molecules. Important receptors recognising the pathogens are the Toll-like receptors (TLRs) and Nod-like receptors (NLRs) (Medzhitov and Janeway, 2000). However, microorganisms evolve more rapidly than their hosts, and this may explain why the cells and molecules of the innate immune system recognize only molecular structures that have reminded unchanged during evolution. In all vertebrates, the primary function of PRRs and the innate immune response is to provide immediate protection from pathogens (Takeda *et al*., 2003; Thoma-Uszynski, *et al*., 2001). This initial recognition of pathogen leads to the production variety of inflammatory mediators, such as chemokines, cytokines, vasoactive amines and eicosanoids

(Medzhitov, 2008). The main and most immediate effect of these mediators is to elicit an inflammatory exudate locally: plasma proteins and leukocytes (mainly neutrophils) that are normally restricted to the blood vessels, access the extravascular tissues at the site of infection or injury (Medzhitov, 2008). In fish, polymorphonuclear granulocytes and mononuclear phagocytes stimulated by PAMPs produce inflammatory cytokines or reactive oxygen and nitrogen species (ROS) (Jault *et al*., 2004; Purcell *et al*., 2006).

Distinct pathogens express different PAMPs, hence a combination of specific sets of PRRs is essential for integrating an immune response against a specific pathogen. Four main types of PRRs have been described in fish: Toll-like receptors (TLRs) (Jault *et al*., 2004; Rodriguez *et al*., 2005), NOD-like receptors (NLR) (Laing, *et al*., 2008), C-type lectin receptors (CLRs) (Soanes *et al*., 2008) and peptidoglycan recognition proteins (PGRPs) (Li *et al*., 2007).

1.3.4.2 DAMPs recognition

In recent years it has been discovered that "self" cells and molecules are capable or triggering immune response. In 1994, Metzinger proposed a Danger model, which suggests that the immune system is more concerned with damage than with foreignness, and is called into action by alarm signal from injured tissues, rather than by the recognition of non-self (Matzinger, 1994). In Danger model, "danger" defines if the immune response must take place or not (Matzinger, 2002; Matzinger 2002). According to this hypothesis, some kind of message should "tell" dendritic cells (DCs) that tissue cells are injured or dying in a violent, stressful or unexpected fashion (Matzinger, 2002; Matzinger, 2002). During damage, infection, necrosis and apoptosis, injured cells can release intra-cellular components such as mitochondrias, DNA, RNA, heat shock proteins, hyaluron or chromatin-associated proteins, and trigger immune response in the surrounding cells (Metzinger, 1994). In that case, any internal molecule that is normally not secreted, be it cytoplasmic, nuclear or membrane, could act as activator of dendritic cells. Thus, any cell damage would be noticed, antigen-presenting cells would be activated and initiate an immune response (Figure 4). If damages were non-infectious, a small autoreactive response might be initiated, followed by inflammatory mechanism to clean up. Recent evidence points to various molecules capable of detecting the alarm, however the search for molecular messages used by the immune system to decipher danger is far from being over. So far, the following molecules are classified as "danger signals":

- Heat shock proteins (Hsps) – scaffold proteins that bind, fold and protect other proteins and peptides inside cell compartments. Many of them are overexpressed under stress. Hsps can

induce maturation of DCs, and on the other hand, they may efficiently transfer antigenic peptides from the cell that is succumbing to the dendritic cells (Palazon *et al*., 2008).

- RNA and DNA from apoptotic cells – under normal circumstances, nucleic acids are confined within the limit of membranes and are not accessible, hence membrane damage abruptly expose them. Moreover, it was shown that upon activation/ death, neutrophils project nets of DNA that trap microbes and may also be considered as endogenous alarm (Wartha and Henriques-Normark, 2008).
- Nucleotides – recent evidence also points to a role of nucleotides in danger signalling, including short-lived extracellular ATP (Piccini *et al*., 2008).
- Extra domain A (EDA) of fibronectin – plays an active role in inflammation and remodelling, and has been found to be expressed in inflamed tissues (Lasarte *et al*., 2007).
- Uric acid – its content increases in injured cells and seems to be a principal endogenous danger signal that stimulates DCs (Shi *et al*., 2003).
- HMGB-1 – a non-histone complex of the nuclear proteasome, ought to be confined into compartments that are not exposed (Rovere-Querini *et al*., 2004). It was shown to have stimulatory effect on macrophages, enhancing TNF-α expression and secretion (Bianchi and Manfredi, 2007).

Vertebrates' antigen presenting cells (APCs) can be activated from their immediate environment and respond to both, endogenous and exogenous signals. Vertebrates and bacteria have shared eons of evolutionary time, and thus receptors for endogenous and exogenous signals may have evolved simultaneously. Indeed, there is evidence that these receptors are often the same molecules. For example TLR4 is a receptor for the bacterial product lipopolysaccharide (LPS), the Hsp70 and the extracellular breakdown products of hyaluron; TLR2 binds bacterial lipoproteins and Hsp60; and TLR9 binds to DNA CpG sequence in all living organisms (Metzinger, 2002). Hence, it appears TLRs actions bind PAMPs and DAMPs (Figure 4).

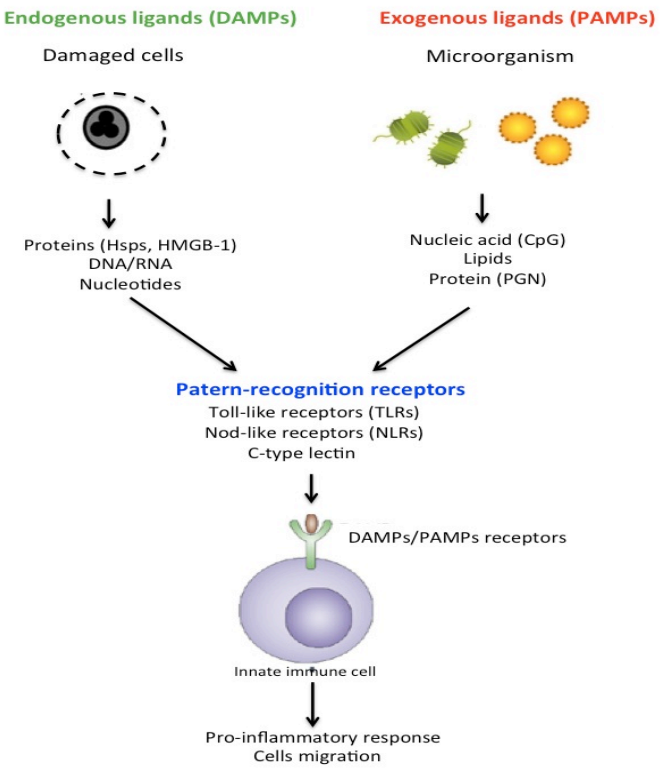


Figure 4. Recognition of DAMPs and PAMPs by immune cells. DAMPs are released from the host cells during injury. PAMPs are recognized by PRR on the surface of immune cells. DAMPs and PAMPs bind to the same receptors on effective cells and trigger an inflammatory response. In current work, wound healing process in relation to DAMPs/PAPMs stimulation was examinated in skin and muscle of common carp (**PAPER 2**). In **PAPER 3**, immunoglobulins expression pattern was examinated following PAMPs intravenous injection into carps' system.

1.4 Stress reaction

1.4.1 Teleost hypothalamo-pituitary-interrenal (HPI) axis

Stress reaction in animals is defines when internal equilibria and proper physiological functioning are disturbed or threatened to become disturbed as result of external or internal stimuli (stressors) (Chrousos and Gold, 1992). Animals respond to stress at different levels: body, tissue and cell (Iwama *et al*., 2004). On the body level the stress response is typically characterized by activation of the hypothalamus – pituitary – adrenal axis (HPA) and release of glucocorticoids and catecholamines.

Teleost fish have a fully functional stress axis. Their stress response is characterized by the activation of hypothalamo-pituitary-interrenal axis (HPI; Figure 5). Neurons in the nucleus preopticus of the hypothalamus (NPO) release corticotropin-releasing hormone CRH, which stimulates corticotrope cells in the pituitary pars distalis (PD) to secrete adrenocorticotropic

hormone (ACTH). Subsequently, ACTH stimulates cortisol release into the blood from interrenal cells in the head kidney (Flik *et al*., 2006). Interestingly, fish head kidney combines endocrine and immune functions, as it is fish major hematopoietic and lymphopoietic organ (Weyts *et al.,* 1998, Flik *et al.*, 2006). This organization suggests that the endproducts of the stress axis have direct paracrine access to the cells of the immune system and vice versa: chemical signals of the immune system e.g. cytokines could exert direct paracrine action on the interrenal (steroid) and chromaffin (catecholaminergic) cells (Weyts *et al*., 1998).

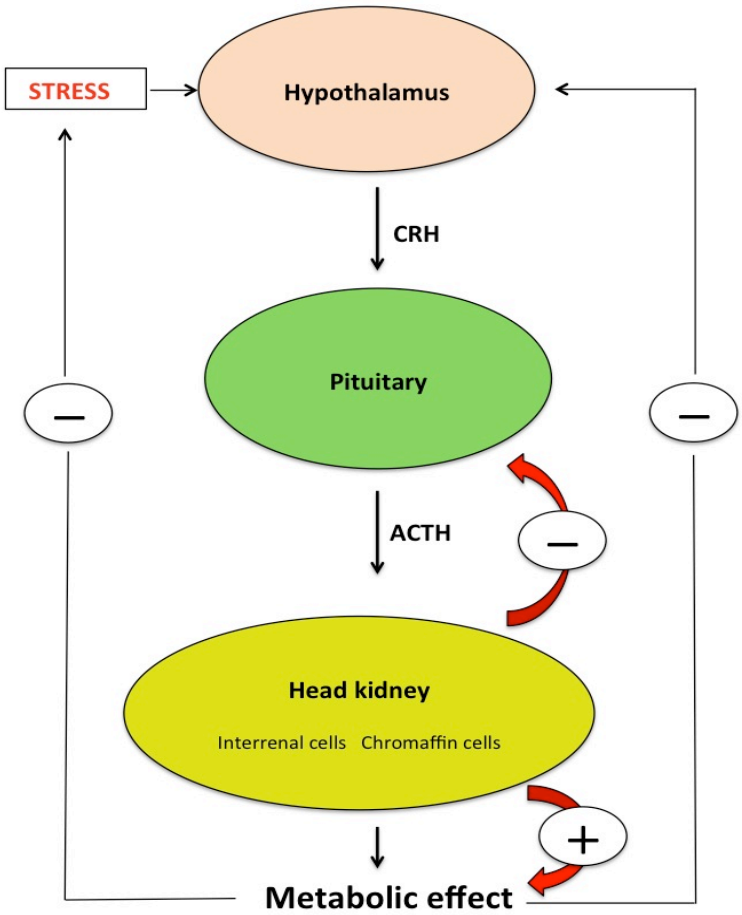


Figure 5. Schematic representation of the hypothalamo-pituitary-interrenal (HPI) axis in teleostean fish. The hypothalamus secretes corticotropin-releasing factor (CRF), which stimulates the pituitary to release adrenocorticotropic hormone (ACTH). The interrenal gland stimulated with ACTH, releases cortisol, the primary stress hormone in fish. A negative feedback system acts on the hypothalamus to ensure homeostatic regulation. In the present work, cortisol level in blood of common carp was investigated following PAMPs intravenous injection (**PAPER 3**).

1.4.2 Glucocorticoids in fish

In vertebrates, the most commonly measured circulating hormone levels, as indicators of stress, are catecholamines and corticosteroids. In teleost, cortisol is the principal glucocorticoid secreted by the interrenal tissue (steroidogenic cells) located in the head kidney (Iwama *et al*., 1999). In the circulation of fish, the bulk (80%) of cortisol is bound to serum steroid globulins, which leaves only 20% of the cortisol in its unbound and bioactive form (Flik and Perry, 1989). Basal plasma cortisol level is species- and stressor-specific, with range from 5-50 ng/ml (Martinez-Porchas *et al*., 2009). In teleost, cortisol is released after HPI axis activation when undergoes stress conditions and rapidly increase, and reach the peak within 30 minutes (Mommsen *et al*., 1999; Tanck *et al*., 2000). However, after cessation of the stressor, plasma cortisol quickly drops again to basal levels. Moreover, once released to the circulation, cortisol is converted and inactivated to cortisone, which lacks the immune regulatory properties of cortisol (Weyts *et al*., 1997). Similar to mammals, glucocorticoids (GRs) are present in fish tissue and are involved in the glucose metabolism, cognition and osmoregulation. This multifunctioning is connected with lack of mineralocorticoid aldosterone in teleosts (Dean *et al*., 2003). Yet, more details of the formation of the GR heterocomplex have to be established in teleost fish, however, it has been assumed, based on the conservative sequence of Hsp70, Hsp90 and the GR ligand-binding region, that comparable processes takes place in all fish (Stolte *et al*., 2006).

Fish immunity is affectd by elevated cortisol level: 1. neutrophilic granulocytes are rescued from apoptosis *in vitro* (Weyts *et al*., 1998b), 2. activated B-lymphocytes show increased apoptosis (Weyts *et al*., 1998a), 3. redistribution of neutrophilic granulocytes from the head kidney into circulation (Huising *et al*., 2003). This stress-induced neutrophilia is considered to improve peripheral surveillance and is reported in mammals as well (Dhabhar *et al*., 1996).

1.5 Antibody response

Immunoglobulins (Igs) are central component of the adaptive arm of the immune system. In mammals, five classes of Igs, including IgG, IgM, IgD, IgA and IgE, have been well identified, whereas in teleosts, IgM and IgD genes have been cloned and characterized (Wilson *et al*., 1990, 1997; Stenvik *et al*., 2000; Hordvik *et al*., 2002; Hirono *et al*., 2003; Saha *et al*., 2004; Savan *et al*., 2005). Although fish equivalents of IgG, IgA and IgE have not been identified, a third teleost Ig heavy chain isotype IgZ (IgZ - zebrafish; IgT – trout; IgH in fugu) has been recently described in fish (Danilova *et al*., 2005; Hansen *et al*., 2005; Gambon-Deza *et al*., 2010; Zhang *et al*., 2010). The basic gene structure of the new class of Ig is conserved, however not every teleost species, e.g. channel catfish seem to have the sequence of IgZ or IgT (Bengten *et al*., 2006).

1.5.1 IgM

The major immunoglobulin of fish blood serum is tetrameric IgM-like molecule (Koumansvandiepen *et al*., 1995). Due to the fact that teleost fish IgM shares some structural and functional characteristics with mammalian IgM, the ability of various teleost fish to produce heterogenous mixtures of IgM polymers, monomr (Rombout *et al*., 1993) and halfmer subunits (Pucci *et al*., 2003) is noteworthy. Due to usage of different methods for IgM detection, comparison of published data is complicated. In general, the first appearance of B-lymphocyte and immunoglobulins is late in marine species compared to fresh water species (Chantanachookhin *et al*., 1991). During larval and organs development IgM, is a pre-dominant immunoglobulin (Ryo *et al*., 2010). In adult fish, IgM is constitutively expressed in renal hematopoietic tissue, spleen and thymus, but expressed at low levels in the skin and gill (Saha *et al*., 2004). Recent gene expression studies in zebrafish have shown IgM as more predominant in both lymphoid and non-lymphoid organs when compared to IgZ (Danilova *et al*., 2005; Ryo *et al*., 2010). More importantly, the higher expression observed in the kidney compared to the spleen and gut might be attributed to the first appearance of IgM positive B-cells in the kidney, which may be transported to spleen, gut and other secondary lymphoid organs (Huttenhuis *et al*., 2006). Recently, it was showed that intravenous injection with β-glucan does not affect IgM expression in HK and skin of common carp (Przybylska and Nielsen, **PAPER 3**).

1.5.2 IgZ

IgZ and IgT were named differently because they were initially discovered in zebrafish and trout, respectively. Although IgZ and IgT share similar characteristics, e.g. heavy chains are composed of four C-regions and are highly homologous, indicating both of which represent the same immunoglobulin isotype, IgZ and IgT genes differ in genome organization and location (Danilova *et al*., 2005; Hansen *et al*., 2005). Functionally, both IgZ and IgT are expressed during embryonic development, indicating it could play a protective role during initial stages of larval development (Danilova *et al*., 2005; Hansen *et al*., 2005). However, in adult fish, IgZ is limited to the primary lymphoid tissue, whereas IgT is expressed in a variety of tissues, suggesting functional differences (Hansen *et al*., 2005). Ryo *et al*. (2010) have recently shown differential expression of the IgZ variants (IgZ1 and IgZ2 chimera) in response to pathogens and suggested that the IgZ subtypes in carp may have mutually exclusive humoral functions. It was shown that IgZ1 is expressed systematically and IgZ2 chimera is preferentially expressed at mucosal sites. IgZ1 and IgZ2 chimera were equally expressed in head kidney, trunk kidney and spleen. In peripheral blood leukocytes, the expression of IgZ1 was significantly higher than in other tissues compared to IgZ2

chimera and IgM, indicating that this antibody might be restricted to pathogens in blood. In contrast, the IgZ2 chimera gene was expressed at relatively higher level in gut and gills, the major routes of infection, thus this antibody might protect the host from epithelial pathogens. IgZ1 mRNA increase was seen during the blood parasite infection, *T. borreli*, as well as during vaccination with *V. anguillarum* antigens, which are rich in lipopolysaccharides, suggesting IgZ1 plays role as Ig that neutralizes foreign antigens in blood (Ryo *et al*., 2010). High IgZ1 gene expression in tissues such as skin and HK was recently reported by Przybylska and Nielsen (**PAPER 3**).

1.6 β-glucans as immune modulators

β-glucans are naturally occurring polysaccharides from cereals (e.g. barley, oat), algae, mushrooms and yeast cell walls. They have various effects in all organisms from plants to vertebrates (Rosenberger, 1976). β-glucans easily form gel containing high-order structures of single spirals or triplet spirals due to its unique primary structure; therefore, its purification is extremely difficult, and consequently crude β-glucan fractions have been used in studies in mammals, rather than purified β-glucan (Kimura *et al*., 2007). In vertebrates, glucan exerts have beneficial effect on a variety of experimental disease states of bacterial, viral, fungal and parasitic origin (DiLuzio and Williams, 1978; Williams *et al*., 1983, Williams and DiLuzio, 1985; Yano *et al*., 1991, Raa *et al*., 1992, Duncan and Klesius 1996, Rodriguez *et al*., 2009, Zhang *et al*., 2009).

1.6.1 Diversity and structure

Glucans are polysaccharides that only contain glucose as structural components. β-1,3-D glucans are chains of D-glucose rings connected at the 1 and 3 positions. The most active form β-1,3-D glucans are those that contain 1,6 side-chains branching from the longer β-1,3 glucan backbone (Akramiene *et al*., 2007). These are referred to as β-1,3/1,6 glucan, whereas 1,3/1,4 β-glucans are linear polysaccharides consisting of β-D-glucosyl residues linked through 1,3- β and 1,4- β-glycosidic linkages, and are localized in the cell wall of cereals, rice and wheat (Figure 6) (Cui, 2001).

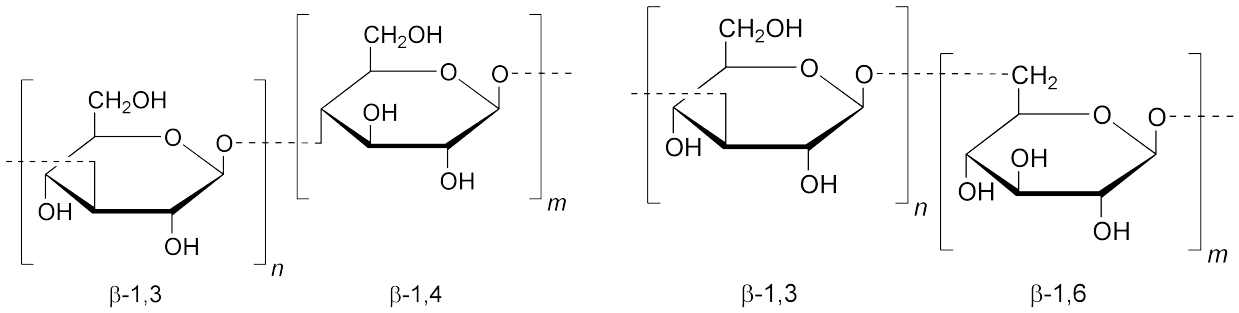


Figure 6. Diagram showing orientation and location of different β-glucan linkages.

β-glucans derived from different sources vary in structure and bioactivity (Akramiene *et al*., 2007). Differences in length of polysaccharide chain, extent of branching, and the length of those branches can result in the difference between material extracted by hot water, insoluble cell wall component and in different molecular weight (Figure 7) (Akramiene *et al*., 2007). It was shown, that insoluble 1,3/1,6 β-glucan, has greater biological activity than that of its soluble 1,3/1,4 β-glucan counterparts (Ooi and Liu, 2000). Another variable is the fact that some of β-glucans exist as single strand chain, while backbones of the β-1,3 glucans exist as double or triple stranded helix chain. Bohn and BeMiller (1995) have described β-glucans as biological response modifiers, in which activity correlates with branching degree and size. They have reported that less branched glucans with molecular weight of 5 kDa-10 kDa were non-active. Conversely, Tanioka *et al*. (2011) have shown that barley-derived β-glucan (~2 kDa) stimulates maturation of mouse dendritic cells. In addition, up-regulation (~ x30) of IL-6 expression in bone marrow cells has been seen when compared to treatment with 40-70 kDa barely β-glucan (Tanioka *et al*., 2011). Przybylska *et al*., (**PAPER 2**) have reported that β-glucans enriched products, MacroGard and 6.3 kDa oat fiber, promote wound healing process in common carp, however with higher wound closure ratio in group treated with MacroGard. Authors concluded it could be related to high branching level.

Final effect of β-glucans immune modulation depends on preparation and used protocol. In present thesis, two different β-glucan enriched products were examinated: MacroGard, provided by Biorigin (Sao Paulo, Brasil) and 6.3 kDa fiber oat provided by Scan Oat (Sweden). In order to dissolve soluble β-glucan fractions, solutions of both products were stirred for 1 h at 90 ^{o}C and autoclaved (121 ^{o}C, 15 min. 1atm.).

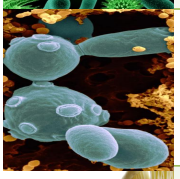
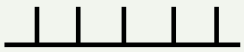
	β-glucan source	Basic structure	Description
	Bacteria		Linear β-1,3-glucan chain, unbranched
	Yeast		Linear β-1,3-glucan chain, with long β-1,6-linked glucan side chains
	Mushrooms		Linear β-1,3-glucan chain, with short β-1,6-linked glucan side chains
	Cereal		Linear β-1,3-glucan chain, with β-1,4-linked glucan side chains

Figure 7. Diversity of β-glucan structure based on source of origin.

1.6.2 Administration routes and its effect on teleost immunity

The immune modulatory effect of β-glucans has been shown in a number of studies (Yano *et al*., 1991; Raa *et al*., 1992; Duncan and Klesius 1996; Rodriguez *et al*., 2009; Zhang *et al*., 2009). These naturally occurring polysaccharides differ in length, extent of branching and bioactivity (Akramiene *et al*., 2007). In vertebrates, β-glucans of similar structure and molecular weight (m.w.) trigger different biological effects in *in vivo* and *in vitro* (William *et al*., 1996; Brown and Gordon 2003; William *et al*., 1996; Nakagawa *et al*., 2003; Bohn and BeMiller, 1995)**.** *In vitro* studies have shown that large molecular weight β-glucans (e.g. zymosan) can directly activate leukocytes and stimulating their phagocytic and cytotoxic activity (Brown and Gordon, 2003). Williams *et al*. (1996) have shown that these carbohydrates can stimulate production of pro-inflammatory cytokines (IL-1β, IL-8, IL-6 and TNF-α). Low or intermediate molecular weight β-glucans (e.g. glucan phosphate) posses biological activity *in vivo*, but their cellular effects are not clear (Williams et al., 1996). Data showed by Nakagawa et al. (2003) suggest that these β-glucans can stimulate production of IL-8 and IL-6, but no Il-1β or TNF-α. Glucans at the size in a range of 5-10 kDa (e.g. laminarin) are considered as inactive in general (Bohn and BeMiller, 1995). On the other hand, even β-glucans of similar structure and molecular weight can show different biological activity in i*n vivo* and *in vitro* studies (Akramiene *et al.*, 2007). β-glucans have been shown to

accelerate the wound healing process in mammals, however not yet in fish (Wolk and Danon, 1985; Hon Kwon *et al*., 2009).

Three different way of administration are available: injection, immersion or feed. Depends on purpose, the mode of immune stimulant application is very important and should be chosen with regard to, for example, labour input or stress factor. Immune stimulants are widely used in aquaculture. β–glucans injections is the most common and efficient route. Yano *et al*. (1991) injected carp (*Cyprinus carpio* L.) with glucan extracts from yeast prior to *Edwardsiell*a *tarda* challenge. All untreated fish died within 3 days, whereas fish injected with β–glucan extract had survival rates of 60-80%. Data showed that the glucan-injected carps had higher activity of the alternative complement pathway. Chen and Ainsworth (1992) injected catfish (*Ictalurus punctatus*) with baker's yeast-derived glucan alone or with *E. tarda*. Data showed increase in non-specific defence parameters of phagocytic cell activity. Selvaraj *et al*. (2005) have demonstrated that intraperitoneal injection of yeast glucan significantly improved the survival rate of *C. carpio* against challenged with *A.hydrophila*. Administration of 500 and 1000 μg glucan/fish elicited 100% survival in carp. Robertson *et al.* (1990) reported that intraperitoneal injection of yeast glucan resulted in increase resistance in Atlantic salmon against challenged with *Vibrio anguillanum*, *Vibrio salmonicida* and *Yersinia ruckeri.* Catfish injected with yeast glucan showed increase resistance against challenge with *Edwardsiella ictaluri* (Chen and Ainsworth, 1992). Taken all together, these data highlight the biggest advantage of injection: direct glucan- organ contact, which induces immune cells to enhance the non-specific cellular immune response consequently.

Feeding trials are the most common, however might be difficult to give full picture of immune stimulant effects due to chemical action of the enzymes and digestive fluids, which would change or reduce immune stimulants effectiveness, or because they would be removed from the body too quickly to have any effect. Nevertheless, Roerstad *et al*. (1992) demonstrated that fish fed with MacroGard supplemented feed, showed increased resistance to infection caused by *Aeromonas salmonicida*. Van der Marel *et al*. (2012) have shown that carp fed with β–glucan supplemented diet had higher mucosal immune response when compared to control group. Moreover, the same study has shown significantly higher expression levels of *β-defensin 2* in gills and both *β-defensin* genes in skin were found.

Immersion method is an alternative, easy and fast route for β–glucans administration to fish in all sizes (Raa *et al*., 1992). However it is extremely expensive to introduce bathing trials to farmed fish species and in consequences, very little information is available on bathing protocols and dosage. The study of Selvaraj *et al*. (2005) where bathing trials were carried out as fish immersion in the

aerated glucan solution for about 90 min., showed that bathing did not increase survival percentage in common carp challenged with *Aeromonas hydrophila,* and no increase in antibody response was observed either. Fish probably cannot absorb glucan particles through the bathing route, as β-glucans are insoluble in nature.

1.7 Methods applied in the study

1.7.1 The damage model

Few studies with damage model involved were performed in order to investigate the immune response in fish. Gonzalez *et al*. (2007) performed mechanical tissue damage in common carp in order to mimic the inflammatory responses to ectoparasite *I. multifiliis.* Ingerslev *et al*. (2010) investigated local tissue response in the musculature of rainbow trout (*Oncorhynchus mykiss*) following mechanical tissue damage and natural infection with *Moritella viscosus*. According to study of Selvaraj *et al*. (2005) immersion in beta glucan solution did not increase survival percentage in common carps challenged with *Aeromonas hydrophila,* and no increase in antibody response was observed. However, biological effect of bath supplemented with immune stimulants on innate immunity in fish was not addressed. In order to study the biological impact of two commercially available β-glucan enriched products on the wound healing process, fish were constantly kept in water supplemented with β-glucans. Five millimetres biopsy punches were used to create wounds on the left side, above lateral line. Using an 8 mm biopsy punch, skin and muscle tissues samples were collected. Apart sampling, an internal controls from non-injured side were taken, which allowed us to study local versus systemic response (Figure).

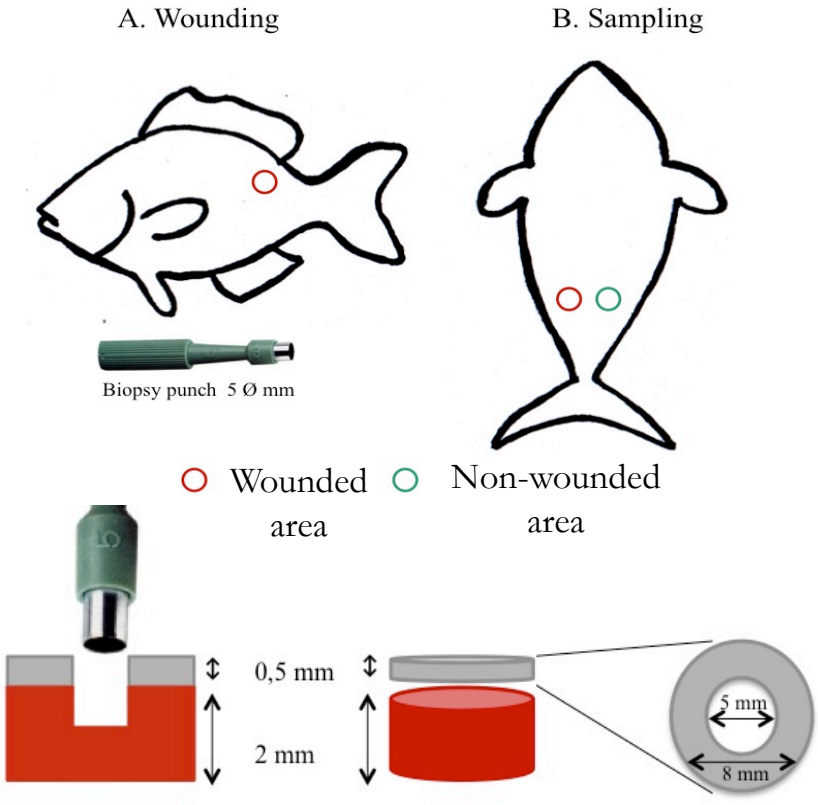


Figure 8. Illustration of wounding (A) and sampling (B-E) procedures (**PAPER 2**). Fish were wounded on the left side above lateral line (red circle) with 5 mm biopsy punch. During sampling, skin and muscle tissue were collected from the wounded site (red circle), as well as from non-wounded site (internal control, green circle) using 8 mm biopsy punch.

1.7.2 Intravenous injection

Three different way of administration are available: injection, immersion or feed. Depends on purpose, the mode of immune stimulant application is very important and should be chosen with regard to, for example, labour input or stress factor. Although, injection is the most time consuming and rough administration route, it is the most efficient route (Robertson *et al*., 1990; Yano *et al*., 1991; Chen and Ainsworth, 1992; Selvaraj *et al*., 2005). These data underlie the biggest advantage of injection, direct glucan – organs contact, which induces cells of immune origin to enhance the non-specific cellular immune response.

Intravenous injection guarantees that injected substance (medication, immune stimulant, etc.) will act more quickly and reach the subcutaneous tissues, the vascular tree, or an organ, therefore immunizing substances or inoculations, are generally given by injection. We investigated if intravenous (i.v.) injection of *Aeromonas hydrophila* bacterin (BSK10) (Nielsen *et al*., 2001) with two dosages of β-glucan derived from Baker's yeast has a biological effect on immune parameters in common carp. Fish were divided randomly into three groups of 23 individuals and all fish were

injected with 100 µl of: 23 fish with 100 µl of bacterin in sterile PBS (B); 23 fish with 100 µl of bacterin + low dose of β-glucan (B-Lβg) and, 23 fish with 100 µl of bacterin + high dose of β-glucan (B-Hβg).

1.7.3 *Aeromonas hydrophila*

Aeromonas hydrophila, a fermentative rod-shaped, with a single polar flagella, is the most common bacteria present in all freshwater environments (Austin, 1999). This Gram-negative bacterium is an opportunistic pathogen that causes a wide range of diseases in fish (Nielsen *et al*., 2001). There are more than 120 serotypes of *A. hydrophila* described and each of them may show different growth in mucus and can produce different kinds of toxins. It does not cause problems in fish populations under normal conditions. Infection with *A. hydrophila* always appears as a secondary infection, when fish are under environmental or physiological stress, injured or infected by other pathogens (Plumb *et al.,* 1976; Pai *et al*., 1995; Fang *et al.,* 2000). The disease caused by *A. hydrophila* is called motile aeromonad septicaemia (MAS) and its clinical signs in fish vary from tissue swelling, necrosis, ulceration and haemorrhagic septicaemia (Hazen *et al*., 1978a; Karunasagar *et al*., 1986; Aguilar *et al*., 1997; Azad *et al*., 2001; Figure 9). When it enters the body of its host, it travels through the bloodstream to the first available organ. This bacterium can digest materials such as gelatin and haemoglobin. It produces aerolysin cytotoxic enterotoxin (ACT), toxin which posses multiple biological activities like ability to lyse red blood cells, destroy tissue and evoke a fluid secretory response in ligated intestinal loop models (Sha *et al*., 2002). It was shown that ACT increases levels of pro-inflammatory cytokines, such as TNF- α, IL-1β and IL-6 but also that many bacterial toxins possess cytokine – inducing activities and therefore the host pro-inflammatory cytokine response to ACT may contribute to the pathogenesis of *Aeromonas* infections (Chopra *et al*., 2000).

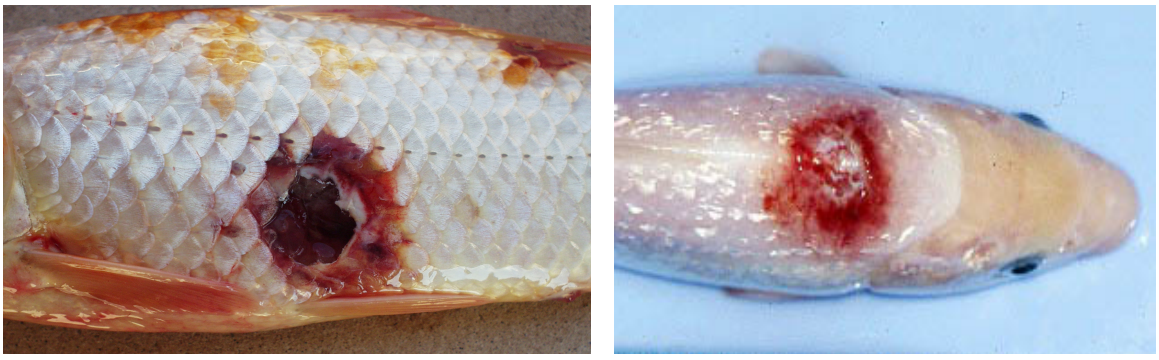


Figure 9. Koi carp infected with *Aeromonas hydrophila* with ulcer in the skin and muscle, and bleeding.

1.8 Objectives of the PhD study

This PhD study aimed to investigate the biological effect of the β-glucan as immune modulator of the mucosal surfaces in common carp (*Cyprinus carpio* L.) in relation to the wound healing process.

Thus, the aims of the current PhD thesis were:

- To examine, if previous infections had an impact on texture quality, and if these changes were detectable after recovery from bacterial infection.
- To create a model in controlled condition, where, without introducing the pathogen, we could mimic naturally occurring wounds, and examine the biological impact of two commercially available β-glucan enriched products on the wound healing process in common carp.
- Examine, if intravenous injection of β-glucan affects the skin mucosal immunity and can introduce an antibody change in *Cyprinus carpio* L.

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

2 General discussion

Fast wound healing process and tissue regeneration is extremely important for all animals. In their natural environment fish are in intimate contact with a high amount of microorganisms and pathogens. Skin, together with mucosa forms the first line of defence against pathogens and is an essential protective barrier in aquatic organisms. Skin damage in fish caused e.g. by pathogens, such ectoparasites as *Ichthyophthirius multifillis* (Gonzalez *et al*., 2007) and lice (Krkosek *et al*., 2011), Gram-negative bacteria as *Moritella viscosus* (Ingerslev *et al*., 2010), as well as mechanical trauma, facilitates entry of pathogens into the underlying tissue and vascular system. Once the protective barrier is broken, the normal (physiologic) process of wound healing is immediately set in motion, and involves a complex series of interactions between different cell types, cytokine mediators, and the extracellular matrix (Singer and Clark, 1999; Kanter and Akpolat, 2008; Gillitzer and Goebeler 2001). The study of the interaction between the parasite and the fish host has been described by Gonzalez *et al*. (2007), however process of wound closure in a molecular perspective have so far not been described in details in common carp. Moreover, nowadays, number of biological compounds has been used to reduce the risk of diseases and improve fish welfare by enhancement the non-specific defence system. Although, among them, β-glucans are some of the most extensively investigated modulators of the immune system, their effect on skin mucosal immunity has not yet been addressed.

2.1 The best use of immune modulators: bath, injection or fed?

Immune modulators are widely used in aquaculture. One of the most frequent doubts is which way of administration is the best and how long the protection will last. The potential use of β-glucans as immune and growth stimulants in the large aquaculture sector has not been fully explored, however research on the use of immune modulators for the improving non-cellular immune response becomes more promising. The limited studies carried out, have been primarily restricted to the cold water fish production e.g. salmonids whilst the application of β-glucan to the large warm water fish and ornamental fish sectors has not been ascertained (Robertsen *et al*., 1990). Three different way of administration are available: injection, bath or oral. Depend on the purpose, the mode of application of immune modulator is very important and should be chosen with regard to labour input and stress factor. Second segment of hindgut in fish is a place where antigens up-take takes place (Rombout *et al*., 1985; Selvaraj *et al*., 2005) and, to a large extent, antibiotics and immune modulators are orally introduced to fish by incorporation into fed. In the present PhD thesis, we were interested in examinating mucosal immune response following direct stimulation with β-glucan.

One of the aims of the present PhD study (**PAPER 2**) was to investigate biological effects of β-glucan enriched products on wound healing process in common carp. Bath trial was an obvious choice for examination if β-glucans can affect wound closure by "outside" stimulation, even though very limited information is available on bathing protocols and dosage, and all up-to date investigations were carried as short immersion in e.g. aerated glucan solution (Selvaraj *et al*., 2005). The idea was to keep wounded fish in constant and direct contact with β-glucans for two experimental weeks and monitor process of wound closure. By using bath treatment, it was also possible to investigate local and systemic immune response due to major stimuli: DAMPs (wounds) or DAMPs/PAMPs (wounds/ β-glucan) combination. The results we obtained clearly showed that β-glucan enriched bath directly affected wound contraction dynamics and faster closure was observed when compared to the control group.
Subsequently, in the current thesis, intravenous injection of β-glucan was introduced (**PAPER 3**) to examine if by presence in the circulation system, β-glucan can cause any biological effects. Our focus was on, if β-glucan i.v. injection affects antibody response in the circulation as well as in skin. Moreover, two different doses of β-glucan were used to investigate any dose-dependent manner or toxic effect. Since injection itself is very stressful for fish, stress response, including cortisol level and expression of Hsp70, was examined. Obtained results showed that effect of direct β-glucan stimulation by i.v. injection in common carp is rigorously correlated with dosage. Moreover, high amount of particulate β-glucan introduced to circulation system of carp was correlated withelevated cortisol level. Furthermore, i.v. injection of β-glucan affects immunoglobulin expression in carp skin: expression of IgM remainded unchanged while IgZ1 expression showed high up-regulation. IgZ1 is considered as mucosal immunoglobulin, and its high expression in skin of fish could increase efficiency in fighting pathogens invading skin layer.

2.2 The use of internal control samples: local vs. systemic reaction

More than two decades ago Wolk and Danon (1985) have emphasized that potential application of glucans to promote wound healing in humans and other heterogenic populations of animals, would require an experimental model in which a wound on one side of the animal could be compared to a symmetrical wound on the other side of the same animal. Ingerslev *et al*. (2010) used a model where during sterile damage in rainbow trout, internal control samples were collected as well. It turned out to be a powerful model to examine local versus systemic response in the fish. Results presented in manuscript of **PAPER 2** clearly showed that there were no significant differences in expression level between the internal controls from wounded fish in comparison to non-wounded fish, and inflicted wounding in a restricted area in the fish muscle resulted in response only in that specific area. In addition, no significant changes have been observed in non-wounded groups, and

the absence of marked differences on the respiratory burst activity in head-kidney cells supports the idea of a localized immune response to the site of injury.

2.3 Inflammatory response during wound healing process: DAMPs and PAMPs stimulation

Wound healing can be defined as the physiology by which the body replaces and restores function to damaged tissues (Tortora and Grabowski, 1996). The classic model of wound healing is divided into three sequential, yet overlapping, phases: (1) inflammatory, (2) proliferative and (3) remodelling, and a set of complex biochemical events takes place in a closely orchestrated cascade to repair the damage (Singer and Clark, 1999; Kanter and Akpolat, 2008; Gillitzer and Goebeler 2001). By wounding procedure that was performed to mimic naturally occurring wounds, danger molecules were released from injured cells of skin and muscle of common carp (**PAPER 2**). Moreover, water bath supplemented with β-glucan was PAMPs source. Immune response can be triggered by DAMPs. On the other hand, some of danger signals e.g. heat shock proteins are involved in myogenesis, and have high constitutive expression in the heart and muscles in fish (Sass *et al*., 1996). Immediate increase in IL-1β, IL-6 and IL-8 cytokines day post-damage in muscle collected from wounded-control group is likely to be the inflammatory response caused by the release of DAMPs. Interestingly, in skin samples from wounded-control fish, different pattern of pro-inflammatory cytokine expression has been noted. No or low IL-1β, IL-6 and IL-8 expression has been detected one day after damage, however no gene expression was investigated at earlier time points. It is possible that stored IL-1β, IL-6 and IL-8 were quickly secreted (hours) in response to wounding and subsequently, new amount of these cytokines needed to be produce. The inversed pattern of gene expression in skin/muscle tissue has been seen in the β-glucan supplemented group as well, with change in expression three days post wounding. In mammals, DAMPs and PAMPs can bind to the same receptors and therefore they may simultaneously effect when combined (Holtick *et al*., 2011). Receptors stimulation with DAMPs/PAMPs resulted in local expression of IL-1β, IL-6 and IL-8 genes, and that local inflammatory response differences in expression pattern dependent on stimulation by DAMPs alone or DAMPs/PAMPs combination (Figure 10). In the i.v. injection experiment (**PAPER 3**) only IL-1β expression was investigated. In fact, present results show no significant changes in IL-1β expression at early phase of experiment. It can be explained by the inhibitory role of cortisol, seing that glucocorticoids control cytokine expression in several ways. One of them is interaction with NF-kB transcription factor, preventing it to attach to kB gene responsive elements (McKay and Cidlowski, 1999) and subsequently down-regulates expression of IL-1β, which expression is regulated via the NF-kB pathway (Engelsma *et al*., 2001; McKay and Cidlowski, 1999). Additionally, also the stability of IL-1β mRNA is decreased by these steroids (Lee *et al*., 1988). On the other hand, we cannot rule out fact that, IL-1β

is main pro-inflammatory cytokine, which reaches peak within minutes after stimulation, so measurement 96 hours after stimulation does not give clear picture. But then again, we observe high and significant up-regulation 12 days post i.v. injection. At that time, cortisol level has dropped to basal line, and expression of IL-1β started. Moreover, fish from B-Hβg injected group appeared to have skin and muscle damage (personal observation) which could enhance IL-1β expression in response to DAMPs. In mammals, corticosteroids have inhibitory effect on monocytes and macrophages (Baybutt and Holsboer, 1990), and if this process is alike in fish, could explain no changes in IL-1β expression, as macrophages are main source of IL-1β.

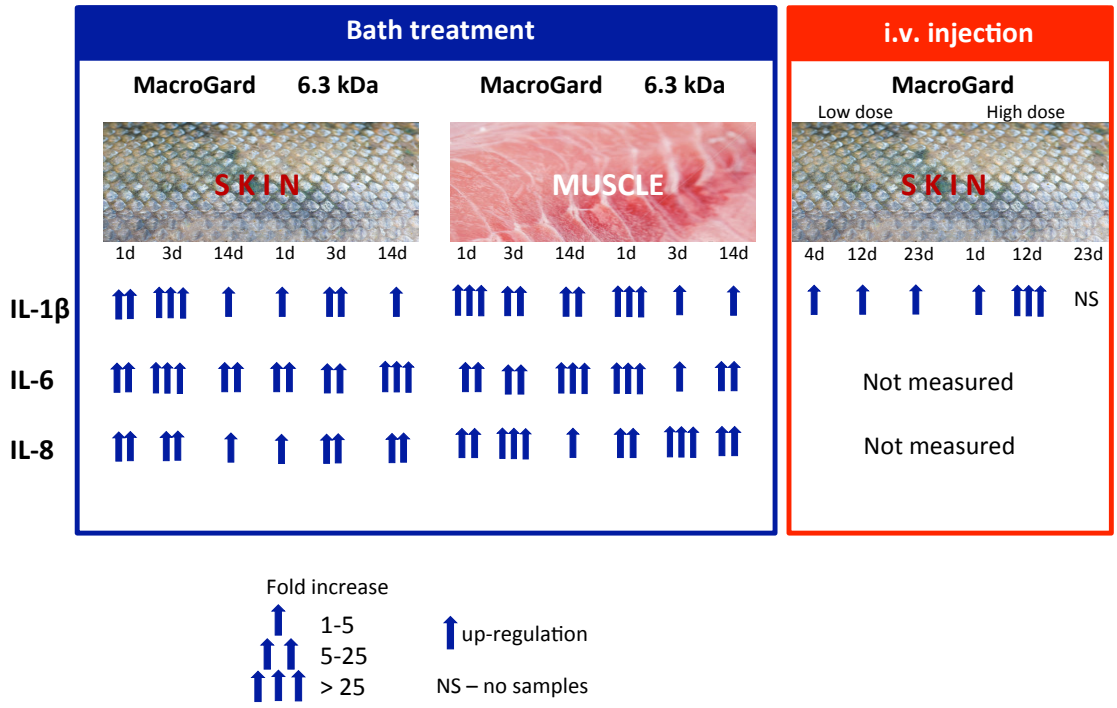


Figure 10. Summary of the expression results in skin and muscle from bath and injection experiment. Sets of one, two and three arrows indicate a low (1-5 fold), intermediate (5-25 fold) and high (> 25 fold) differences in gene expression respectively. NS indicates no samples were tested. Results compiled from **PAPER 2** and partly from **PAPER 3**.

2.4 β-glucan immune modulator effect on non-immune cells

Skin injury (DAMPs) activates mucosal immune cells as well as epithelial resident cells. It has been proven, not only "typical" immune cells of haematopoietic origin, such as neutrophils or macrophages are involved in wound closure and cytokines production (Ingerslev *et al*., 2010). Chen *et al*. (2005) have described human fibroblasts as the non-conventional cell type having immune regulation capabilities. Recent studies in human have shown that fibroblasts can not only respond to cytokines, but also generate these signals (IL-1β, IL-4, IL-6, IL-8, IL-10, IL-12, TNF-α and IFN-γ) and influence the behaviour of immunocompetent cells trafficking to the injury site (Chen *et al*., 2005; Ruiz *et al*., 2007). In fish, several fibroblast cell lines have been established and

characterised (Collet and Secombes, 2001; Ossum *et al*., 2004). Furthermore, Ingerslev *et al.,* (2010) reported that fish fibroblasts are susceptible to DAMPs. In the wounding experiment (**PAPER 2**), high up-regulation of IL-1β and IL-6 in skin samples from wounded-MacroGard fish three days post wounding could be explained by the ability of fibroblasts to express genes of immune functions before the more potent immune cells have been recruited. Presence of DAMPs/PAMPs could potentiate immune-like performance of fibroblasts, as well as having a direct effect on the resident cells present in skin, such as epithelial cells, resident macrophages and lymphocytes. Glucans' ability for direct stimulation of human fibroblasts has been shown by Wei *et al.* (2002).

Another cell type that was shown to be involved in wound healing process is goblet cells (Geggel *et al*., 1984; Kanter and Akpolat, 2008). Goblet cells, cells responsible for the production and maintenance of the protective mucus blanket, are exclusively lodged in epithelia. Due to their location, we expected that bath supplemented with β-glucans would have an effect on their function in common carp, and subsequently changes in Muc5b expression should be detected. High Muc5b up-regulation three and 14 days post-wounding has, however not been restricted to the wound area, but was a general response of the skin mucosa to the injury. Our results can be compared to work presented by van der Marel *et al*. (2010) where carp kept in water with increased bacterial load did not cause clinical symptoms, however rapid skin mucosa respond have been seen, even if the bacteria involved were considered to be non-pathogenic. Moreover, boblet cells differentiation and functioning in mammalian system is affected by mucosal immunity and gel-forming mucins can be regulated by inflammatory cytokines such as interleukins (IL-1β, -6, -17), tumour necrosis factor α (TNF-α) or exogenous factors (Ciacci *et al*., 2002; Roussel and Delmotte, 2004; Chakraborty *et al*., 2010; van der Marel *et al*., 2012) and similar mechanism could be present in fish. Therefore, during wound closure in common carp, high local inflammation and IL-1β expression could hamper Muc5b expression at the site of injury. Furthermore, in humans, cells may differ in the density of mucin granules, suggesting a diversity of the mucins synthesized even by an individual cell (Roussel and Delmotte, 2004; Kesimer *et al*., 2010; Sheehan and Thornton 2000). Considering that based on mucins content, several different goblet cell types have been described, we cannot assure, that β-glucans bath did not have any effect on expression of other mucin granules present in mucous or goblet cells (Fletcher et al., 1976).

2.5 Skin – perfect tissue to follow β-glucan actions?

Carp skin is a complex structure composed of the outer stratified, relatively thin epithelium (epidermis) and the inner layer (dermis) composed of dense fibrous connective tissue, which is attached to the underlying muscles, by a layer of loose connective tissue. However, in contrast to

terrestrial vertebrates, cells from fish skin are non-keratinised and consequently metabolically active (Bullock and Roberts, 1974). During two conducted experiments, bath and i.v. injection trial, effects of used immune modulant were detected in skin. In the bath treatment (**PAPER 2**), direct and constant contact of β-glucans caused positive effect on wound closure. In the injection trial (**PAPER 3**), indirect effect was observed. Injected into vein β-glucan reached all internal organs, as well as skin.

Skin and mucucs blanket are the first layers between fish and an environment, and beside mechanical barrier function, immune system was developed. Powerful mucosal immune system increases efficiency in fighting pathogens invading the host. One of the arms that could be used for that purpose is antibody. Thgoughout evolution, immunoglobulins diversified into several isotypes with specialized roles in innate and adaptive responses, in the systemic and mucosal compartments. In teleost, the most primitive vertebrates with immunoglobulins, due to lack of evidences of immunoglobulin specialization, IgM was regarded as the only functional antibody in systemic and mucosal compartments. However, in 2005, a new immunoglobulin isotype, called IgZ was found in zebrafish (IgZ - in zebrafish; also called IgT – trout; Solem and Stenvik, 2006; Danilova *et al*., 2005) and furthermore, existence of two distinct B cell lineages, with mutuall expression either IgM or IgZ, was suggested, while, the possible class-switch recombination process between IgM and IgT was rouled out (Danilova *et al*., 2005; Hansen *et al*., 2005). In 2010, Zhang *et al*. provided evidence for the existence of a previously unrecognized B cell lineage that expressed only IgT. In the same study, IgT^{+}B cells have been showed to be potent phagocytic cells with ability for killing rainbow trouts' gut parasite *Ceratomyxa shasta*. In fish that survived infection, authors reported the substantial accumulation of IgT^{+} cells in the gut concomitantly with considerably increased (~51-fold) gut mucus IgT. In contrast, the number of IgM^{+} B cells and concentration of IgM did not change in surviving fish. Further, substantial upregulation (~733-fold) of IgT gene expression was detected in gut of these fish, with almost negligible upregulation of IgM expression. Such a high expression of IgT in gut and protective role against gut parasite indicates, that it is the first nontetrapod immunoglobulin specialized in mucosal immunity (Zhang *et al*., 2010). In fish, skin, mucus, gills and gut together, form mucosal immune system. In present PhD thesis, IgM and IgZ1 antibody response following intravenous injection with different doses of β-glucan was examinated in skin and head kidney of common carp (**PAPER 3**). We observed high expression of IgZ1 in skin 12 and 23 days post injection (injection with B-Lβg; ~390- and ~310-fold respectively) with no significant changes in IgM expression (Figure 11). Taking under consideration mucosal functions of IgT(Z) reported by Zhang *el al*. (2010) and further by Ryo *et al*., (2011), high IgZ1 expression detected in the injection experiment (**PAPER 3**) leads to the conclusion that $IgT(Z)^{+}$ B cells are major B cells in skin of common carp, with low cell count for IgM^{+} B cells. Zhang *et al*., (2010)

found the IgT(Z)$^+$ B cell population constituted 16-28% of all trout B cells in the blood, spleen, head kidney and peritoneal cavity, while in gut they represented the main B cell subset. Results obtained from the i.v. injection experiment show that IgZ1 expression was significantly higher in common carp skin than in head kidney, suggesting that either IgT(Z)$^+$ B cells have the majority among cells in carp skin, or β-glucan injection enhanced proliferation of these cells. IgM$^+$B cells are highly phagocytic and have intracellular killing abilities (Li *et al*., 2006). However Zhang *et al*. (2010) compared the phagocytic ability of IgT(Z)$^+$ and IgM$^+$ B cells, and they seemed to be alike, as well as capacities to proliferate and secrete immunoglobulin in response to microbial stimulation. Even so, in our injection experiment, only IgT(Z) gene expression was up-regulated, with no changes in IgM expression (Figure 11). That draws the conclusion that only IgT(Z)$^+$ cells present in the skin, respond to i.v. injection with β-glucan, or that phagocytic activity of two distinct B cell lineages differs. That could confirm previous findings that IgT(Z)$^+$ B cells are able to ingested and kill bacteria intracellularly (Zhang *et al*., 2010). Furthermore, IgZ(T)$^+$ B cells response to β-glucan might suggests presence of the receptor(s) through which, any actions with β-glucan will be more pronounced in fish skin. Hence, to obtain more data, studies on the fish cell line should be carry on.

Throughout the bath and intravenous injection, in spite of administration route, effect of β-glucan stimulation was always observed in skin of common carp. That raises a new hypothesis about β-glucan actions as immune modulant in fish immunity. β-glucans as polysaccharides that contain only glucose as structural components are "part" of fungi, and fungal infection in fish always occurs on skin surfaces. To clarify this hypothesis further studies on fish mucosal immunity following immune modulation are needed.

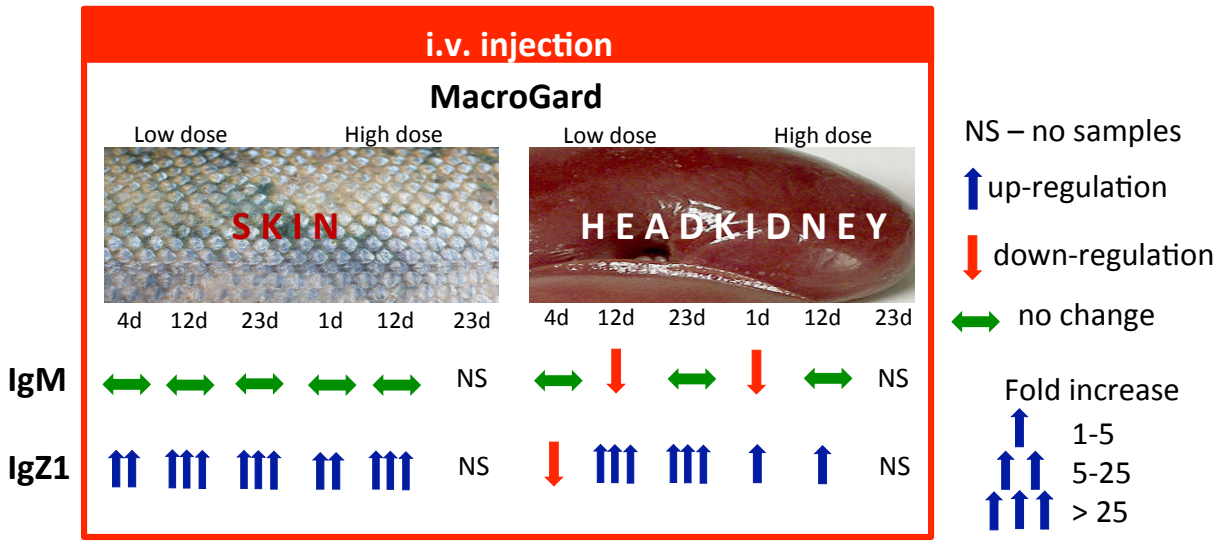


Figure 11. Summary of the antibody expression results in skin and head kidney from injection experiment (paper 3). Sets of one, two and three arrows indicate a low (1-5 fold), intermediate (5-25 fold) and high (> 25 fold) differences in gene expression respectively. NS indicates no samples were tested.

3 Conclusions

1. Fish diseases have an impact on product quality-associated parameters, as the structural changes can be associated with the development of scars as a consequence of the regeneration process.

2. β-glucan enriched products, MacroGard and 6.3 kDa oat fiber, promote wound healing process in common carp, however with higher wound closure ratio in group treated with MacroGard.

3. The immunological and regenerative response following stimulation with DAMPs (wounding procedure) and PAMPs (β-glucan) in controlled conditions, without the exposure to pathogens results in an inflammatory response by activating main pro-inflammatory cytokines such as IL-1β, IL-6 and IL-8. Moreover, these changes have a local character and depend on receptor(s) mediated stimulation with major stimuli: DAMPs alone or DAMPs/PAMPs combination.

4. DAMPs/PAMPs stimulation affected the inflammatory phase in wound healing process in common carp, and therefore bathing in β-glucans, specifically MacroGard and 6.3 kDa oat fiber, enhanced the wound closure in common carp.

5. Fish skin injury introduces DAMPs and activates cells present in mucosal layers, these of the immune function, as well as resident cells such as fibroblasts and goblet/mucous cells. High local inflammation and IL-1β expression could hamper Muc5b expression at the site of wound, which might explain why Muc5b expression was not higher in MacroGard treated group as would have expected. Moreover, stimulation with DAMPs/PAMPs possibly increased the immune-like performance of fibroblasts.

6. Effect of direct β-glucan stimulation by intravenous injection in common carp is rigorously correlated with dosage: high amount of particulate β-glucan introduced to circulation system of carp correlates with elevated level of cortisol.

7. Intravenous administration of β-glucan affects immunoglobulin expression in carp skin: no changes in IgM gene expression were observed, with high expression of IgZ1. It could suggest that $IgZ(T)^{+}$ B cells lineage posses receptor, through which stimulation with β-glucan occurs.

8. Intravenous injection of *Aeromonas hydrophila* bacterin alone or together with β-glucan does not elevates production of antibodies against *A.hydrophila* if fish were earlier primed to *A. hydrophila*. Slight elevation in specific antibody level could be a short-term alert in response to injected PAMPs (bacterin and β-glucan) and temporarily affect antibody production.

4 Perspective for future research

The work conducted in my thesis has provided new insight into quality related issue in relation to previous diseases. Further, biological impact of β-glucan was investigated in relation to wound healing process in common carp. To continue with investigation of immune modulators effect on fish immune system, intravenous injection experiment was performed. Nowadays, fish mucosal immunity is broadly investigated area and new captivating opportunities arise. In continuation of these studies, several interesting subjects are worth examining:

(1) *Fluorescent-labelled antibodies against β-glucans – a perfect tool for β-glucans localization?*

Although the mechanism, by which β-glucan triggers immune response in teleost fish remains unknown, all three ways of introducing immune modulators, bath, injection and oral, are proved to stimulate immune system in common carp. Research in area of mucosal immunity is progressing fast and recently monoclonal antibodies were raised against 1,3/1,4- β-glucan, which could be useful tool in the quantitation and immunocytochemical location of 1,3/1,4)- β-glucan. Fluorescent-labelled antibodies against β-glucans could show how deep β-glucan-enriched products such as MacroGard, penetrate wounded tissue. We have showed that bath in MacroGard and 6.3 kDa oat fiber enhanced the wound healing process when compared to non-treated group, but immune response differs in skin and muscle tissue. Usage of fluorescent-labelled antibodies against β-glucans could contribute to the discussion if there is any correlation between size of immune modulants and their variable immune potency. Similar issue is addressed in fed trials. It remains unclear, under what changes β-glucan goes in the gut of fish, and subsequently triggers an immune reaction that can be detected in e.g. skin of fish.

(2) *Further investigation of IgM/IgZ1 expression in skin of common carp following immune stimulation/modulation.*

Among circulating lymphocytes in fish, B cells count for approximately 70%, however some resident B cells can be found e.g. in skin of common carp. Immunoglobulin M (IgM) and Z1 (IgZ1) are mutually exclusive on B cells. We showed high expression of IgZ1 in skin accompanying by no changes in IgM expression. However, it was showed that IgM is present in goblet cells in rainbow trout skin. It was also showed that no immunoglobulin class switching takes place in fish. Goblet cells staining against IgM and IgZ1 could provide more information about content of goblet cells before, during and after immune stimulation. Moreover, new assay in which separate examination of IgM/IgZ1 expression in skin resident B cells and goblet cells should be established, as recent studies have proven the existence of two distinct B cell lineages. Furthermore, seems like $IgZ(T)^{+}B$

cells of common carp skinrespond to immune modulation. Hence, further research should focuse on work with cell lines, looking for receptor(s) of interest through which β-glucan triggers the immune modulation in fish skin.

(3) *Does β-glucan work in dose dependent manner?*

Immune modulants are valuable alternative for medical treatment in the control of fish diseases and may be useful in fish culture, as drug choices for the treatment of common infectious diseases are becoming increasingly limited and expensive and, in some cases, unavailable due to the emergence of drug resistance in bacteria and fungi. As mention in the introduction of this PhD thesis, three routes of administration of the immune modulants are possible. The most effective method of administration of immune modulants to fish is by injection. Many studies have reported so-called "optimal dose" for fish injection, however, the dosage range is wide. Oral and immersion methods have also been successfully used in fish, however, their efficacy decreases with long-term administration. Moreover, still little information about dosage and protocols is available, especially for bath treatments. Since dose-dependent side effects of immune modulants have not been well studied in fish, it is interesting to obtain more data on potential dose-related effects of injected β-glucan, including the timing, dosages, method of administration and the physiological condition of fish.

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Dominika A.Przybylska

June 2012

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PAPER I

Previous bacterial infection affects textural quality parameters of heat-treated fillets from rainbow trout (*Oncorhynchus mykiss*)

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PREVIOUS BACTERIAL INFECTION AFFECTS TEXTURAL QUALITY PARAMETERS OF HEAT-TREATED FILLETS FROM RAINBOW TROUT (*ONCORHYNCHUS MYKISS*)

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ABSTRACT

Sensory quality of fish meat is influenced by many parameters prior to slaughter. In the present study, it was examined if previous infections or damages in the muscle tissue influence product quality parameters in fish. Fillets from rainbow trout (*Oncorhynchus mykiss*) reared in seawater at a commercial fish farm were sensory evaluated for more than a year after recovery following physical tissue damage or infection by the bacterial pathogens *Yersinia ruckeri* and *Vibrio anguillarum*. The effect of vaccination was also included as some fish were vaccinated before bacterial challenge. The fish fillets were sensory examined as heat-treated and cold-smoked. Heat-treated fillets from nonvaccinated fish previously infected by *V. anguillarum* had changed textural characteristics and were less flaky, had a lower oiliness and a higher toughness and fibrousness in comparison with control fish. This article was the first to describe a correlation between previous infections in fish and changes in sensory-quality parameters.

PRACTICAL APPLICATIONS

This work contributes with knowledge about sensory-quality parameters of fish meat after recovery from infections and physical-tissue damage. Because the results demonstrate an influence on the texture from previous disease, the practical potentials of the results are valuable for the aquaculture industry. In order to minimize the effects of previous diseases on the sensory quality regarding the texture, these fishes should be processed as cold-smoked instead of being sold as raw meat. The established correlation between disease history stresses the importance of disease prevention in aquaculture production, e.g., vaccination of the fish.

INTRODUCTION

Fish farming is an expanding worldwide industry, and the amount of produced fish and the number of species is increasing every year (Fao 2008). Many biological and nonbiological parameters such as welfare, rearing, stress level at slaughter and the amount of fat in the fillet have been shown to influence subsequent quality parameters of farmed fish postslaughter (Sigurgisladottir *et al.* 1997; Poli *et al.* 2005; Ashley 2007; Morkore *et al.* 2007; Pettersson *et al.* 2009; Merkin *et al.* 2010). Some of the quality parameters influenced by these factors are the texture of the fillet (meat) as well as the content of connective tissue and coloration of the fillet (Sigholt *et al.* 1997; Sigurgisladottir *et al.* 1997).

Disease outbreaks occur in fish farms, and the pathogens isolated can be a wide range of bacteria, viruses, parasites and fungi (Dalsgaard and Madsen 2000; Bondad-Reantaso *et al.* 2005; Pedersen *et al.* 2008). In general, infections are seen in both freshwater and seawater, and in all developmental stages of the fish. Survival and recovery of infected fish depends, however, on the type of infection, vaccination and veterinary treatment (Coyne *et al.* 2006; Raida and Buchmann 2008a).

Previous studies from commercial fish farms have reported changes in the tissue structure of the fillet of Atlantic salmon (*Salmo salar* L.) following bacterial and viral infections (Lunder *et al.* 1995; Lerfall *et al.* 2012). These changes develop as a result of heavy damages and bleedings in the musculature caused by pathogens (Moran *et al.* 1999). The structural

changes can be associated with the development of scars as a consequence of the regeneration process (Pedersen *et al.* 1994). Occasionally, examples of infections leading to tissue damage and bleedings are seen in Norwegian-salmon farming, when Atlantic salmon (*S. salar*) becomes infected by the bacterium *Moritella viscosa* or by the virus causing pancreas disease (PD) (Mcloughlin *et al.* 1996; Benediktsdottir *et al.* 1998). Recently, Lerfall *et al.* (2012) showed using a texture analyzer that cold-smoked fillets from infected or previous infected fish had a higher firmness relative to healthy fish. Usually, fish recovered from a *M. viscosa* or PD infection are downgraded from "superior quality" to "production fish," causing a lowered sales price on the market (Salte *et al.* 1994; Aunsmo *et al.* 2010). In addition to infections, physical tissue damage of the skin may also occur under fish farming conditions in connection to handling, transporting and crowding. In a number of studies, the structure and texture of meat have been directly linked to quality-related aspects (Trigo *et al.* 2008; Wolowiny 2008; Lonergan *et al.* 2010). However, until now it has not been shown whether previous infection or physical tissue damage may influence the sensory quality of fish meat regarding the texture of the fillet from recovered fish.

In Denmark, bacterial pathogens causing the most serious economical problems in the aquaculture industry are the gram-negative bacteria *Yersinia ruckeri* and *Vibrio anguillarum* (Larsen and Pedersen 1999; Tobback *et al.* 2007). *Y. ruckeri* usually causes infections in fry in freshwater, while *V. anguillarum* is mostly associated with larger fish in brackish- and saltwater. Both bacteria can lead to high mortality and the clinical signs include ulcerations and punctual bleedings in the musculature of the fish. Earlier findings have reported development of scars following infection by *V. anguillarum* (Pedersen *et al.* 1994; Stone *et al.* 1997). Usually, high ratio of fillets from slaughtered rainbow trout are sold as either fresh or smoked, making it relevant to examine those products. The aim of this study was to examine if physical tissue damage or previous infections of rainbow trout by *Y. ruckeri*, *V. anguillarum* or a combination of both influence sensory parameters of the fillet after recovery from disease. The studies were performed under regular farming conditions using both vaccinated and non-vaccinated fish.

MATERIALS AND METHODS

Overall Experimental Setup

The experimental setup was designed to mimic a natural farming situation implying an initial freshwater period and a later seawater-rearing period. The freshwater phase in the current experiment was subdivided into two parts, A and B. Facility A was located at the Technical University of Denmark and was used from fry and until a fish size of about 50 g. At this location, the fish were experimentally infected by *Y. ruckeri* and *V. anguillarum* and further medically treated. Another group of fish underwent a local, mechanical damage of the musculature. After recovery, all fish were transferred to a second freshwater facility, facility B, for further growth before being transferred to seawater cages (facility C).

Rearing of Fish at Facility A and Experimental Groups of Fish

Initially, unvaccinated rainbow trout (0+) fry ($n = 4{,}300$) reared at Horns Herred Ørredopdræt (Skibby, Zealand) were brought to the rearing facilities at facility A, the National Food Institute, Technical University of Denmark (DTU Food) in the Autumn of 2007. The experimental facilities at DTU Food consisted of two separate units: A1 and A2. Unit A1 was used for control fish and consisted of two 1,200-L tanks that were connected to a bio-filter with recirculated water and temperature control. Unit A2 was used for infection of fish and was composed of four 400-L tanks that were also connected to a bio-filter with recirculated water, temperature control and exposure of circulating water by UV light. Both rearing facilities were supplied daily with 10% freshwater and the content of ammonium and nitrite were measured in order to ensure good water quality. The water temperature was kept at 15C in both units and the fish were exposed to 16 h of light and 8 h of darkness. Ten different experimental groups of fish were obtained at facility A after vaccination and challenge with *Y. ruckeri* and *V. anguillarum*: (1) control fish; (2) *Y. ruckeri* challenged; (3) *Y. ruckeri* vaccinated + challenged; (4) *V. anguillarum* challenged; (5) *V. anguillarum* vaccinated + challenged; (6) *Y. ruckeri* challenged + *V. anguillarum* vaccinated; (7) *Y. ruckeri* challenged + *V. anguillarum* challenged; (8) *Y. ruckeri* vaccinated + challenged + *V. anguillarum* challenged; and (9) *Y. ruckeri* vaccinated + challenged + *V. anguillarum* vaccinated + challenged. Another group (10) was obtained after mechanical damage of the skin and musculature. Figure 1 shows how the different experimental groups were obtained. The procedures for vaccination, challenge and mechanical tissue damage are described later.

Each of the surviving fish from groups other than control were in August 2008 individually tagged by injection of electronic passive integrated transponder (PIT) tags into the peritoneal cavity (Product no. AB10400, Loligo Systems, Tjele, Denmark). Hence, all groups of fish could later be reared in the same cage. The individual tag numbers were read using an electronic PIT reader (Product no. AB10625, Loligo Systems).

Rearing of Fish at Facility B and C

In September 2008, the fish had reach a size of approximately 50 g and all experimental groups of fish (groups 1–10;

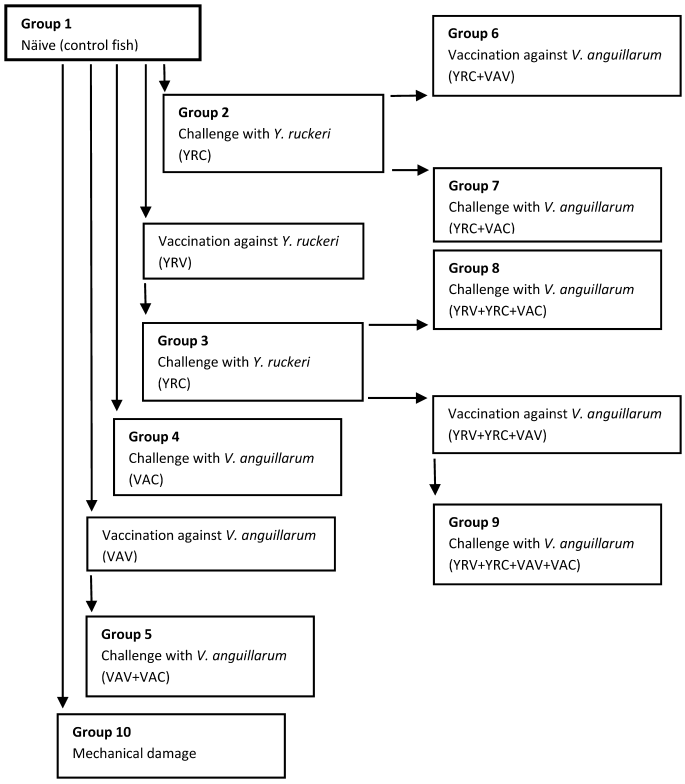

FIG. 1. OVERVIEW OF THE DIFFERENT EXPERIMENTAL GROUPS OF FISH OBTAINED AFTER VACCINATION, CHALLENGE AND MECHANICAL TISSUE DAMAGE AT FACILITY A
Control fish were not challenged. YRV, *Yersinia ruckeri* vaccinated; YRC, *Yersinia ruckeri* challenged; VAV, *V. anguillarum* vaccinated; VAC, *Vibrio anguillarum* challenged.

$n = 1{,}500$) were moved from facility A to the second freshwater facility (facility B) in Bisserup, Denmark. Facility B was composed of four 2,000-L circular tanks containing freshwater and connected to a bio-filter. The water quality parameters were equal to the conditions at facility A. The fish were equally distributed in the tanks upon arrival. Here, the fish were reared until transferred to one seawater cage with a diameter of 40 m and a depth of 4 m in the Spring of 2009. The size of the fish when transferred to seawater was approximately 150 g. The fish were reared in the cages until slaughter in November 2009.

Procedures for Vaccination against *Y. ruckeri* and Challenge

At a size of 3 g ($n = 30$, subsampling), 2,150 näive fish (group 1) were bath-vaccinated against *Y. ruckeri*. The bath-vaccination was performed using the Ermogen Vet. vaccine (Aqua Health, Novartis, Charlottetown, Canada) in October 2007. The procedures were in accordance with the manufacturer's instructions by diluting the vaccine 1:10 into water and further 30 s of bathing of the fish.

At a mean size of 14 g ($n = 30$, subsampling), 733 previously *Y. ruckeri*-vaccinated and 754 nonvaccinated, näive fish (group 1) were challenged with *Y. ruckeri* in March 2008 using the 99.8.60 strain (serotype O1). The strain was kindly provided by Dr. Inger Dalsgaard, National Veterinary Institute in Denmark and had previously been isolated from infected rainbow trout in a Danish fish farm. Bacteria were grown for 18 h at 25C in Luria Bertani (LB) medium with shaking until a final concentration of 10^9 bacteria/mL. 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 1× phosphate buffered saline (PBS). Based on these results, 10^6 bacteria were chosen for challenge of experimental fish. The bacteria were intraperitoneally (i.p.) injected (100 µL) in the fish using a syringe. When mortality was registered, the surviving fish were treated with antibiotics.

Procedures for Vaccination against *V. anguillarum* and Challenge

At a mean size of 30 g ($n = 30$, subsampling), approximately 1,200 naïve fish (group 1) and 150 previously *Y. ruckeri* challenged fish (group 3) were vaccinated against *V. anguillarum* in May 2008 using the AquaVac™ Vibrio vet. vaccine (Schering-Plough Animal Health). The fish were i.p. injected using 100 μL of vaccine per fish according to the manufacturer's instructions.

At a mean size of 40 g ($n = 30$, subsampling), 119 previously *Y. ruckeri*-vaccinated and challenged (group 3) plus 149 *V. anguillarum*-vaccinated fish were challenged with *V. anguillarum* in July 2008 using the strain 070602-1/2A (serotype O1). The strain was kindly provided by Dr. Inger Dalsgaard, National Veterinary Institute in Denmark and had previously been isolated from infected rainbow trout in a Danish fish farm. Bacteria were grown in LB medium containing 1% $NaCl_2$ for 24 h at 25C with shaking until a final concentration of 10^9 bacteria/mL. A challenge test was also set up for *V. anguillarum*. Based on this, a dose of 10^5 bacteria per fish was chosen for challenge of experimental fish. The bacteria were i.p. injected (100 μL) into the fish using a syringe. Treatment with antibiotics was initiated 2 days postchallenge.

Treatment of *Y. ruckeri*- and *V. anguillarum*-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 with Aquavet feed (Biomar, Brande, Denmark, 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 postchallenge and for the following 8 days. For *V. anguillarum*, feeding was started 2 days postchallenge and continued for 4 days.

Bacteriology of Challenged Fish

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*. Both species were incubated at 20C.

Plating out was also performed from noninfected control fish in order to verify that they were free of infection. Later in the experiment, after antibiotic treatment and recovery and prior to movement of the fish from the facilities at facility A, the fish were again screened for the presence of bacteria. This was performed in order to state the fish free of pathogens and not to bring bacterial pathogens from the laboratory facilities to the sea cages.

Mechanical Damage of the Musculature

Mechanical punctual damage of the skin and musculature was performed on the left side of 50 fishes 2 weeks prior to transfer to seawater using a specially constructed device. This has previously been shown to be a good control of muscle damage, which can be compared with following a bacterial infection (Ingerslev *et al.* 2010). The fish had not been vaccinated or challenged prior to the damage. Within minutes before the damage, the fishes were anesthetized using a dose of 50 mg/L of MS-222 (Sigma-Aldrich, St. Louis, MO). The device contained 8×4 (32) needles (25 g, Becton Dickinson, Brøndby, Denmark) equally distributed on an area of 2.7×6.3 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. The 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.

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 100 g the fish were fed with a diet of DAN-EX trout feed (Danafeed, Horsens, Denmark). Once transferred to the freshwater facilities at Bisserup and for the remaining experiment the fish were fed with Aqualife/EFICO trout feed and Ecolife Pearl (Biomar).

Enzyme-Linked Immunosorbant Assays for Detection of *Y. ruckeri* and *V. anguillarum*

An enzyme-linked immunosorbant assay (ELISA) was used to detect the presence of the *Y. ruckeri* O1 and *V. 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 (*Y. ruckeri* O1 and *V. anguillarum* O1) bacteria were grown for 12 and 24 h at room

temperature in LB media, respectively. The bacteria were then centrifuged for 13 min at 1,800 rpm and resuspended in coating buffer (pH 9.6).

Initially, the wells of microtiter plates (Immunosorb, Nunc, Roksilde, Denmark, product no. 269620) were coated using 200 µL of antigen solution diluted in coating buffer (10^6 bacteria/well) and further incubated at 4C over night. Unbound bacteria were removed by three washes in PBS-Tween, pH 7.2. The following day, the plates were blocked using 200 µL of 0.5% bovine serum albumin (BSA) in PBS and shaken for 15 min. 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 min. The wells were then washed in order to remove unbound plasma. Furthermore, 100 µL of rabbit-anti trout Ig diluted 1:3,000 in PBS with 0.1% BSA (Sigma Aldrich) was then added to the wells and the plates were gently shaken for 10 min and the wells were thereafter washed three times. Subsequently, 100 µL of horseradish peroxidase conjugated goat-anti rabbit antibody (Bio-Rad, Hercules, CA, product no. 172-1019) diluted 1:3,000 in PBS with 0.1% BSA was added to the wells followed by shaking for 10 min. 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 min. The reaction was stopped using 50 µL of stop solution (1 M H_2SO_4). It was added when a strong color in the wells was obtained or after maximally 15 min of incubation. The absorbance was when measured at 450 nm using a Synergy 2 multi-mode microplate reader (BioTek Instruments, Winooski, VT).

Fish Slaughtering and Fillet Smoking

Prior to slaughtering, fishes were killed by sharp blows to the head. They were further gill-cut and bled for 5 min in ice-water. After slaughter and gutting, the fishes were stored on ice until the next day when they were filleted in a postrigor state. The fillets were further 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 heat-treated fish.

The fish fillets were smoked at Bisserup Havbrug according to standard procedures the same day as the fish were filleted. Initially, the fillets were dry-salted for 3 h followed by a short dripping of water. Smoke was generated using a mix of peat litter from beech and crowberry heather. The fillets were then smoked for 40 h at 28C and subsequently vacuum packed and stored at 2C prior to sensory evaluation.

Sensory Profiling

The trout fillets were evaluated for textural characteristics by sensory profiling either as heat-treated or cold-smoked. Because of loss of some of the experimental fish, only four groups in addition to the control fish were sensory evaluated. These were: (1) previously *V. anguillarum*-challenged fish (group 4); (2) previously mechanically damaged fish (group 10); (3) previously *Y. ruckeri*-vaccinated + challenged + *V. anguillarum*-challenged fish (group 8); and (4) previously *Y. ruckeri*-vaccinated + challenged + *V. anguillarum*-vaccinated + challenged (group 9). The fillets evaluated as heat treated and were stored on ice for 3 and 7 days before steaming. The cold-smoked fillets were evaluated 9 days after smoking. A sensory panel that consists of nine (for heat-treated) or eight (cold-smoked) assessors, who had been selected, tested, and specifically trained in descriptive analysis (Iso 1988, 1993) of rainbow trout, evaluated the fillets (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 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 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, Couternon, France). One fillet from each fish was analyzed 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 50 g. The fish samples for steaming were heated in a prewarmed convection oven (RATIONAL Combi-Dämpfer CCM, Neuruppin, Germany) with air circulation for 15 min at 100C. After heat treatment, the samples were immediately served for the panel. The sensory attributes for analyzing the textural characteristics of heat-treated fillets were: flakiness, juiciness, firmness, oiliness, toughness and fibrousness. For appearance, they were: bright, colored and discolored (Hyldig 2009b). For the cold-smoked fillets, they were: elastic (with a fork), elastic, firm, juicy, tough, crispy and oily. For appearance, they were: discolored, colored and iridescent (Hyldig 2009b). Each sample was cut in slices (3 mm wide) and three pieces were put into individual Petri dishes for each assessor. All evaluations were performed in separated booths under normal daylight and at ambient temperature.

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 Fisher's exact test in order to compare the experimental groups with control fish. The specific test was

used in order to give a statistically qualitative result because of the relatively small sample numbers. 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 4.0c (GraphPad Software, San Diego, CA) for Macintosh software (GraphPad Software, La Jolla, CA).

RESULTS

Challenge by *Y. ruckeri*

Mortality following challenge by *Y. ruckeri* was initiated on day 3 postchallenge and continued until day 17 postchallenge (Fig. 2). Antibiotics was given from day 6 postchallenge and continued until the mortality had ceased according to veterinarian regulations. A final survival of 63.8 and 59.5% was obtained in vaccinated and naïve fish challenged by *Y. ruckeri*, respectively. No significant difference was seen between these two groups ($P > 0.05$). No mortality was observed for control fish.

Challenge by *V. anguillarum*

A number of 149 previously *Y. ruckeri* vaccinated + *Y. ruckeri* challenged + *V. anguillarum-vaccinated* fish were challenged by *V. anguillarum*. Furthermore, 123 previoulsy *Y. ruckeri* vaccinated + *Y. ruckeri* challenged and 124 nonvaccinated fish were challenged by *V. anguillarum*. Mortality was initiated on day 2 following challenge in naïve fish. The survival of this group following challenge by *V. anguillarum* was 82.4% (Fig. 3). This was significantly higher compared with nonvaccinated fish challenged against *Y. ruckeri* (59.5%, $P < 0.05$; see Figs. 2 and 3). In addition, vaccinated fish were protected against the bacterium because only 0.7% in total of vaccinated fish died following challenge (Fig. 3). For fish that were previously vaccinated against and infected by *Y. ruckeri*, the final survival was 79.0%. Between this group and naïve fish challenged by *V. anguillarum*, there was no significant difference in survival ($P > 0.05$), but the survival of these two groups was significantly lower than for vaccinated fish ($P < 0.05$).

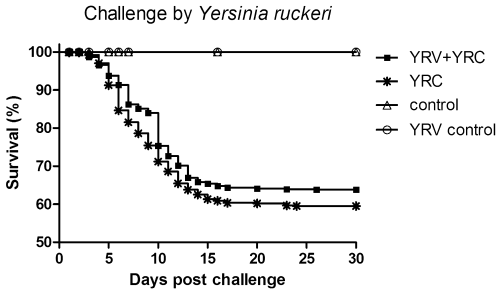

FIG. 2. CHALLENGE OF RAINBOW TROUT BY *YERSINIA RUCKERI*
Control fish were not challenged. YRV, *Yersinia ruckeri* vaccinated; YRC, *Yersinia ruckeri* challenged.

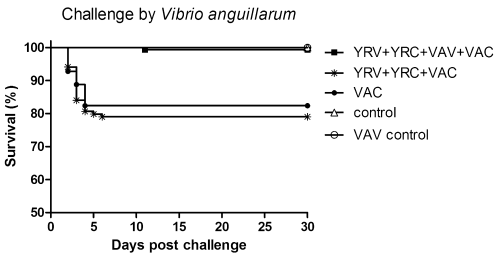

FIG. 3. CHALLENGE OF RAINBOW TROUT BY *VIBRIO ANGUILLARUM*
Control fish were not challenged. YRV, *Yersinia ruckeri* vaccinated; YRC, *Yersinia ruckeri* challenged; VAV, *Vibrio anguillarum* vaccinated; VAC, *Vibrio anguillarum* challenged.

Clinical Signs of Infected Fish

Both bacteria gave rise to the classical clinical signs of disease as reported in earlier studies (Poppe and Bergh 1999). For *Y. ruckeri*, bleedings were observed in the region of the mouth and some fish showed bleedings in the fins. Internal signs were pale liver, bleedings and ascites. The clinical signs for *V. anguillarum*-infected fish were seen as external bleedings in the skin, musculature and at the base of the fins. For some fish, bleedings were visible in the musculature the whole way from the skin and into the peritoneal cavity. Internal signs included ascites, pale liver and bleedings in the peritoneal cavity.

Bacteriology following Challenge and Recovery

Following bacterial challenge, subsequent dead fish were immediately screened for the presence of bacteria. Plating of blood on agar plates from either *Y. ruckeri* or *V. anguillarum*-infected fish appeared as white colonies. Furthermore, *V. anguillarum* were hemolytic on blood agar (results not shown).

After antibiotic treatment and subsequent recovery, a number ($n = 10$, subsampling) of previously infected fish were examined for the presence of pathogens before the fish were transferred to the sea cages. All examined fish were stated clear of both *Y. ruckeri* and *V. anguillarum* (results not shown).

Detection of *Y. ruckeri* and *V. anguillarum* by ELISA

The results of the ELISAs for detection of *Y. ruckeri* and *V. anguillarum* showed presence of antibodies against both bacte-

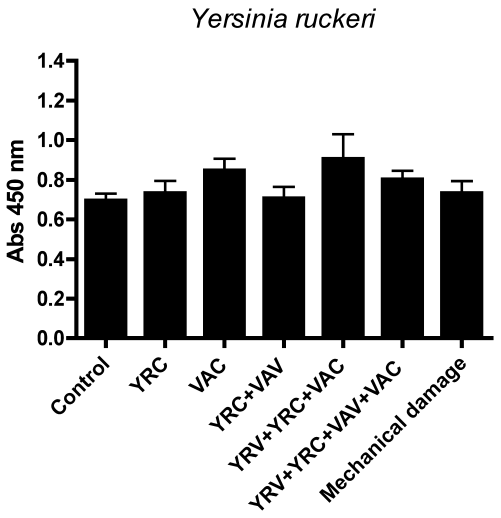

FIG. 4. ANTIBODIES AGAINST *YERSINIA RUCKERI*
Antibody levels were measured in the experimental groups 1, 3, 4, 6, 8, 9 and 10. The results are shown as absorbance at 450 nm + standard error of the mean. Control fish were not challenged. $n = 9$ fish in each group analyzed. YRV, *Yersinia ruckeri* vaccinated; YRC, *Yersinia ruckeri* challenged; VAV, *Vibrio anguillarum* vaccinated; VAC, *Vibrio anguillarum* challenged.

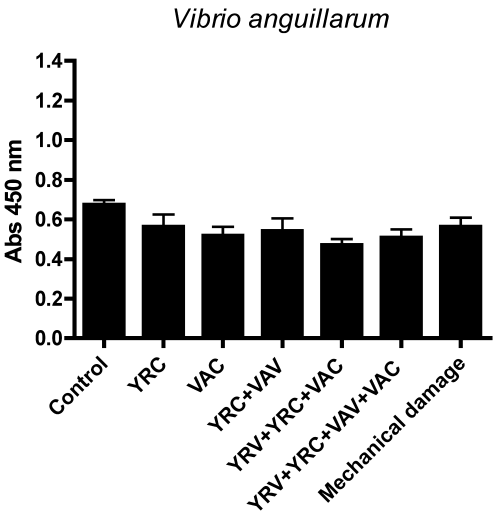

FIG. 5. ANTIBODIES AGAINST *VIBRIO ANGUILLARUM*
Antibody levels were measured in the experimental groups 1, 3, 4, 6, 8, 9 and 10. The results are shown as absorbance at 450 nm + standard error of the mean. Control fish were not challenged. $n = 9$ fish in each group analyzed. YRV, *Yersinia ruckeri* vaccinated; YRC, *Yersinia ruckeri* challenged; VAV, *Vibrio anguillarum* vaccinated; VAC, *Vibrio anguillarum* challenged.

ria in all groups of fish (Figs. 4 and 5). For both bacteria, there was no statistical difference between any of the groups in antibody titre (*t*-test, $P > 0.05$).

Size of Fish at Termination of the Experiment

The fish were reared in the sea cages until slaughter in November 2009. The average weight (g ± standard deviation [SD]) and length (cm ± SD) of the fish at slaughter were 667.1 ± 103.9 and 36.8 ± 1.8, respectively ($n = 111$). Furthermore, there was no significant difference in size between fish from the different experimental groups ($P > 0.05$).

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. Because of this, the subsequent sensory analysis was reduced to the experimental groups described in Table 1 (groups 4, 8, 9 and 10). The numbers of fish from each group used for sensory analysis were 8 and 9 for cold-smoked fillets and heat-treated fillets, respectively. Significant differences were seen for fish stored for 7 days on ice before heat-treating regarding texture (Table 1). For these, previously *V. anguillarum* infected fish (group 4) were significantly less flaky and had a lower oiliness in comparison with control fish ($P < 0.001$). Furthermore, they had a significantly higher toughness in comparison with control fish ($P < 0.01$). Fish previously vaccinated against and challenged with *Y. ruckeri* and later *V. anguillarum* challenged (group 8) did also have a significantly higher toughness, but also a higher fibrousness in comparison with control fish ($P < 0.001$). Fish vaccinated and challenged with *Y. ruckeri* and *V. anguillarum* (group 9) and mechanically damaged fish (group 10) did not show significant changes for any of parameters analyzed in comparison with control fish ($P > 0.05$). For heat-treated fish after 3 days of ice-storage and cold-smoked fillets, no difference was seen for any of the parameters tested ($P > 0.05$, results not shown).

DISCUSSION

The results presented herein establish a linkage between previous infections and sensory-quality parameters in rainbow trout recovered from disease. Fillets from previously infected and mechanically damaged rainbow trout were examined for sensory analysis as heat-treated and smoked in order to

TABLE 1. SENSORY ANALYSIS OF HEAT-TREATED FILLETS STORED ON ICE FOR 7 DAYS

		Texture						Appearance		
Group number	Experimental treatment	Flakiness	Juiciness	Firmness	Oiliness	Toughness	Fibrousness	Brightness	Color	Discolor
4	VAC	–	n.s.	n.s	–	+	n.s.	n.s.	n.s.	n.s.
8	YRV + YRC + VAC	n.s.	n.s.	n.s.	n.s.	+	+	n.s.	n.s.	n.s.
9	YRV + YRC + VAV + VAC	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.
10	Mechanical damage	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.	n.s.

Experimental groups shown are 4, 8, 9 and 10. All experimental groups are compared with the control group using one-tailed Fisher's exact test. "+" indicates significantly higher level relatively to control fish ($P < 0.001$); "–" indicates significantly lower level relatively to control fish ($P < 0.001$). $P > 0.001$.

$n = 9$ fish evaluated in each of the groups.

n.s., nonsignificant; VAV, *Vibrio anguillarum* vaccinated; VAC, *Vibrio anguillarum* challenged; YRV, *Yersinia ruckeri* vaccinated; YRC, *Yersinia ruckeri* challenged.

examine the usual consumer products industrially processed from that species. The time points of 3 and 7 days of ice-storage of heat-treated fillets prior to the sensory analysis were chosen in order to make the results comparable with what could be expected within a natural situation in fish farming. Furthermore, the texture of fish meat is known to undergo changes together with storage time, making it obvious to do measurements at different time points (Faergemand *et al*. 1995). Only fish that were previously challenged by *V. anguillarum* and processed as heat-treated were significantly different regarding the texture in comparison with control fish. Fillets from this group were less flaky, had a lower oiliness and a higher toughness in comparison with control fish. Furthermore, fish previously vaccinated against and challenged with *Y. ruckeri* and later *V. anguillarum*-challenged had a significantly higher toughness and fibrousness compared with control fish. These results show that previous infection has an impact on the resulting sensory quality of the fish meat from recovered fish. The findings are further supported by recent studies of Atlantic salmon infected or previous infected by PD (Lerfall *et al*. 2012). Here, the texture, although measured instrumentally, was directly influenced by the infection.

Usually, 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 characterized 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). Inflammation together with the later stages of tissue regeneration typically leads to establishment of scarring in affected regions, as seen for farmed Atlantic salmon following infection by *M. viscosa* (Salte *et al*. 1994). Scarring is a result of deposition of collagen between the live cells and this is believed to account for the textural differences between previously infected fish 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). In beef meat, a correlation between meat tenderness and the collagen content has been shown, pointing out a significant influence of collagen on the textural characteristics of meat (Riley *et al*. 2005). The preparation of the heat-treated fillets was performed at a temperature of 100C. Previous experiments with similar treatments of fish meat of the same size have shown that the resulting core temperature thus reach approximately 70C (Green-Petersen and Hyldig 2010). Usually, at much lower temperatures about 35C, fish collagens denature and become soluble (Saito *et al*. 2001). Thus, it could be believed that the collagen content in meat would not have an influence on textural characteristics when meat is processed at those temperatures. However, in earlier studies it has been shown that also denatured (soluble) collagens in meat contribute to the textural characteristics (Lepetit 2008). The mechanical damage performed may have had limited impact on the fish. A less heavy inflammatory response could be expected following sterile physical damage in comparison with infected fish because of the absence of pathogens in the tissue (Ingerslev *et al*. 2010). Furthermore, nonvaccinated, *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 with 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 natural route of entry by bacterial pathogens in the host is through the outer surfaces such as the skin and gills (Lovoll *et al*. 2009). However, in this study the fish were 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 with a natural route of infection because

the bacteria spread systemically through the blood (Høie 1999; Larsen and Pedersen 1999). Because of the small size of the fish when vaccinated against *Y. ruckeri*, an immersion vaccine was chosen. In contrast to the vaccine used against *V. anguillarum*, there was no effect of the *Y. ruckeri* vaccine, which is in line with earlier observations when fish are i.p. challenged following an immersion vaccine (Raida and Buchmann 2008b). In contrast, the fish were i.p. vaccinated against *V. anguillarum* due to a larger size at the time of vaccination, and for these fish with almost full protection of the vaccine was seen. The ELISA data showed no significant difference to any of the different groups either for *V. anguillarum* or *Y. ruckeri*. A survey on the occurrence of typical bacterial pathogens from Danish marine rainbow trout farms has revealed that both species of bacteria are isolated from fish in there (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 because of this have developed antibodies (Ellis 1988). The time from the last infection and antibiotic treatment 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 shorter-term effects would be more prominent because the signs of disease are expected to diminish over time (Lerfall *et al*. 2012).

Overall, from the results it should be concluded that disease has an impact on product quality-associated parameters. The results may be useful knowledge for the fish industry because knowledge about previous history of disease may be used in the choice of strategy regarding processing of the fish following slaughter. Thus, it might be beneficial to use previously infected fish for smoked products. Lastly, the findings underline the importance of effective vaccination in order to prevent subsequent quality changes.

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PAPER II

β-glucan enriched bath directly stimulates the wound healing process in common carp (*Cyprinus carpio* L.)

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Abstract

Wound healing is a complex and well-organized process in which physiological factors and immune mechanisms are involved. A number of different immune modulators have been found to enhance the non-specific defence system in vertebrates, among which β-glucans are the most powerful and extensively investigated.

The aim of the present study was to investigate the biological impact of two different commercially available β-glucan containing products on the wound healing process in carp. Throughout a two week experiment fish were kept either untreated (control), or in water supplemented with the two different types of β-glucans. The wound healing process was monitored using a multispectral visualisation system. The correlation between wound closure and immune response was investigated by measuring the gene expression patterns of IL-1β, IL-6, IL-8 and Muc5b, and measurement of production of radical oxygen species. PAMPs/DAMPs stimulation caused by the wounding and or β-glucans resulted in an inflammatory response by activating IL-1β, IL-6 and IL-8 and differences in the expression pattern were seen depending on stimuli. IL-1β, IL-6 and IL-8 were activated in all wounds regardless of treatment. Expression of all three interleukins was highly up regulated in control wounded muscle already at day 1 post-wounding and decreased at subsequent time-points. The reverse was the case with control wounded skin, where expression increased from day 1 through day 14. The results for the β-glucan treated wounds were more complex. The images showed significantly faster wound contraction in both treated groups compared to the control. The obtained results clearly demonstrated that a β-glucan enriched bath promotes the closure of wounds in common carp and induce a local change in cytokine expression.

Keywords: carp, *Cyprinus carpio* L., β-glucan, wound healing, gene expression

1. Introduction

Skin, together with the mucus, forms the first line of defence against pathogens and is an essential protective barrier in aquatic organisms. Mucus can entrap foreign particles and microorganisms, before they can interact with the epithelial surface and cause damage [1]. The main mucus components are water and large, highly glycosylated glycoproteins called mucins. Mucins and mucus have rheological properties (viscosity and elasticity), which are important for their physiological function [2]. Based on biochemical characterisation, 19 mucin genes have been identified in humans and gene structure as well as their role in the infection process has been described [3-5]. To date, only two mucin genes, Muc2c and Muc5b, have been cloned and sequenced in fish [6].

Wounds in fish can be caused by pathogens such as ectoparasites or Gram-negative bacteria, as well as by physical trauma [7-9]. In vertebrates reduced skin integrity or mucus production facilitates entry of pathogens into the underlying tissue and vascular system, therefore rapid reaction at the wound site is essential [10]. Wound healing is a complex and well-organized process which can be roughly divided into three overlapping phases: inflammation, tissue formation and remodeling, in which blood cells, soluble mediators, resident cells (fibroblasts, endothelial cells, goblet cells) and extracellular matrix components are involved [10-12].

The immunomodulatory effect of β-glucans has been shown in studies on several taxonomic groups including fish [13-18]. These naturally occurring polysaccharides differ in length, molecular weight, extent of branching and bioactivity, and in vertebrates trigger different biological effects [19-24]. β-glucans have been shown to accelerate the wound healing process in mammals, however such effect remains to be proven in fish [25, 26].

β-glucans are pathogen-associated molecular pattern molecules (PAMPs) and are recognized by pattern recognition receptors (PRRs) such as toll-like receptors (TLR) or NOD-like receptors (NLRs), and activate transcription of pro-inflammatory genes [27]. Furthermore, PRR signaling can also be activated by “danger signals” – damage associated molecular patterns (DAMPs), which are endogenous molecules such as nucleic acids, high-mobility group box 1-protein or heat shock proteins released from cells during tissue damage, or hyaluronan products degraded during said process [28, 29]. Hence, introducing PAMPs and DAMPs simultaneously may have a combined impact [30].

Mammalian models have shown that in response to PAMPs and DAMPs, numerous cytokines are secreted and subsequently, will guide and selectively activate leukocyte subsets [12]. Some

cytokines have shown to be highly important during wound healing. Mammalian interleukin 1β (IL-1β) is strongly up-regulated during the inflammatory phase of healing [31, 32]. The main IL-1β sources are polymorphonuclear leukocytes (constitute nearly 50% of all cells at the wound site in first few days post-wounding) and macrophages, as well as some resident cells [12, 31]. In addition, human recombinant IL-1β is reported to affect mucus release in mice [33]. Interleukin 8 (IL-8) is a known regulator of neutrophil trafficking and augments angiogenesis in mammals [12, 32, 34-36]. Carp possess two CXCL8 lineages, which appear to be functional homologs to mammalian IL-8 [37]. Expression of IL-8 in mammals is correlated with interleukin 6 (IL-6) [38]. IL-6 is produced and secreted by macrophages, neutrophils, as well as resident cells [12, 31]. Experiments on diabetic or IL-6 knockout mice have shown a reduction in neutrophil and macrophage number, as well as a decrease in collagen production and deposition [39, 40]. Orthologues of the mammalian IL-6 have been identified in fish [41-44]. Moreover, PAMPs and DAMPs induce production and secretion of hydrolases, complement components, and reactive oxygen and nitrogen species (ROS and RNS) by macrophages in vertebrates [45-48].

In the present study, we have examined the direct biological effect of β-glucan enriched products on the wound healing process in common carp in a controlled environment. This effect has been verified by monitoring visual wound contraction dynamics and gene expression patterns of three pro-inflammatory cytokines: IL-1β, IL-6 and IL-8, as well as measurement of ROS production. Additionally, as Muc5b is the major gel-forming mucin in the protective mucous matrix of mammals, its expression was investigated [49].

2. Materials and methods

2.1 Animals

One-year-old common carp (*Cyprinus carpio* L., R3xR8, WUR, The Netherlands) were kept in 30 L aquariums filled with tap water adjusted to 21°C and fed a commercial carp feed (Trouvit, Nutreco) at 2% of their estimated body weight per day. Fish were exposed to a 12/12 light/dark cycle. The fish were divided into six groups (Table 1).

2.2 Preparation of β-glucans

For stock solution preparation, 0.5 g of MacroGard (yeast, >60% pure, Biorigin, Brasil) or 6.3 kDa fiber (oat, 50% pure, Scan Oat, Sweden) was dissolved in 500 ml MilliQ water. In order to dissolve the soluble β-glucan fractions, solutions of both products were stirred for 1 h at 90°C and autoclaved (121°C, 15 min, 1 atm). In the experimental setup, water was changed daily and the β-glucan product concentrations were adjusted to 0.1 μg/ml.

2.3 Wounding and sampling procedure

Carp were anaesthetised by immersion in 0.01% benzocaine (VetPharm, Belgium). 5 mm Ø biopsy punches (Miltex, Inc, USA, Figure 1.) were used to mechanically damage areas of the skin and musculature (left side, above the lateral line, three wounds per fish). At day one, three and fourteen post-wounding four individuals from each group were anaesthetised and, using 8 mm biopsy punches, the following tissue samples from the edges of the wounds were collected: 1. Muscle tissue from the wounded area; 2. Muscle tissue from a non-wounded area (right side); 3. Skin tissue from the wound area; 4. Skin tissue from non-wounded area (right side); 5. Muscle and skin tissue from all control fish (left side, Figure 1). Samples were collected and immediately frozen in liquid nitrogen for further analysis. This experiment was conducted according to Danish legislation and by scientists accredited by the Federation of Laboratory Animal Science Associations (FELASA).

2.4 RNA isolation and cDNA synthesis for qPCR

The previously frozen in the liquid nitrogen samples (50 mg) were mixed with 500 µl of buffer (2-Mercaptoethanol in lysis buffer) and sonicated (30 s, pulse 2, amplitude 70 %, using a soVCX-130 sonicator CiAB, Sweden) as it is considered as more straightforward method than homogenization [50]. Following the manufacturer's protocol, RNA was isolated using GenElute Mammalian™ Total RNA Miniprep Kit (Sigma-Aldrich, Denmark) and subsequently treated with DNase-I (Sigma-Aldrich, Denmark) to remove any genomic DNA. RNA purity and quantity was determined by OD260/280 measurements on a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Germany). 400 ng of total RNA was primed with random hexamers and reverse transcribed using TaqMan® Reverse Transcription reagents (Applied Biosystems, Denmark) in a final volume of 20 µl. The synthesized cDNA was diluted 1:10 in MilliQ water and stored at -20 °C until further analysis.

2.5 Real-time quantitative PCR

Based on sequences deposited in the GenBank (refer to Table 2) and using the program Primer3, PCR primer sets specific for 40S, IL-1β, IL-6 and IL-8, were designed [51]. The mucin 5b (Muc5b) primer set was provided by Fish Disease Research Unit at the University of Veterinary Medicine, Hanover, Germany. The subunit S11 of the ribosomal gene 40S was selected as a reference gene based on previous work [7]. The assays for examined genes were run using SYBR® Green JumpStart™ *Taq* ReadyMix™ (Sigma-Aldrich, Denmark). Quantitive RT-PCR was performed using a Stratagene MX3000P™ real-time PCR system (Integrated Sciences, Australia). Master mixes for each PCR run were prepared as follows: 12.5 µl Brilliant SYBR® Green JumpStart™ *Taq*

ReadyMix™, 1 µl of each primer and 5.5 µl ultra pure water. Finally 5 µl of diluted cDNA was added to 20 µl of master mix. The cycling conditions for the reaction were: incubation step of 10 min at 95 °C, followed by 45 cycles of 95 °C for 30 s and 60 °C for 1 min. At the end of each reaction, DNA melting curve analysis was performed in order to confirm the specificity of the PCR products. All samples were run in triplicate, and for each primer set, control reactions without cDNA were included in order to detect any non-specific amplification. The expression results were analyzed using the $-2^{-\Delta\Delta Ct}$ method [52]. Data are shown as fold expression relative to non-wounded internal control site, and the analysis was carried out using Mx ProTM qPCR software (Integrated Sciences, Australia). The threshold values (Ct) were determined manually for each run.

2.6 Isolation of head-kidney (HK) cells and measurement of respiratory burst

Anaesthetised fish were bled from the caudal vein. Both head-kidneys (HK) were excised and placed on a 100 µm nylon cell strainer (BD Falcon, New Jersey, USA). A cell suspension was obtained by pressing the head-kidneys with a plunger through the cell strainer, and rinsing them with phenol red-free Hank's balanced salt solution with 0.1 % gelatine (g-HBSS). The HK cells were counted using a Bürker chamber. Cell viability was assessed by Trypan exclusion and cell concentration was adjusted to $3x10^6$ HK cells/ml in g-HBSS. Subsequently, the respiratory burst activity was measured using luminol-amplified chemiluminescence, modified from the protocol described by Vernho *et al*. (2005) [53]. To elicit the respiratory burst response, *Aeromonas hydrophila* were incubated overnight. The bacteria was washed twice with HBSS, OD_{600} was set to 0.5, and 50 µl was added to each well in a white 96-well plate (Sigma-Aldrich P8616, Denmark). Next, a mixture of 40 µl of 10mM luminol in 0.2 M borate buffer (pH 9.0) and 100 µl of HK cells were added. The total well volume was adjusted with g-HBSS to 300 µl. The chemiluminescence emission from the HK cells was measured with a luminometer (Synergy2, Biotek) every 3 minutes for 210 min. at 26°C. Results are expressed as the integral of the relative light units (RLU) recorded by the luminometer (Max RLU).

2.7 Visual analysis of wound closure

Prior to sampling of the head kidney, skin and muscle, images of the wounds were acquired using a VideometerLab (Videometer A/S, Hørsholm, Denmark). The fish were placed in a special container in order to exclude the ambient light as the VideometerLab provides diffuse illumination from light-emitting diodes to capture 20 images with unique spectra between ultra violet and near infra red of the electromagnetic spectrum. The multispectral imaging facilitated the visual detection of the wound edge. The distance from the camera to the right (intact) side of the fish was kept constant. Differences in width of individual carp could affect the image capture area of the wound.

However, given the long distance from the camera to the subject (~40cm) and narrow size range of the fish, this had no measurable effect on wound size estimations. The wound edges on day 14 post-wounding were outlined and the resulting open wound area determined using a script in MATLAB (The MathWorks Inc., Natick, MA, USA).

2.8 Statistics

A non-parametric Mann-Whitney test was used to compare results from gene expression and respiratory burst. Differences between groups were regarded as significant at $p < 0.05$. Results from visual wound closure were tested with a two-way ANOVA using the Prism software, version 4.03 (Macintosh, GraphPad Inc., La Jolla, CA, USA).

3. Results

3.1 The visual healing of the wounds

At day 14 post-wounding, the wound size was significantly ($p < 0.01$) reduced in both β-glucan treated groups in comparison to control fish, but significantly more in the MacroGard-treated group. Figure 3 shows the differences in open wound area 14 days post-wounding from the experimental groups.

3.2 Gene expression during wound healing process

No significant changes were observed in non-wounded groups.

3.2.1 Interleukin 1β (IL-1β)

In skin collected from the W-C group, IL-1β showed a tendency to increase over time (Figure 4A). Muscle samples taken one day post-wounding showed a high and significant up-regulation ($p < 0.05$) (Figure 4B).

In skin collected from the W-MG group, a significant up-regulation ($p < 0.05$) three days post-wounding was observed (Figure 4A). No significant changes in muscle were seen (Figure 4B).

In skin collected from the W-6.3 group, IL-1β expression was low but significant ($p < 0.05$) one day post-wounding (Figure 4A). In muscle, high and significant up-regulation ($p < 0.05$) was observed one day post-wounding (Figure 4B).

3.2.2 Interleukin 6 (IL-6)

In skin collected from the W-C group, IL-6 showed a tendency for up-regulation three and fourteen days post-wounding (Figure 4C). In muscle, significant up-regulation ($p < 0.05$) was noted on day one post-wounding (Figure 4D).

In skin collected from W-MG fish, significant IL-6 up-regulation ($p < 0.05$) was seen three days post-wounding (Figure 4C). In musculature tissue, no significant changes were seen (Figure 4D).

In skin collected from W-6.3 fish, a tendency for up-regulation was observed (Figure 4C). In muscle, a high and significant up-regulation ($p < 0.05$) was detected one day post-wounding (Figure 4D).

3.2.3 Interleukin 8 (IL-8)

In skin collected from the W-C group, three and fourteen days post-wounding, a high and significant up-regulation ($p < 0.01$) of IL-8 was observed (Figure 4E). In muscle, a high and significant up-regulation ($p < 0.01$) was noted one day post-wounding (Figure 4F).

In skin collected from W-MG fish, IL-8 showed a tendency to be down-regulated over time (Figure 4E). In muscle, a high and significant up-regulation ($p < 0.01$) was detected three days post-wounding (Figure 4F).

In skin collected from W-6.3 fish, a tendency for IL-8 up-regulation was seen fourteen days post-wounding (Figure 4E). In muscle, significant up-regulation ($p < 0.05$) was detected three days post-wounding (Figure 4F).

3.2.4 Mucin 5b (Muc5b)

In skin collected from W-C fish, a significant up-regulation ($p < 0.05$) of Muc5b was observed fourteen days post-wounding (Figure 4G). In muscle samples taken fourteen days post-wounding, a tendency for up-regulation was observed (Figure 4H).

In skin collected from the W-MG group, no significant differences in Muc5b expression were observed between wounded and non-wounded sides (Figure 4G). In muscle, a tendency for up-regulation was noted fourteen days post-wounding (Figure 4H).

In skin collected from the W-6.3 group, a significant up-regulation ($p < 0.05$) in Muc5b expression was observed three days post-wounding with tendency to be up-regulated fourteen days post-

wounding (Figure 4G). In muscle a tendency for up-regulation was seen fourteen days post-wounding (Figure 4H).

3.3 Measurement of respiratory burst

Some significant differences between groups were observed (grey color), however they were inconsistent with no regular pattern. The absence of marked differences on ROS production in head kidney leukocytes was a sign of a localized immune response (refer to Table 3).

5. Discussion

Fish mucosal immunity is an extensively investigated area [54]. In contrast to terrestrial vertebrates fish skin is not keratinised and is hence metabolically active [55, 56]. Following wounding, DAMPs activate mucosal immune cells as well as epithelial resident cells, e.g. in vertebrates, fibroblasts have been shown to possess immune regulation capabilities [57-59]. Several fibroblast cell lines have been established and characterised in fish, and Ingerslev *et al.,* (2010) have reported that fish fibroblasts are susceptible to DAMPs [57, 60, 61]. In the present study, high up-regulation of IL-1β and IL-6 in skin samples from W-MG fish three days post-wounding could be explained by a boost of the immune-like performance from fibroblasts, elicited by the presence of DAMPs/PAMPs. Wei *et al*., (2002) presented the first evidence that glucans directly stimulate human fibroblasts [62]. Therefore fish fibroblasts, which could be activated in a similar way to immune competent cells, might directly stimulate the resident cells present in skin, such as epithelial, resident macrophages and lymphocytes, and amplify cytokine profiles leading to tissue regeneration. In contrast, fish Malpighian cells, regarded as the counterpart of the mammalian keratin-containing keratinocyte, which actively participate in wound healing process in fish, were shown not to react *in vitro* to yeast cells obtained from *Saccharomyces cerevisiae* [63].
Few studies have emphasized the role of goblet cells and mucus in wound healing process in mammals [11, 64]. Goblet cell differentiation and function in mammalian systems are affected by mucosal immunity, and gel-forming mucins can be regulated by different cytokines or exogenous factors [2, 6, 33, 65, 66]. Van der Marel *et al*., (2012) have shown that Muc5b is exclusively expressed in skin and gills of common carp, therefore the Muc5b expression was expected to change in carp subjected to β-glucan bath [6]. High Muc5b up-regulation three and fourteen days post-wounding was, however, not restricted to the wound area but was a general response of the skin mucosa to the wounding. It is in agreement with work presented by van der Marel *et al*. (2010) where water with increased bacterial load did not induce clinical symptoms in carp, however a rapid skin mucosal response was observed even if the bacteria involved were considered to be non-pathogenic [67]. Moreover, high local inflammation and IL-1β expression could hamper Muc5b

expression at the site of wounding which is in accordance with work presented by Cohan *et al*. (1991). This could also explain why Muc5b expression was not higher in the MacroGard treated group as would have been expected. However, although Muc5b in vertebrates is one of the largest gel-forming glycoprotein in the body, individual cells may differ in mucin composition [2, 49, 68]. Thus it cannot be excluded that β-glucan supplemented bathing induced expression of other mucins in the skin of carp, which were not included in the present study. According to work presented by van der Marel *et al.* (2012), Muc5b was exclusively expressed in skin and gills of common carp [6]. However, in the present study, expression of Muc5b was detected in muscle samples collected fourteen days post-wounding. It was suggested by Cheng and Leblond (1974), and further by Paulus *et al*. (1993) and Kanter and Akpolat (2008), that columnar and goblet cells in mammals originate from a common stem cell located at the crypt base [11, 69, 70]. If that is the case in fish model, Muc5b detected in muscle could be an effect of goblet/mucus cell migration toward newly differentiating muscle layers.

Tissue damage and release of danger signals such as RNA/DNA or heat shock proteins influence expression of pro-inflammatory cytokines [12, 27]. High levels of IL-1β, IL-6 and IL-8 cytokines one day post-wounding in muscle collected from the W-C group is likely to be the inflammatory response caused by the release of DAMPs. Interestingly, in skin samples from W-C fish, different patterns of pro-inflammatory cytokines expression has been noted. No or low IL-1β, IL-6 and IL-8 expression was detected one day post-wounding. However, gene expression was not investigated at earlier time points. It is possible that stored IL-1β, IL-6 and IL-8 have been secreted quickly (hours) in response to wounding and subsequently, these stores needed to be replenished. The temporally inversed pattern of gene expression in skin/muscle tissue has been seen in both β-glucan supplemented groups, with change in expression three days post-wounding. It is consistent with work done on rodents by Wolk and Danon (1985) [25]. According to these authors, the most pronounced differences in wound closure between glucan treated and control groups took place between 48 and 96 hours. All changes occurred earlier in the glucan treated group, and included proliferation and arrangement of fibroblasts as well as deposition of the collagenous matrix.

Yeast β-glucan is known as a powerful immune modulator and many studies have described its positive effect on various vertebrates [14-18, 25]. Bohn and BeMiller (1995) described β-glucans as biological response modifiers with activity correlated to the branching degree and size [24]. Accordingly, no biological activity/effect was detected in mammalian models when less branched glucans with molecular weights of 5 kDa-10 kDa were used. In contrast, Tanioka *et al*. (2011) have shown that barley-derived β-glucan (~2 kDa) stimulates maturation of mouse dendritic cells [71]. In addition, up-regulation (~ x30) of IL-6 expression in murine bone marrow cells has been seen

when compared to treatment with 40-70 kDa barley β-glucan [71]. Present results show that both β-glucans, MacroGard and 6.3 kDa oat fiber, promote the wound healing process in common carp and therefore 6.3 kDa oat fiber can be considered bioactive.

To our knowledge, this is the first investigation of the biological effect of β-glucans on the wound healing process in fish. Furthermore it is the first experimental design that allows the open wounds to stay in direct and constant contact with the β-glucan supplementation during the entire trial. Many studies have described the positive effect of β-glucans on the fish immune system [13-18]. However, the mechanism by which β-glucans enhance the wound closure remains unclear [14-18, 72]. Wolk and Danon (1985) have emphasized that application of glucans to promote wound healing in vertebrates would require an experimental model in which a wound on one side of the animal could be compared to a symmetrical wound on the other side of the same animal [25]. In our study, internal controls from non-wounded site of the wounded fish have been studied. This allowed us to eliminate external factors (e.g. temperature) and focus on obtaining more data about local and systemic response in carp during the wound closure process.

Overall, our results show that both β-glucans promote wound healing process in common carp when compared to control fish. We have concluded that bathing in β-glucans has direct positive effects on the wound closure in common carp and it can suggestible be related to high branching level due to fish being bathed in MacroGard supplemented water showed higher wound closure ratio in comparison to 6.3 kDa supplemented bath. We have shown the immunological and regenerative response following stimulation with β-glucans (PAMPs) and wounding (DAMPs) in controlled conditions without exposure to pathogens. PAMP/DAMP stimulation resulted in an inflammatory response by activating IL-1β, IL-6 and IL-8, and local differences in expression patterns depended on major stimuli: DAMPs or DAMPs/PAMPs combination. In addition, the absence of marked differences on the respiratory burst activity in head-kidney cells supports the notion of a local immune response at the site of wound.

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Figure legends

Figure 1. Illustration of wounding (A) and sampling (B) procedures and biopsy punches. Fish were wounded on the left side above the lateral line (red circle). During sampling, skin and muscle tissue were taken from the wounded site (red circle), as well as from non-wounded site (internal control, green circle).

Figure 2. Images of the wounded area fourteen days post-wounding acquired with the VideometerLab. Top row – control, middle row – MacroGard, botton row – 6.3 kDa oat fiber. NS – no sample.

Figure 3. Statistical representation of the wound sizes fourteen days post-wounding from wounded-control, wounded-MacroGard and wounded-6.3 kDa groups. Bars show mean values (pixels) + SD of n=4 (n=3 14 days in wounded-MacroGard group). “a” and “b” depict statistical significance ($p < 0.01$) between control and MacroGard groups, and control and 6.3 kDa groups subsequently. “c” depicts statistical significance ($p < 0.05$) between MacroGard and 6.3 kDa groups.

Figure 4. Quantitative real-time PCR for mechanically wounded fish. Expression of the genes interleukin 1β (IL-1β), interleukin 6 (IL-6), interleukin 8 (IL-8) and mucin 5b (Muc5b) is shown in skin (A, C, E, F) and muscle (B, D, F, H) of common carp. Results are obtained by qPCR and expressed relative to the internal control at each time point. The data are normalized relative to 40S and analyzed using the $-2^{-\Delta\Delta Ct}$ method. Bars represent fold expression + SD relative to non-wounded side of n=4 (n=3 14 days in wounded-MacroGard group). cnt – control; ND – not detected. * Depicts statistical significance between the wounded site and internal control site (*$p < 0.05$; **$p < 0.01$).

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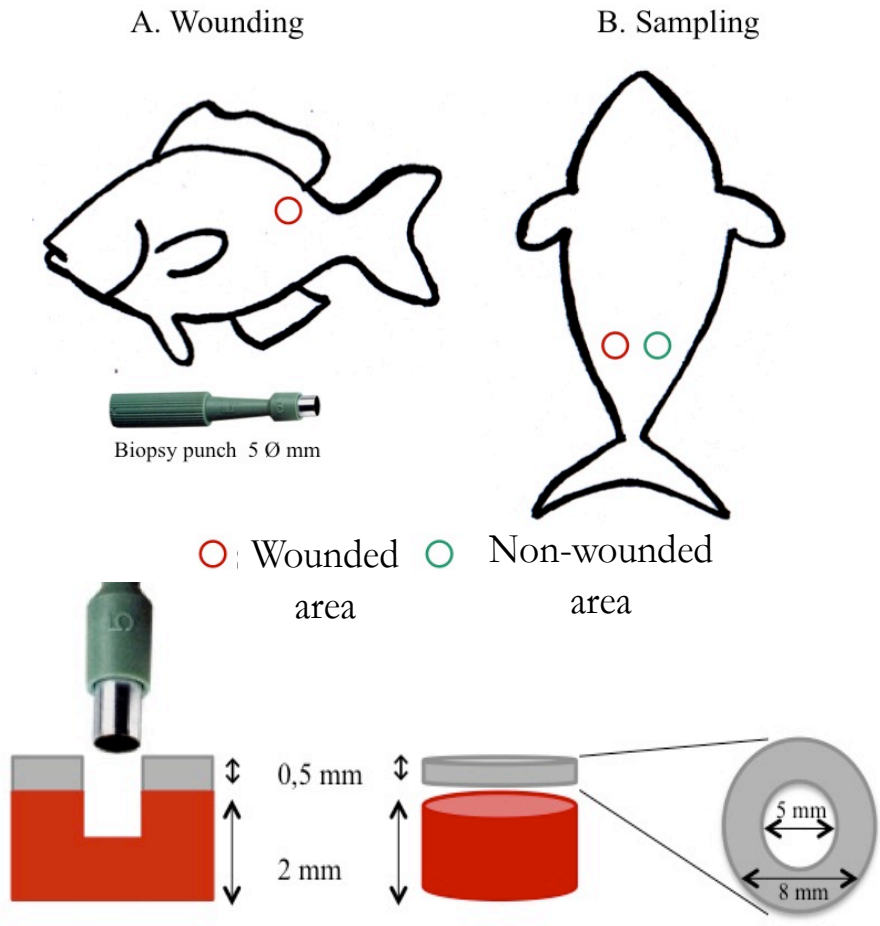
A. Wounding
B. Sampling
Biopsy punch 5 Ø mm
Wounded area
Non-wounded area
0,5 mm
2 mm
5 mm
8 mm

Figure 1.

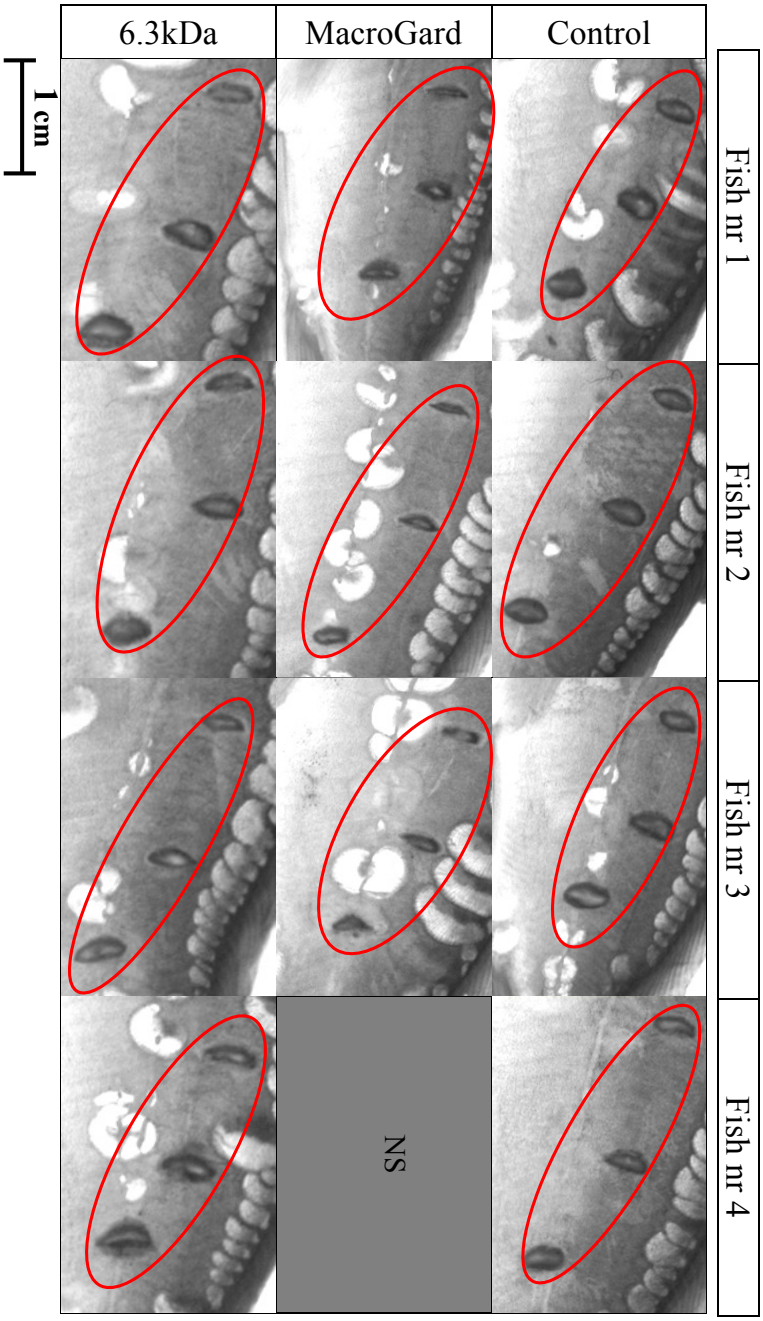
Control
MacroGard
6.3kDa
Fish nr 1
Fish nr 2
Fish nr 3
Fish nr 4
1 cm
NS

Figure 2.

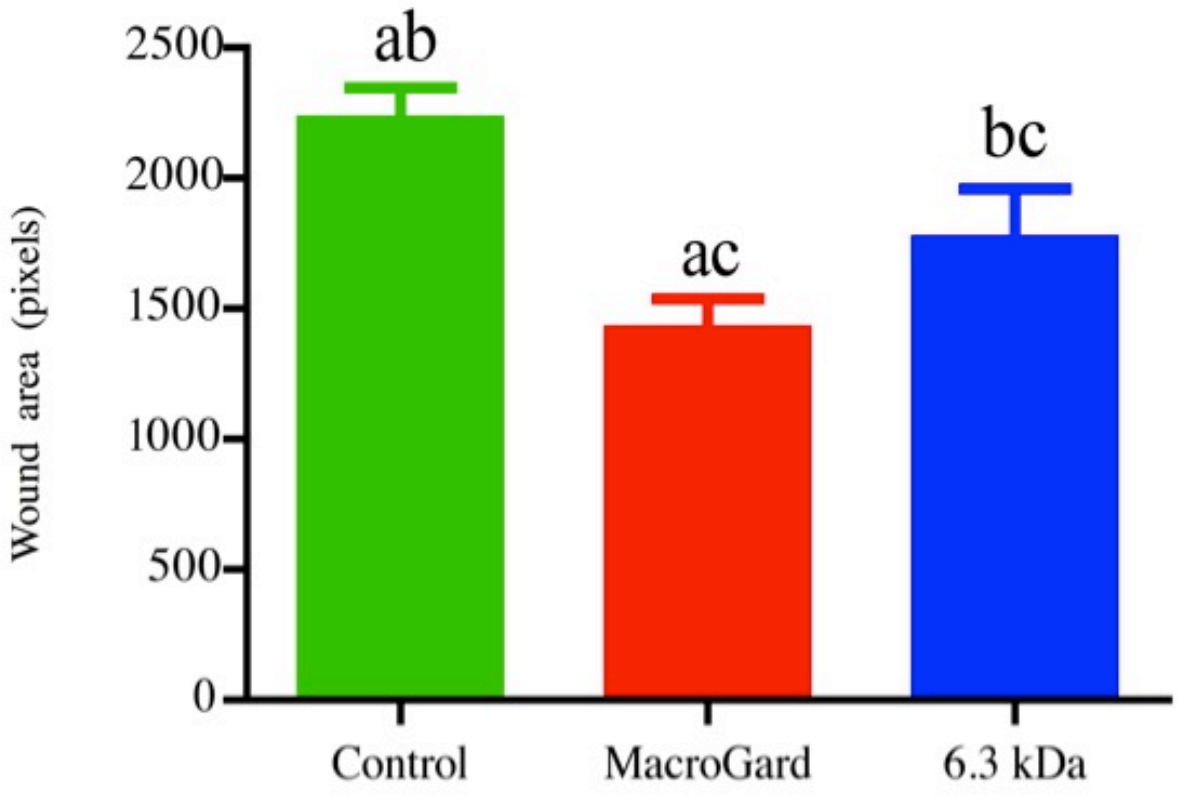
Wound area (pixels)
2500
2000
1500
1000
500
0
ab
ac
bc
Control
MacroGard
6.3 kDa

Figure 3.

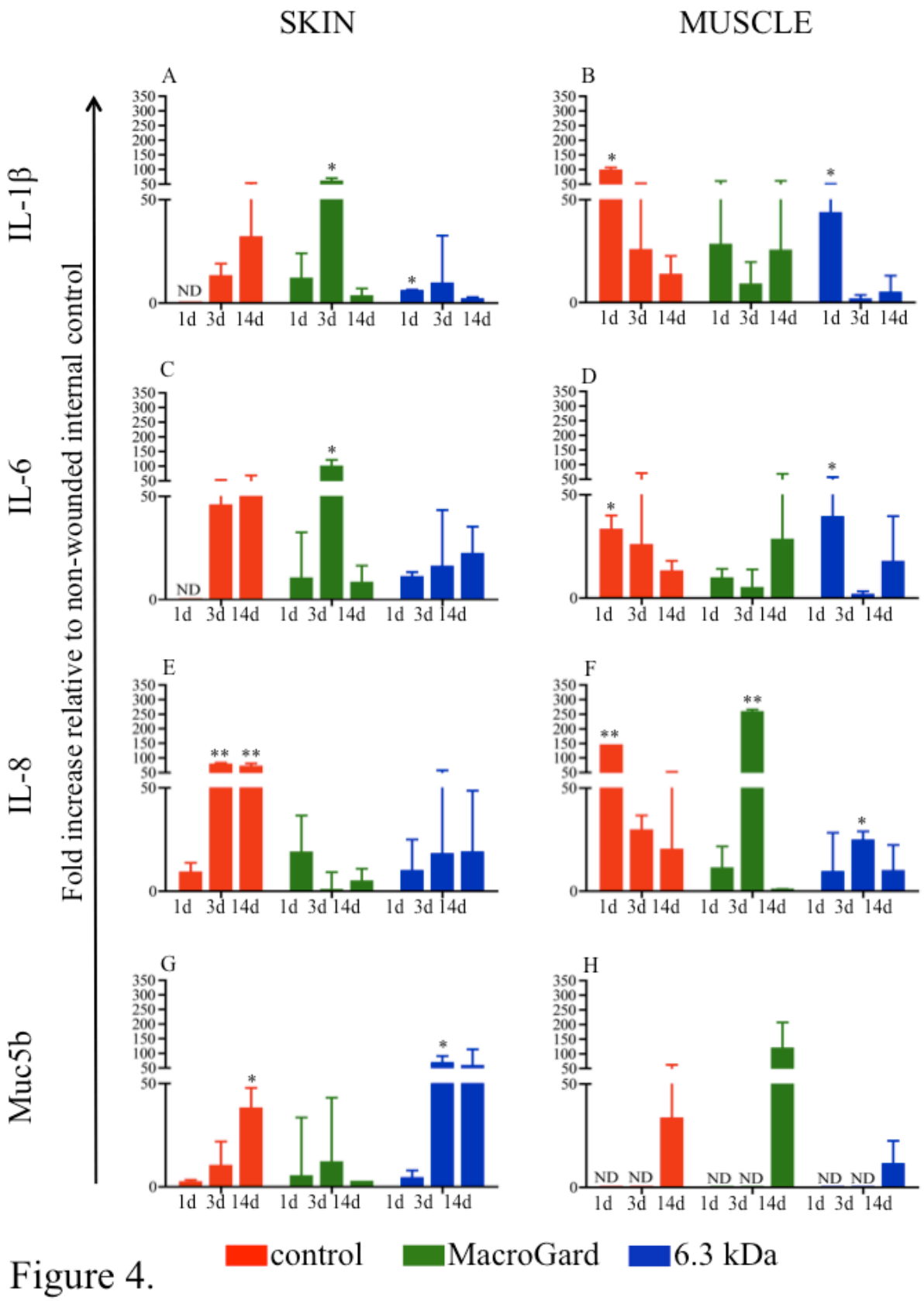
SKIN
MUSCLE
IL-1β
IL-6
IL-8
Muc5b
Fold increase relative to non-wounded internal control
A
B
C
D
E
F
G
H
ND
1d 3d 14d
control
MacroGard
6.3 kDa

Figure 4.

Table 1. Experimental groups.

Wounding	Water Condition: Control	Water Condition: MacroGard	Water Condition: 6.3 kDa fiber
No wound	12	12	12
5 mm Biopsy	12	12	12

Table 2. Sequences of primers used for real-time PCR.

Gene	Primer	Sequence (5' - 3')	GeneBank acc. No.
40S	Forward	GTTGAAGGAAGTGGCAAGGA	AB012087
	Reverse	AGAATACGGCCTCTGATGGA	
IL-1β	Forward	AAGGAGGCCAGTGGCTCTGT	AJ245635
	Reverse	CCTGAAGAAGAGGAGGCTGTCA	
IL-6	Forward	CCGCACATGAAGACAGTGAT	AY102632
	Reverse	GGGTATATTTGGCTGCAGGA	
IL-8	Forward	TGGAGCTCTTCCCTCCAAG	EU011243
	Reverse	AGGGTGCAGTAGGGTCCAG	
Muc5b	Forward	CAGCCCTCTTCCTCTTTCATC	JF343438
	Reverse	CCACTCATCTTTCCTTTCTCTTC	

Table 3. Respiratory burst significant differences within or among treatments, measured by luminol-amplified chemiluminescence of carp head kidney (HK) leukocytes. The gray color indicates the only combinations with a significant difference (p value= 0.0286). 1) non-wounded control (1dpw), 2) non-wounded MacroGard (1dpw), 3) non-wounded 6.3 kDa (1dpw), 4) non-wounded control (3dpw), 5) non-wounded MacroGard (3dpw), 6) non-wounded 6.3 kDa (3dpw), 7) non-wounded control (14dpw), 8) non-wounded MacroGard (14dpw), 9) non-wounded 6.3 kDa (14dpw), 10) wounded-control (1dpw), 11) wounded-MacroGard (1dpw), 12) wounded-6.3 kDa (1dpw), 13) wounded-control (3dpw), 14) wounded-MacroGard (3dpw), 15) wounded-6.3 kDa (3dpw), 16) wounded-Control (14dpw), 17) wounded-MacroGard (14dpw), 18) wounded-6.3 kDa (14dpw).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1																		
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PAPER III

Intravenous injection of β-glucan in a dose-related manner stimulates IgZ1 expression in skin of common carp (*Cyprinus carpio* L.)

Przybylska DA and Nielsen ME.

(In preparation)

Abstract

β-glucans, naturally occurring polysaccharides are found in the cell wall of plants, bacteria and fungi. They have been proven to enhance the immune system and pharmacologically they are classified as biological response modifiers (BRM). However, there are also toxicological effects associated with the systemic administration of these agents.

The purpose of this study was to determinate if intravenous (i.v.) injection of *Aeromonas hydrophila* bacterin (BSK10) with two dosages of MacroGard has a biological effect on immune parameters in carp. Hence, 0,1 g/ml (low β-glucan dose; B-Lβg) and 1 g/ml β-glucan (high β-glucan dose; B-Hβg) in PBS were mixed with bacterin and i.v. injection was performed. To follow stress reaction blood samples from all fish were collected every three days for measurement of cortisol level. Additionally, ratio for specific antibody against *A. hdrophila* was examined. Furthermore, 4, 12 and 23 days post i.v. injection, skin and head kidney (HK) samples were collected from seven random representatives of each group to determinate gene expression of IL-1β, Hsp70, IgM and IgZ1. ELISA for specific antibody against *A. hydrophila* showed no significant differences between experimental groups. Results obtained from cortisol assay showed high stress level in B-Hβg group which was correlated with high level of IL-1β and Hsp70 in skin and head kidney. B-Hβg and B-Lβg groups showed high level of IgZ1 over IgM in skin. Obtained data indicate that the systemic administration of β-glucan affects immunoglobulin expression in carp skin. Moreover, high dose of MacroGard injected intravenously may elevate cortisol level.

Keywords: carp, *Cyprinus carpio* L., β-glucan, i.v. injection, IgM, IgZ

1. Introduction

β-glucans, are well known from their ability to modulate immune system in all organisms from plants to vertebrates, including fish [1-6]. Pharmacologically they are classified as biological response modifiers (BRM; [7]). Depends on the purpose, three ways of administration are available: oral, bath and injection. In the case of fish farms, the most convenient route for antibiotics and immune modulators administration is feed supplementation [3]. On the other hand, bath is an easy and fast route for β–glucans administration to fish in all sizes, and recently, it has been shown that bath in beta glucans supplemented water promotes faster wound closure [3, 8]. Injection is time-consuming and stressful for fish, however it is considered to be the most common and efficient route, due to fact it can reach all internal organs [2]. In fish, the immune system is linked to nervous system, due to double function of head kidney [9]. Fish head kidney is the main hematopoietic and endocrine organ; the major corticosteroid is cortisol, which is produced by the internal cells of the pronefros or head kidney [10-12]. Such organization suggests that the immune processes in fish may be under direct, paracrine hormonal control and, vice versa, chemical signals of the immune system e.g. cytokines could exert direct paracrine action on the interrenal (steroid) and chromaffin (catecholaminergic) cells [12]. That is why immune modulation administered to fish, not only promotes more effective immune response to infectious agents, but also acts upon effects of stress [13]. Teleost fish produce stress signals via their hypothalamus–pituitary–interrenal (HPI) axis that is analogous to the mammalian HPA axis [14]. In response to stressor, fish will undergo a series of biological and physiological changes [15]. In fish, glucocorticoids secretion cause changes in plasma and tissue ion and metabolite levels, hematological features and heat shock proteins (Hsp) and hampers the immune response, resulting in increase of susceptibility to diseases, suppression lymphocytes, macrophages and neutrophils function, decreased antibody production and inflammatory cytokine expression inhibition *in vitro*, [14, 16-25]. Upon duration and magnitude of stress, fish may be affected at all levels, from biochemical and molecular to population, including ultimately survival [26-28].

In the current study we investigated the biological effect of intravenously injected β-glucan on immune and stress response in common carp. Blood samples were collected from all fish to establish if handling and β–glucan injection together affect cortisol level, as elevated cortisol has profound and differential effects on the immune system [19, 29]. Additionally, ratio for specific anty-*Aeromonas hydrophila* antibody was measured to investigate if systemically introduced β–glucan affects B cells in carp blood stream. Furthermore, gene expression of main pro-inflammatory cytokine IL-1β, damage associated molecular pattern (DAMP) and chaperone protein

- heat shock protein 70 (Hsp70), major fish immunoglobulin IgM and recently discovered mucosal immunoglobulin IgZ1 were examined in collected skin and head kidney.

2. Material and methods

2.1. Animals

Sixty-nine, two-year-old common carp (*Cyprinus carpio* L., R3xR8, WUR, The Netherlands, 50 g mean body weight) were kept in 120 L tap water aquariums at 22 ^{o}C and fed a commercial carp feed (Tetra, Melle, Germany) at 2 % of their estimated body weight per day. Fish were exposed to a 12/12 light/dark cycle. All experimental groups were kept in one aquarium. Additional fin clips were made in order to distinguish between treatments.

2.1 Preparation of *Aeromonas hydrophila* bacterin

Aeromonas hydrophila strain BSK10 [30] was cultured in 150 ml of LB medium (Merck, Darmstadt, Germany) overnight at 28 ^{o}C. Subsequently, 15 ml aliquots were spin at 1800 RCF for 10 min. Supernatant was discarded and pellet was dissolved in PBS. A bacterium was killed through heating for one hour at 60 ^{o}C. Aliquots were spin down and pellet was re-suspended in PBS. Subsequently, aliquots were pooled and OD measured at 450 nm determined bacterial concentration. Bacterin at final concentration of 10^6 was injected into fish. Bacterin was plate on agar to confirm no presence of live *Aeromonas hydrophila* cells.

2.2 Preparation of β-glucan

Two doses of MacroGard were chosen: 0,1 g/ml (low dose of β-glucan, Lβg) and 1 g/ml (high dose of β-glucan, Hβg). To obtain high dose of β-glucan, 0.4 g of MacroGard (Biorigin, Sao Paulo, Brasil) was suspended in 10 ml MilliQ water. In order to dissolve the soluble β-glucan fractions, solution was stirred for 1 h at 90 °C and autoclaved (121 °C, 15 min. 1 atm.). To obtain low β-glucan concentration, dilution of high dose β-glucan was made. β-glucan and bacterin solutions were mixed prior to injection.

2.3 Fish and samples recognition

All experimental groups were kept in one aquarium. During sampling procedure individuals were recognized by visual analysis based on scales shape determined by script in MATLAB (The MathWorks Inc., Natick, MA, USA).

2.4 Injection and sampling procedure

Fish were acclimatized one week prior to experiment. Two days before i.v. injection, blood samples were collected from all carp. Additionally, skin and head kidney (HK) samples from seven, randomly chosen individuals were collected. Prior to i.v. injection, fish were anesthetized by immersion in 0.01 % benzocaine solution (VetPharm, Brussels, Belgium). The speciments were divided randomly into three groups of 23 fish and all fish were injected with 100 µl of: 23 fish with 100 µl of bacterin in sterile PBS (B); 23 fish with 100 µl of bacterin + low dose of β-glucan (B-Lβg) and, 23 fish with 100 µl of bacterin + high dose of β-glucan (B-Hβg). Blood samples (300 µl) were collected from all individuals one, 4, 12 and 23 days post i.v. injection from caudal vein. Plasma was obtain after centrifugation at 15000 g x for 5 min. Tissue samples (skin and HK) were collected from seven random representatives of each group, 4, 12 and 23 days post i.v. injection. Samples were collected and kept in RNA later for further analysis. Experiment was conducted according to Danish legislation and by scientists accredited by the Federation of Laboratory Animal Science Associations (FELASA).

2.5 RNA isolation and cDNA synthesis

Sampled tissues (50 mg) were sonicated (30 s, pulse 2, amplitude 70 %, using a soVCX-130 sonicator CiAB, Sweden), as it is considered as more straightforward method than homogenization [Yu C and Cohen LH. Tissue Sample Preparation - Not the Same Old Grind. Sample Preparation Perspectives 2004; 2: 2-6]. Following the manufacturer's protocol, RNA was isolated using GenElute Mammalian™ Total RNA Miniprep Kit (Sigma-Aldrich, Denmark) and subsequently treated with DNase-I (Sigma-Aldrich, Denmark) to remove any genomic DNA. RNA purity and quantity was determined by OD260/280 measurements on a NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Germany). 400 ng of total RNA in a 20 µl, was primed with random hexamers and reverse transcribed using TaqMan® Reverse Transcription reagents (Applied Biosystems, Denmark). The synthesized cDNA was diluted 1:10 in MilliQ water and stored at -20 °C until further analysis.

2.6 Real-time quantitative PCR

Based on sequences deposited in the Genebank (refer to Table 1) and using the program Primer3, PCR primer sets specific for 40S, IL-1β, IgM and IgZ1, were designed [31]. The subunit S11 of the ribosomal gene 40S was selected as a reference gene based on previous work [32]. The assays for examined genes were run using SYBR® Green JumpStart™ *Taq* ReadyMix™ (Sigma-Aldrich, Denmark). Quantitive RT-PCR was performed using a Stratagene MX3000P™ real-time PCR

system (Integrated Sciences, Australia). Master mixes for each PCR run were prepared as follows: 12.5 µl Brilliant SYBR® Green JumpStart™ *Taq* ReadyMix™, 1 µl of each primer and 5.5 µl ultra pure water. Finally 5 µl of diluted cDNA were added to 20 µl of master mix. The cycling conditions for the reaction were: incubation step of 10 min at 95 ^{o}C, followed by 45 cycles of 65 ^{o}C for 30 s and 65 ^{o}C for 1 min. At the end of each reaction, DNA melting curve analysis was performed in order to confirm the specificity of the PCR products. All samples were run in triplicates, and for each primer set, control reactions without cDNA were included in order to detect any non-specific amplification. The expression results were analyzed using the $-2^{-\Delta\Delta Ct}$ method [33]. Data are shown as fold expression relative to group i.v. injected with 100 µl of bacterin in sterile PBS (B), and analysis was carried out using Mx ProTM QPCR software (Integrated Sciences, Australia). The threshold values (Ct) were determined manually for each run. All samples were blind-tested.

2.7 ELISA assay for cortisol

Ninety-six well plates for cortisol ELISA (Demeditec Diagnostics GmbH, Germany) were used following manufacturers' protocol. The absorbance was measured at 450 nm using a Synergy 2 Multi Mode microplate reader (BioTek Instruments, USA). Samples were analyzed in triplicates and blind.

2.8 ELISA assay for detection of *Aeromonas hydrophila*

ELISA assay was used to detect the presence of the antibody against *Aeromonas hydrophila* (BSK10) in the plasma collected from fish. All samples were blind-tested, run in triplicates according to previously described protocol [34]. Prior to samples analysis, standard curve was generated to obtain accurate results in calculating concentrations of unknown samples and working samples dilution of 1:160 was chosen. In the following, all steps in the protocol were carried out at room temperature. Antigen (*A. hydrophila*) was prepared as described in 2.2 section. Initially, the wells of microtiter plates (Immunosorb, Nunc, Denmark) were coated using 200 µl of antigen solution diluted in coating buffer (10^6 bacterin / well) and further incubated at 4 °C overnight. An unbound bacterin was removed by three washes in PBS-Tween. Subsequently, the plates were blocked using 200 µl of 0.5 % bovine serum albumin (BSA) in PBS and shaken for 15 min. Wells were then further washed three times in PBS-Tween and frozen in -20 ^{o}C for further work.

To each well, 100 µl of carp serum diluted 1:160 in PBS with 0.1 % BSA was added and the plates were gently shaken for 30 min. Wells were then washed in order to remove unbound plasma. Further, 100 µl of polyclonal rabbit-anti carp Ig diluted 1:2000 in PBS with 0.1% BSA (Sigma

Aldrich, Denmark) was added to the wells and the plates were gently shaken for 10 min. followed by washing step. Subsequently, 100 µl of polyclonal HRP conjugated goat-anti rabbit antibody (BioRad, Denmark) diluted 1:3000 in PBS with 0.1 % BSA was added to the wells followed by shaking for 10 min. Wells were then washed three times. Lastly, 100 µl of enzyme substrate (TMB; Sigma Aldrich, Denmark) was added to the wells and the plates were incubated for 5 min. The reaction was stopped using 50 µl of stop solution (0.5M H_2SO_4). The absorbance was when measured at 450 nm using a Synergy 2 Multi Mode microplate reader (BioTek Instruments, USA).

2.9 Statistics

A non-parametric Mann-Whitney test was used to compare results from all experimental groups. Differences between groups were regarded as significant at $p < 0.05$. Survival analysis was done with Kaplan-Meier estimator [35].

3 Results

3.1 Mortality

One day post i.v. injection, in group injected with high dose of β-glucan, 26% mortality was observed and raised up to 65% two days post injection (Figure 1). No mortality was observed either in B-injected or B-Lβg-injected group, or further during experiment.

3.2 ELISA assay for cortisol

Values from all experimental groups are presented in Table 2. Significantly elevated plasma cortisol was observed in all experimental groups (B-injected: 463,51 ± 46,90; B-Lβg: 722,86 ± 16,55; B-Hβg: 1158,78 ± 13,80) one day post i.v. injection (Figure 2). The significant difference ($p < 0.001$) in cortisol level between the B-injected and B-Hβg group, as well as between B-Lβg and B-Hβg group ($p < 0.01$) was observed one day post injection (Figure 2). In all experimental groups, cortisol level went back to the basal level 12 days post injection and stayed stable till the end of experiment (Figure 2).

3.3 ELISA assay for detection of *Aeromonas hydrophila*

The level of specific antibodies against *Aeromonas hydrophila* was measured in triplicates and blind, in all experimental groups. Results have shown presence of antibodies against *Aeromonas hydrophila* in all experimental groups, with peak in antibody ratio, four days post i.v. injection. However, no significant difference in antibody titer between treatments was observed (Figure 3).

3.4 Gene expression

3.4.1 Interleukin 1β (IL-1β)

In skin and HK samples collected from B-Lβg group a tendency for up regulation was observed 23 days post i.v. injection (Figure 4A and Figure 4F). In skin and HK collected from B-Hβg high, significant ($p < 0.001$) up regulation was detected 12 days post i.v. injection (Figure 5A and Figure 5F subsequently).

3.4.2 Heat shock protein 70 (Hsp70)

In skin collected from B-Lβg group, no significant changes were observed at any time point, although tendency for up regulation was observed 12 days post i.v. injection (Figure 4B). In HK, significant up regulation was observed four ($p < 0.01$) and 12 days ($p < 0.001$) post i.v. injection (Figure 4G). In skin collected from B-Hβg injected fish, significant ($p < 0.001$) up regulation was observed four and 12 days post i.v. injection (Figure 5B). In HK, significant ($p < 0.001$) up regulation was observed four days post i.v. injection (Figure 5G).

3.4.3 Immunoglobulin M (IgM)

In skin collected from B-Lβg group no significant changes in IgM expression at any time point were observed (Figure 4D). In HK, significant ($p < 0.01$) down regulation was noted 12 days post i.v. injection (Figure 4I). In samples collected from B-Hβg group, significant ($p < 0.05$) down regulation in HK four days post i.v. injection was noted (Figure 5I).

3.4.4 Immunoglobulin Z1 (IgZ1)

High and significant ($p < 0.001$) up regulation was observed 12 and 23 days post i.v. injection in skin and HK samples collected from B-Lβg group (Figure 4E and 4J respectively). High and significant ($p < 0.01$) up regulation was observed four days post i.v. injection in skin and HK samples collected from B-Hβg (Figure 5E and 5J respectively). Significant ($p < 0.001$) IgZ1 up regulation was seen in skin 12 days post i.v. injection (Figure 5E).

4 Discussion

An intriguing difference between mammals and fish is fish tolerance to high concentration of endotoxins and resistance to septic shock [36-39]. In their natural environment fish are in intimate contact with a potentially high amount of microorganisms. If a high inflammatory reaction was triggered after each contact with external putative pathogens, fish simply could not survive [40]. β-glucans are biological extracts and pharmacologically they are classified as biological response

modifiers (BRMs; [7]). The immune modulatory effect of β-glucans has been shown in several studies including fish [2-6, 8, 41]. However, in vertebrates, there are some side and toxicological effects associated with the systemic administration of these agents. Long-term feeding regime of peptido-glucans decreased the immune response in rainbow trout when challenged with *Vibrio anguillarum*, as well as in catfish, indicating a negative feedback effect of β-glucan [42-44]. In the current study we observed 65% mortality two days post injection in B-Hβg-injected group. No mortality was observed either in B-injected or B-Lβg-injected group, or further during experiment. Such high mortality in only one experimental group suggests toxicological, dose-related effect of β-glucan, which is recognized as pathogen associated molecular pattern (PAMP). In vertebrates, effects of immune modulation occur due to increased activity of macrophages, neutrophils and other immunocytes [3, 45-47]. High dose of injected β-glucan together with bacterin could potentially overwhelm the immune system of carp. Moreover, Williams and co-workers has showed in a mice model, that intravenous administration of glucan water-insoluble micro particulates is associated with hypertrophy and hyperplasia of macrophage-rich organs such as liver, lung and spleen [48-50]. Furthermore, many of the BRMs were proved to exhibit significant toxicity in mammals, including vasodilatation, microvascular hemorrhage and circulatory collapse [51-53]. Interestingly, it was reported that if water-insoluble forms of β-glucans are converted to a water-soluble form, the activity of murine immune system is preserved, but the undesirable side effects are eliminated [43, 54].

In the present study i.v. injection with high dose of β-glucan was found to be fish stressor factor which potentially could elevate cortisol level and in subsequently, contribute to high mortality seen in one of the experimental groups (B-Hβg). It is in agreement with results presented by Williams et al., (1988) where i.v. injection of soluble glucan in doses ranging from 40 to 1000 mg/kg did not induce mortality, appearance or behavioral changes in mice or rats [54]. In fact, preliminary studies in that laboratory have indicated that the systemic administration of soluble glucan in the dose of 2000 mg/kg did not induce any side effects in mice and rats (unpublished data). In contrary to that are results presented by Maeda et al. (1984) that showed vascular dilatation and hemorrhage in mice 24 hours following a single i.p. injection of 10 mg/kg [51]. However, in rat model, intraperitoneal injection is being considered more effective route of drugs administration [55]. Results of the present study showed that dose 1 g/ml intravenously injected into carp elevates cortisol level and increase expression of Hsp70. Heat shock proteins, have been recognized among the mammalian primary defense cellular mechanisms during acute stress [56-59]. Moreover, in mammals, Hsp70 is one of the “danger signals” – damage associated molecular patterns (DAMPs), released endogenous structures that can activate immune reaction. [60]. We believe, that faster and higher reaction in B-Hβg injected group is dose-related.

Fish head kidney (HK) is an intriguing link between immune and endocrine systems [10-12]. HK is also main source of macrophages and neutrophilic granulocytes and therefore, actively participates in the clearance of antigens from bloodstream [61,62]. Chadzinska et al., (2008) reported migration of inflammatory leukocytes from HK into peritoneum during intraperitoneal injection of zymosan particles. Constitutive expression of IL-1β in peritoneal leukocytes (PTL) was two-fold higher than in HK, however during zymosan-induced peritonitis, IL-1β expression showed twice higher expression in peritoneal leukocytes (PTL) than in HK 6 hours after injection. IL-1β peak in HK was observed 96 hours post injection [63]. In our study, we observed a tendency for IL-1β up-regulation in B-Lβg injected group, and significant up-regulation 12 days post injection in B-Hβg injected group. Besides HK, we measured IL-1β expression in skin and raising overtime expression in B-Lβg, as well as high expression in B-Hβg group, could strengthen hypothesis of leukocytes leaving HK and locating other tissues. Moreover, fish from B-Hβg injected group appeared to have skin and muscle damage (personal observation) which could enhance IL-1β expression in response to DAMPs. On the other hand, *in vitro* studies in common carp showed inhibition of inflammatory cytokine due to acute stress, to prevent damage due to an excessive inflammatory response [64]. In mammals, corticosteroids often have inhibitory effect on monocytes and macrophages, what could explain no changes in IL-1β expression [65]. *In vitro* study showed that proliferation of RTS11, a monocyte/macrophage rainbow trout cell line is inhibited by cortisol [66,67]. As macrophages are main source of IL-1β, inhibition in their proliferation could effectively inhibit IL-1β expression. Furthermore, it cannot be excluded that, IL-1β is main pro-inflammatory cytokine and reaches peak within minutes after stimulation, so measurement 96 hours after stimulation does not give clear picture.

Aeromonas hydrophila is the most common Gram-negative bacteria present in all freshwater environments and causes a wide range of diseases in fish [30]. There are more than 120 serotypes of *A. hydrophila* described, however it does not usually cause problems in fish populations under normal conditions: infection with *A. hydrophila* in fish appears as a secondary infection, under environmental or physiological stress, injury or infection with other pathogens [68, 69]. It is in accordance with work presented by van der Marel and co-workers (2010) where water with increased bacterial load did not induce clinical symptoms in carp, however rapid skin mucosa respond have been seen, even if the bacteria involved were considered to be non-pathogenic [70]. The present study revealed that i.v. injection of dead *A. hydrophila* bacteria alone or together with β-glucan did not elevate production of antibodies against *A.hydrophila* as was originally expected. Handling, injection and dose of injected β-glucan caused stress reaction in all groups, and we expected it would have an effect on specific antibody production. According to Selvaraj et al. (2005) the minimum amount of glucan required for enhanced production of antibodies against

A.hydrophila was 500 µl/ fish [71]. Following that, we expected group injected with 1 g/ml/ fish, to have significantly higher antibody level in comparison to bacterin or 0,1 g/ml/ fish injected group. Surprisingly, the basal level of antibody (2 days pre to i.v. injection) was high in all three groups, with no difference among them. This draws a conclusion of fish being previously primed with *A.hydrophila* and producing high amount of antibody. Elevation in specific antibody level four days post i.v. injection, could be a short-term alert in response to injected PAMPs (bacterin and β-glucan) and slightly affect antibody production.

Up to date, only IgM has been shown to be functionally involved in protective immunity in fish [72,73]. B cells comprise 25-35% of all blood lymphocytes in rainbow trout, 50% in puffer fish and 70% in common carp [11,74,75]. Recently described IgT/IgZ seem to be involved in fish mucosal immunity [6, 76, 77]. Ryo et al. (2010) have shown that the highest induction of IgZ1 mRNA expression was seen during the blood parasite infection*, T. borreli*, as well as during vaccination with *V. anguillarum* antigens, which are rich in lipopolysaccharides [77]. The same authors reported that IgZ1 functions as antibody that neutralizes foreign antigens in the fish blood [77]. It could explain high expression of IgZ1 in current study – if mechanisms triggered by LPS and β-glucan are alike, IgZ1 level should increase to clear circulation from injected β-glucan. In teleost, IgM and IgZ are mutually exclusive on B-cells as reported by Li et al (2010; [78].). More evidence is accumulating that, the antibodies in the mucus, although antigenically identical to the serum Igs, may differ in other aspects. Lobb and Clem (1981b) identified three types of Igs in the mucus of the *Archosargus probatocephalus* sheepshead bile: one type of tetrameric high molecular weight Ig, identical to the serum counterpart and two types of low molecular weight dimeric Ig [79]. The existence of three different types of Igs seems to suggest three distinct ways of producing them, systemically or locally. Our results indicate absence of IgM expression, with high IgZ1 expression in skin and HK samples collected from B-Lβg injected group. High expression of IgZ1 in skin, with lower but significant up-regulation in HK, indicates presence of resident B-cells and/or antibody-secreting cells (ASC) in skin of common carp, which were not affected by high cortisol level. Moreover, such a strong expression in skin indicates systemic response of these cells to injected PAMPs. It is strengthen by fact, that further experiments in our laboratory have demonstrated (data not showed) that fish gained “tolerance” and were able to survive i.v. injection with high dose of β-glucan.

Three routes of antibiotics, vitamins, immune modulators and others administration are possible: injection, oral and bath. The best one should be chosen depends on the aim of experiment. However, we can observe an interesting pattern when it comes to beta glucans administration in common carp. Despite of the administration route, effects can be observed in carp skin. Van der

Marel et al. (2012) have reported that β-glucan feeding regime had an effect on the expression of mucus-related genes in carp. In addition to the mucin encoding genes, β-glucan feeding affected the expression of carp β-defensins genes, which were significantly higher when compared to group fed without β-glucan [80]. This suggests that, by feeding β-glucans, not only gut mucosal system is affected, but also changes in skin mucosa might be an important to monitor fed-related improvement of fish health. Recently, Przybylska et al. (submitted) showed that β-glucan enriched bath, specifically MacroGard and 6.3 kDa oat fiber have a direct positive effects on the wound closure in common carp and promote faster wound healing compared to non-treated fish [8]. Results of the current study show that intravenous injection of β-glucan, once again affects skin mucosa.

In conclusion, we show that effect of direct β-glucan stimulation by intravenous injection in common carp is rigorously correlated with dosage. Moreover, high amount of particulate β-glucan introduced to circulation system of carp elevates cortisol level, and at high dose can be toxic for fish. Furthermore, systemic administration of β-glucan affects immunoglobulin expression in carp skin with immunoglobulin switch-like process, where presence of main fish immunoglobulin IgM is overtaken by recently described, mucosal immunoglobulin IgZ1.

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Figure legends

Figure 1. Kaplan-Meier curve of survival percentage among all groups during experiment period. B - bacterin injected group; B-Lβg - bacterin + low dose of β-glucan injected group; B-Hβg - bacterin + high dose of β-glucan injected group.

Figure 2. Plasma cortisol concentrations for B-injected, B-Lβg-injected and B-Hβg-injected group. The results are shown as mean (n=7) ± standard deviation (SD). Significantly elevated level of plasma cortisol was observed in all experimental groups. The significant difference (Δ) in cortisol level between the B-injected and B-Hβg groups was observed one day post injection. At the same time point, significant difference (***) between B-Lβg and B-Hβg group was noted. B - bacterin injected group; B-Lβg - bacterin + low dose of β-glucan injected group; B-Hβg - bacterin + high dose of β-glucan injected group. *** $p < 0.001$; Δ depicts significant difference between B and B-Hβg injected groups.

Figure 3. Antibodies against *Aeromonas hydrophila*. The results are shown as mean absorbance at 450 nm + standard deviation (SD). Results have shown presence of antibodies against *Aeromonas hydrophila* in all experimental groups, although no significant differences in antibody titre between treatments were observed.

Figure 4. Quantitative real-time PCR in group injected with bacterin + low dose of β-glucan (B-Lβg). Expression for genes interleukin 1β (IL-1β), Heat shock protein 70 (Hsp70), immunoglobulin M (IgM) and immunoglobulin Z1 (IgZ1) in skin (A-D) and head kidney (E-H) of common carp. Results are obtained by RQ-PCR and expressed relative to B-injected group at each time point. The data are normalised relative to 40S and analysed using the $-2^{-\Delta\Delta Ct}$ method. Bars represent fold expression (n = 7) + SD. * depicts significant differences in comparison to B-injected group (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

Figure 5. Quantitative real-time PCR in group injected with bacterin + high dose of β-glucan (B-Hβg). Expression for genes interleukin 1β (IL-1β), Heat shock protein 70 (Hsp70), immunoglobulin M (IgM) and immunoglobulin Z1 (IgZ1) in skin (A-D) and head kidney (E-H) of common carp. Results are obtained by RQ-PCR and expressed relative to B-injected group at each time point. The data are normalised relative to 40S and analysed using the $-2^{-\Delta\Delta Ct}$ method. Bars represent fold expression (n = 7) + SD. * depicts significant differences in comparison to B-injected group (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$).

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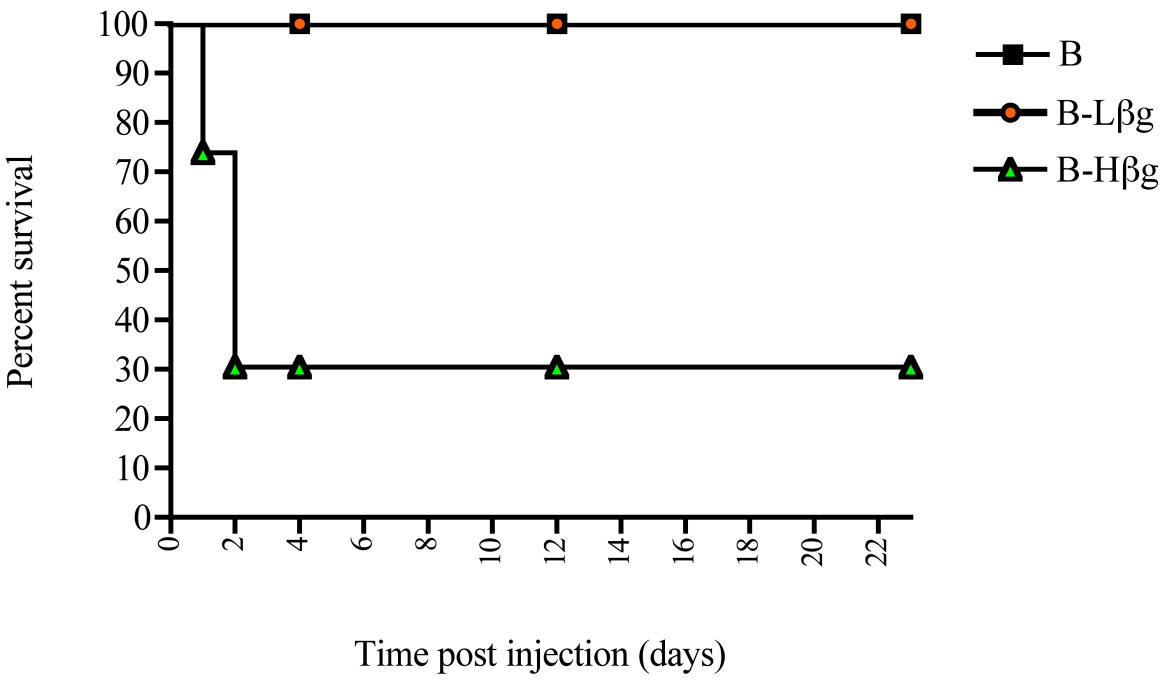
Percent survival
100
90
80
70
60
50
40
30
20
10
0
0
2
4
6
8
10
12
14
16
18
20
22
Time post injection (days)
B
B-Lβg
B-Hβg

Figure 1.

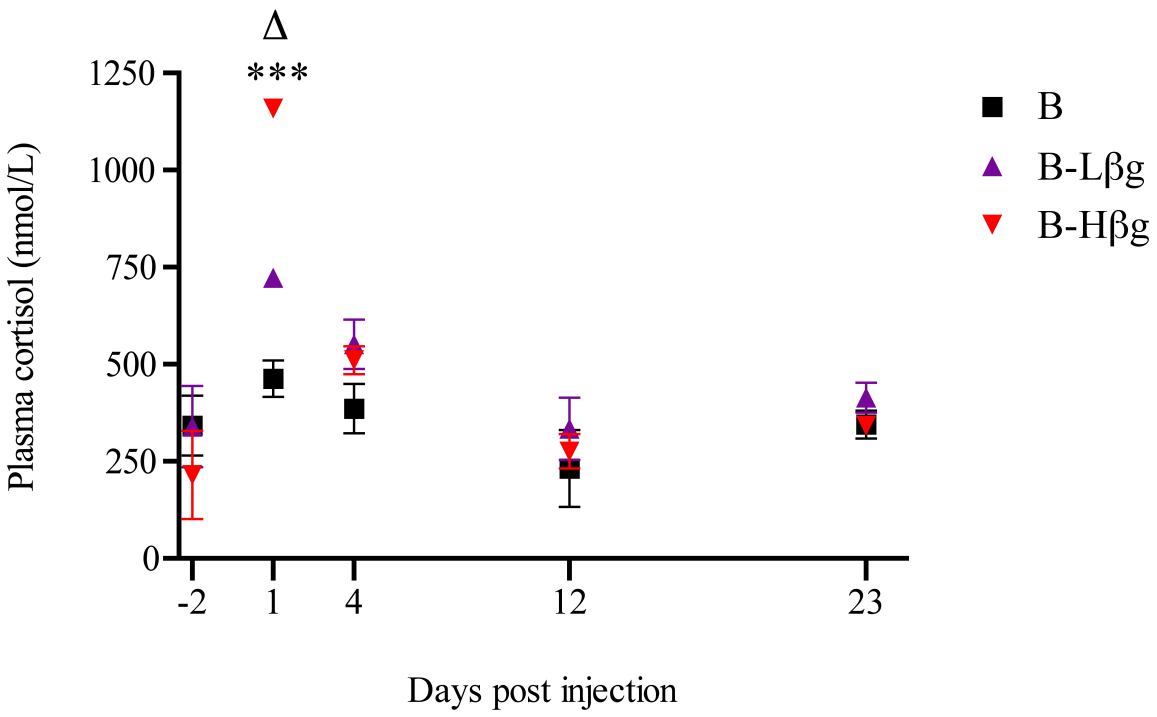
Δ
***
1250
1000
750
500
250
0
Plasma cortisol (nmol/L)
-2
1
4
12
23
Days post injection
B
B-Lβg
B-Hβg

Figure 2.

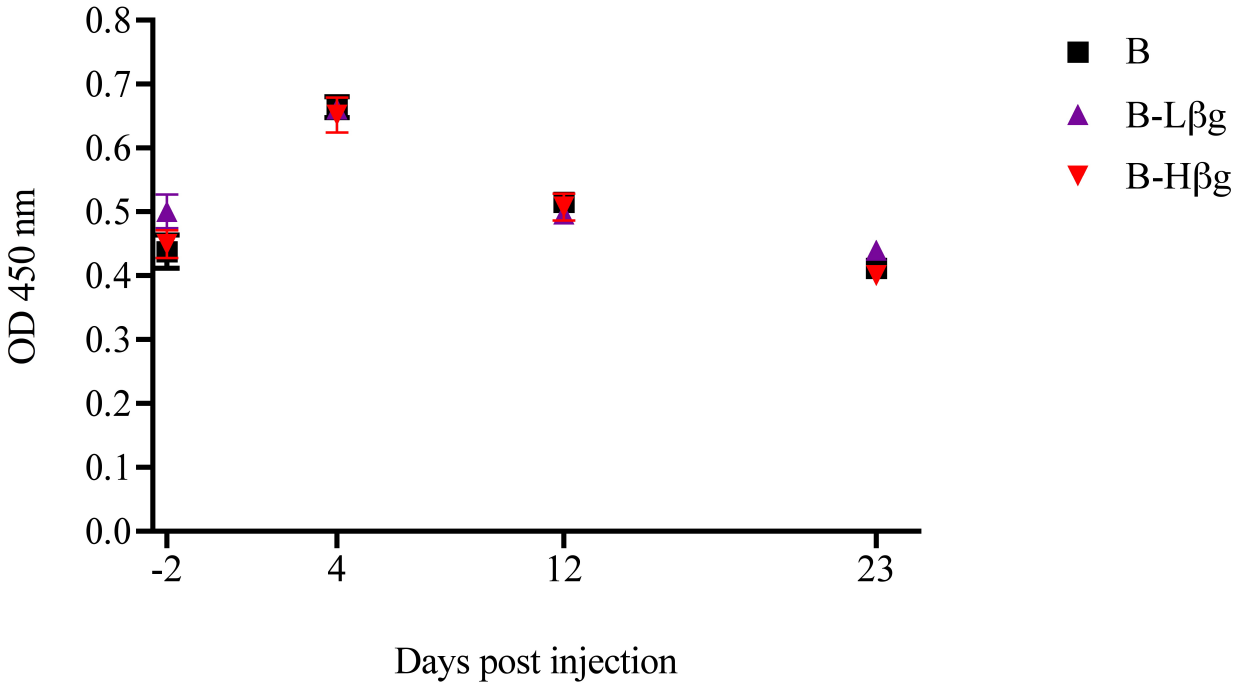
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
OD 450 nm
-2
4
12
23
Days post injection
B
B-Lβg
B-Hβg

Figure 3.

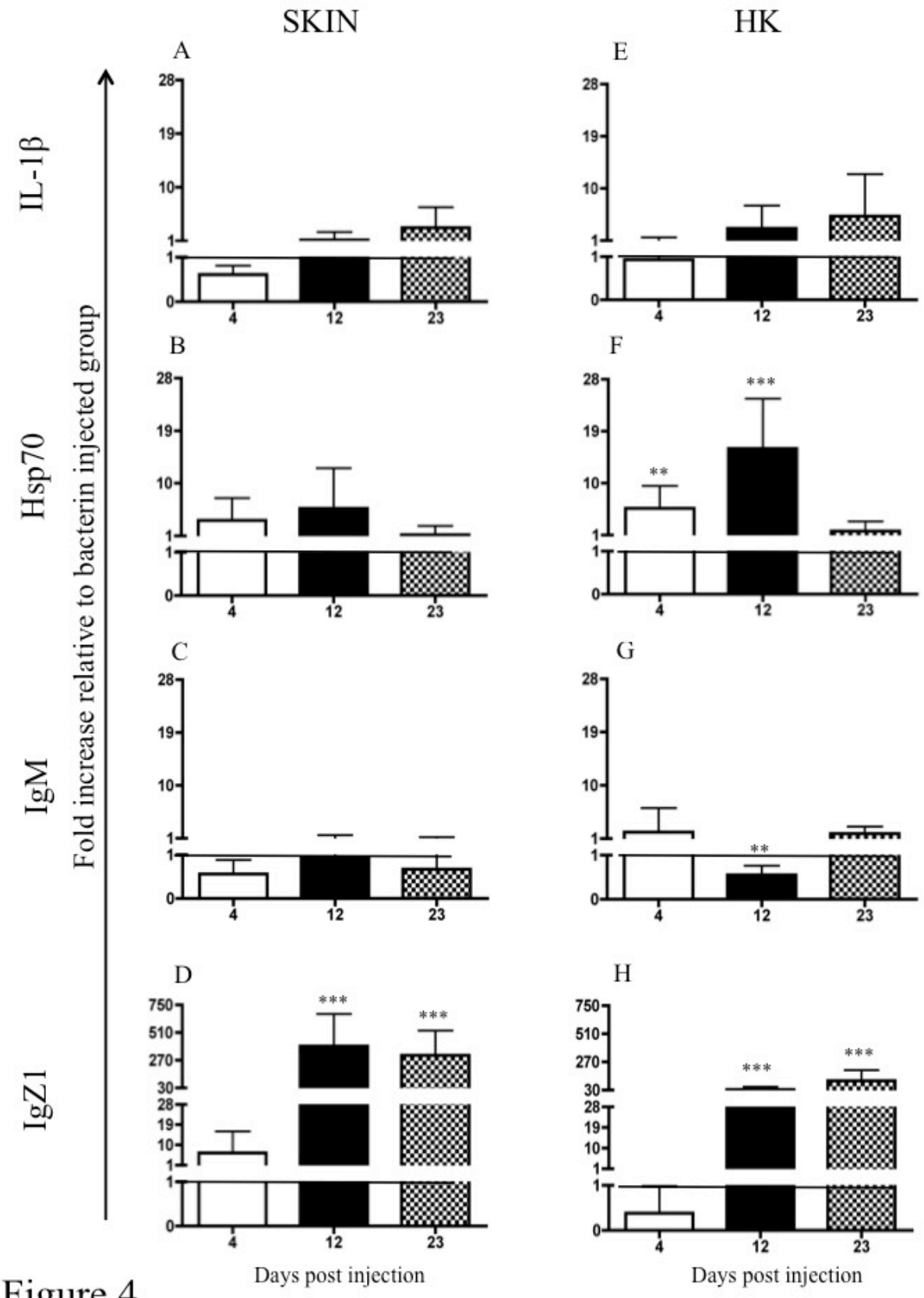
SKIN
HK
A
E
B
F
C
G
D
H
IL-1β
Hsp70
IgM
IgZ1
Fold increase relative to bacterin injected group
Days post injection
Days post injection

Figure 4.

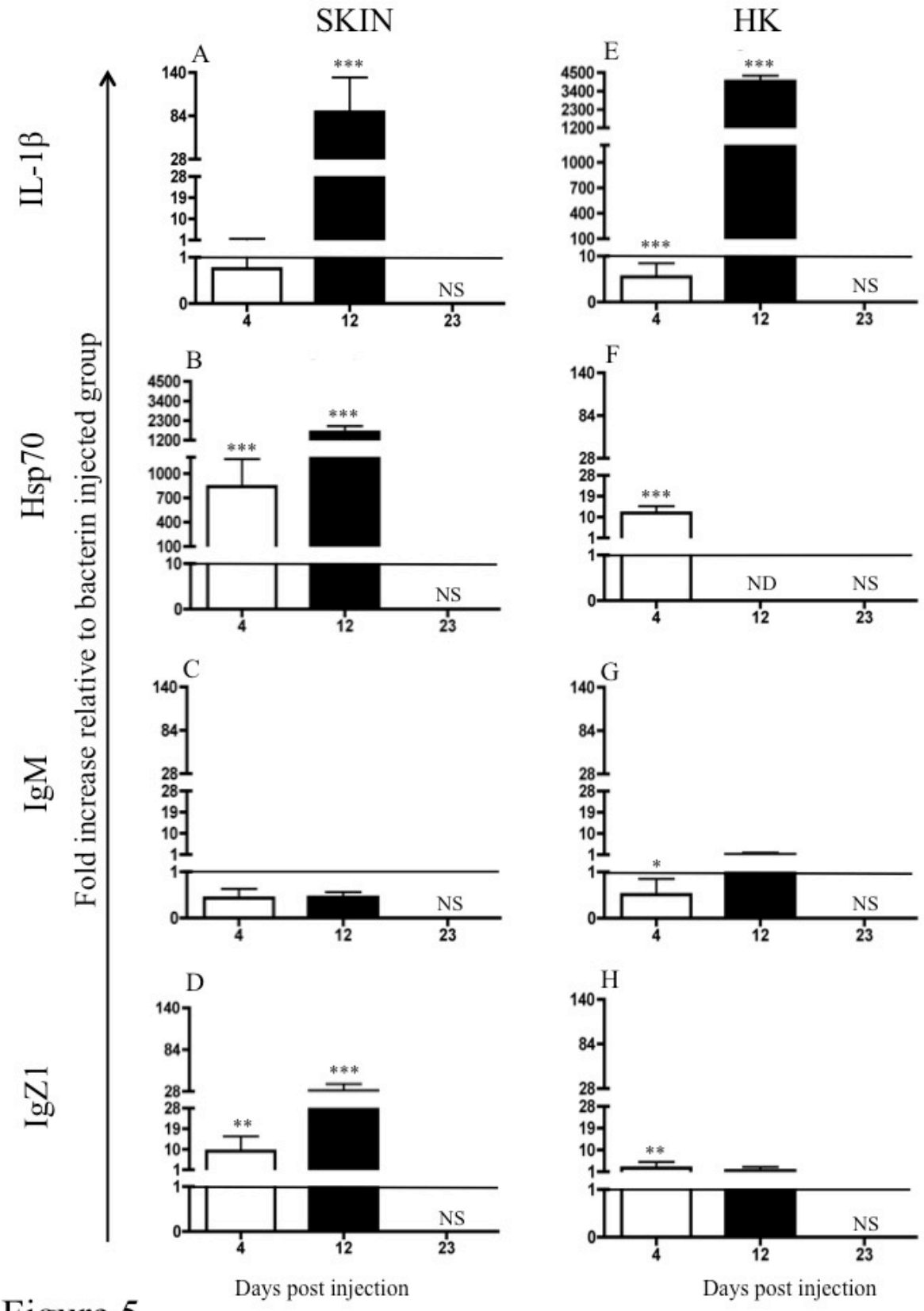
SKIN
HK
IL-1β
Hsp70
IgM
IgZ1
Fold increase relative to bacterin injected group
A
B
C
D
E
F
G
H
***
**
*
NS
ND
4
12
23
Days post injection
Days post injection

Figure 5.

Table 1. Sequences of primers used for real-time PCR.

Gene	Primer	Sequence (5' – 3')
40S	Forward	GTTGAAGGAAGTGGCAAGGA
	Reverse	AGAATACGGCCTCTGATGGA
IL-1β	Forward	AAGGAGGCCAGTGGCTCTGT
	Reverse	CCTGAAGAAGAGGAGGCTGTCA
Hsp70	Forward	GGCAGAAGGTGACAAATGCA
	Reverse	TGGGCTCGTTGATGTTCTCA
IgM	Forward	CACAAGGCGGGAAATGAAGA
	Reverse	GGAGGCACTATATCAACAGCA
IgZ1	Forward	ACTCCCTGGTGTGTGACCTC
	Reverse	TACAAACAGCATGAGCCAGCTA

Table 2. Plasma cortisol (nmol/L)

	Days post injection					
	-2	**1**	**4**	**7**	**12**	**23**
B	342,12 ±77,25	463,51 ±46,9	386,26 ±63,46	813,91 ±19,31	231,76 ±99,30	344,86 ±35,87
B-Lβg	339,36 ±104,84	722,86 ±16,55	551,8 ±63,46	800,11 ±22,1	333,84 ±80	413,85 ±38,63
B-Hβg	215,2 ±113,12	1158,78 ±13,8	510,42 ±35,8	744,93 ±13,8	257,9 ±44,14	342,12 ±8,28

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