Apoptosis and its association with immunomodulation and disease in common carp (*Cyprinus carpio* L.)

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Abstract

Stimulating the immune system of fish by oral administration of immunomodulatory substances can prevent disease outbreaks in aquaculture. Yeast β-(1,3/1,6)-glucan, the active ingredient of the commercially available feed supplement MacroGard®, has been associated with production of microbicical and cytocidal oxygen radicals and the induction of apoptosis in human cancer cells. Hence it was hypothesized that the immunosuppressive effects of this substance, which were observed by some authors, could be caused by induction of apoptosis in immune cells due to oxidative stress. Utilizing molecular and immunohistochemical staining techniques it has been shown that although MacroGard® can induce apoptosis *in vitro* it is not associated with this form of cell death *in vivo*. However dietary MacroGard® influences the expression of apoptosis-related genes in a time and organ dependent manner.

Apoptosis is also associated with disease and can be modulated by both the host as a means of controlling infection, and by pathogens in an attempt to avoid the host immune system. It was thus hypothesized that bacteria (*Aeromonas salmonicida*) and viruses (koi herpes virus (KHV) and spring viremia of carp virus (SVCV)) can modulate apoptosis in carp and that this can be affected by oral immunostimulation. In this thesis it was established that the bacterial pathogen *A. salmonicida* and the SVC virus induce apoptosis and that this is associated with changes of apoptosis-related gene expression. KHV in contrast appeared to supress apoptosis during early stages of the infection but induced it during the later stages possibly as a means to disseminate the virus. MacroGard® enhanced gene expression in response to SVCV infection and exposure to virus- and bacteria-associated molecular patterns (i.e. Poly(I:C) and LPS). In conclusion, MacroGard® can

influence apoptosis-related gene expression but does not appear to induce apoptosis on its own.

Contribution to work conducted

The work presented in this thesis has been conducted within the EU funded ITN Network NEMO and the studies described in chapter 5 – 7 have been conducted in collaboration with partners from this project. Below it is stated in which form the collaborators contributed to the work presented here.

Joanna Miest conducted all analyses of apoptosis (immunohistochemistry and molecular) described in this thesis and Professor Dave Hoole provided constant helpful advice throughout all experiments presented in this thesis. Dr. Alberto Falco assisted in the setup of the molecular analysis of apoptosis-related genes.

The *in vitro* work conducted in chapter 3 was performed with advice from Prof. D. Hoole, Gwyn Williams and Mirna Mourtada-Maarabouni and was carried out by J. Miest.

Chapter 4 described a feeding and PAMP exposure study which was conducted at Keele University. During this study J. Miest, Dr. A. Falco and Nicolas Pionnier were involved in the experimental design, fish husbandry and execution of the experiment. Injections were performed by Prof. D. Hoole. Sampling of fish was carried out by Dr. A. Falco and N. Pionnier, while J. Miest analysed the apoptosis in pronephric cells. Preparation of samples for molecular analysis was carried out jointly by J. Miest and Dr. A. Falco.

The work described in chapter 5 was carried out at the Institute of Ichthyobiology & Aquaculture of the Polish Academy of Sciences, in Golyz, Poland. The experiment was designed by Dr. A. Falco, J. Miest, N. Pionnier, Patrick Frost and Dr. Ilgiz Irnazarow. Fish husbandry and feeding was carried out by Patrick Frost and bacterial injections were performed by Mikolaj Adamek and Graham Brogden. Fish were sampled by Dr. A. Falco and M. Adamek. Morphological analysis of apoptosis was performed by J. Miest.

Preparation of organ samples for molecular analysis was carried out equally by Dr. A. Falco and J. Miest.

Five weeks were spent at the Veterinary University Hanover in Germany and the work conducted there is described in chapter 6. During this time Prof. Dieter Steinhagen and M. Adamek contributed to the experimental design and M. Adamek was involved in the setup of the Flow Cytometer and the antibody staining against SVCV and KHV. He was also responsible for infecting the cells with the viruses and prior to the experiment cells were cultured by Birgit Lockhart. The experiment including MacroGard® exposure, sampling, acridine orange and CaspaTag staining, flow cytometry and in part SVCV/KHV antibody staining was carried out by J. Miest. Sample preparation for the molecular analysis and the analysis of gene expression was carried out by J. Miest. M. Adamek in addition assisted with the primer design for Caspase 9.

The first part of chapter 7 described an experiment conducted at National Veterinary Research Institute (NVRI) Pulawy, Poland. This project was designed by J. Miest and M. Adamek and carp were organized by M. Adamek. Fish husbandry and MacroGard® feeding was carried out by the staff of the NVRI. Viral infection was carried out by M. Adamek and Dr. Marek Matras. Sampling was carried out by M. Adamek, N. Pionnier and J. Miest and apoptosis was analysed by J. Miest. Virus load in the samples was carried out by M. Adamek.

The second KHV infection study described in chapter 7 was carried out by Kristof Rakus, M. Adamek and others and organ samples were provided by M. Adamek. Samples were prepared for molecular analysis mainly by Joanna Miest.

The analysis of the KHV genome for viral Bcl-2 was conducted by J. Miest in discussion with M. Adamek.

Acknowledgements

First of all I would like to thank Prof. Dave Hoole for giving me the opportunity of conducting this thesis and for his never-ending patience, support and advice. Dave had always an encouraging word even when challenges (not problems) needed to be tackled and especially during these last months he has been a bastion of calm and an incredible quick proof-reader. I will also fondly remember the many social hours spend at Dave's and Jan's home and his rich pool of stories from all over the (science) world.

I would also like to thank Prof. Gwyn Williams and Dr. Sheila Hope, who have supervised and advised me over the last years. They always managed to open up new discussion points and research ideas by sharing their expertise with me.

I would also like to thank my examiners Dr. Anne Loweth and Prof. Alexandra Adams for a fair and lively discussion during my viva.

I have been very lucky to be part of the NEMO network, which has been a truly fantastic team. Not only have I made great friends but I also have to thank Geert Wiegertjes, Michael Nielsen and Dieter Steinhagen for sharing their expertise and to always care about us young scientists. I have to thank the team in Golyz (Ilgiz Irnazarow, Patrick Frost etc.) for assisting and helping us with the *Aeromonas* experiment and the Hanover team (Dieter Steinhagen, Mikolaj Adamek) for their advice and help during my stay in Hanover and Pulawy. I especially have to thank Mikolaj for his assistance and help with the virus studies and for always pushing (*praca, praca!!*). I cried, I was ill, but it was worth it. Thank you also for the encouragement, jokes and the many discussions on science and non-science.

In Keele I was fortunate to work in a great team with Dr. Alberto Falco and Nicolas Pionnier. Alberto introduced me to molecular research ("First step: Turn radio on") and has

always been a great help and a fantastic colleague. I have to thank Nicolas for his assistance during the fish sampling processes and travels and his wonderful French accent. I also would like to thank the technicians at Keele University for their support and for keeping our fish fit and healthy and a big thank you also goes out to Mirna Mourtada-Maarabouni for introducing me to the apoptosis stainings.

Before I came to Keele I was apprehensive if I would find friends but I shouldn't have worried. Everyone here was extremely welcoming and I have met some lovely friends. Thank you Lynsey, Amy, Clare and Doug for the many cups of tea, the chats, and for always being there. I will miss you guys!

Dan (meu macaco), you have managed to keep me as sane as possible during the last years and especially during the last months no matter how far apart we were. You did not mind putting up with my endless thesis worries, statistic questions, my moods when you told me to re-write a day's work and you would then still happily read my new version. Thank you for always encouraging and supporting me in everything I wanted to do (even though you now have to learn German) – and you always make me laugh.

I would like to blame my family for this thesis. Probably since I was a baby they have always encouraged my thirst for knowledge and my interest in nature. As they believe that one is only good in what one loves they have always supported me during my studies and without it I might have strayed off the science path and tried and probably failed to do something safer. My parents have always been my stronghold and have always kept my feet on the ground and my head above the water (and sometimes in the clouds).

I therefore dedicate this thesis to my parents Anne and Jochen, without your love I would not be where and who I am today, and to my late grandfathers, who have been such a great inspiration. Danke!

Contents

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Abbreviations

aa	amino acid
ANOVA	analysis of variance
Apaf-1	apoptosis protease activating factor-1
Bcl-2	b-cell lymphoma-2
BH	bcl-2 homology domain
BLAST	basic local alignment tool
bp	base pair
CCB	*Cyprinus carpio* brain cell line
cDNA	complementary DNA
CLC	carp leucocyte cell line
CO_2	carbon dioxide
Ct	threshold cycle
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
FADD	fas associated protein with death domain
FITC	fluorescein isothiocyanate
FS	forward scatter
g	gram(s)
GALT	gut associated lymphoid tissue
h	hour
i.p.	intraperitoneally
IAP	inhibitor of apoptosis family of proteins

IL	interleukin
iNOS	inducible NO synthase
J	joule(s)
KHV	koi herpes virus
LPS	lipopolysaccharide
M	molar
Mcl-1	myeloid cell leukemia-1
min	minute(s)
ml	mililiter(s)
mRNA	messenger RNA
ng	nanogram(s)
nm	nanometer(s)
NO	nitric oxide
ORF	open reading frame
p.i.	post infection/injection
PAMP	pathogen associated molecular pattern
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PI	propidium iodide
Poly(I:C)	polyinosinic:polycytidylic acid
qPCR	quantitative PCR
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Roswell park memorial institute 1640

RT-PCR	reverse transcription PCR
s	second(s)
SEM	standard error of means
SS	Side scatter
SVCV	spring viremia of carp virus
TNF-α	tumor necrosis factor α
UV	ultraviolet
µl	microliters
Mm	micrometers

1 General introduction

According to the report of the Food and Agriculture Organization of the United Nation (FAO) in 2012 a new all-time high of annual fish consumption of 128 million tons produced by capture fisheries and aquaculture had been reached. This increasing demand reflects the input of a rapidly expanding aquaculture sector (6 % annual growth) (FAO, 2012).

In freshwater aquaculture the common carp is the dominating fish species. In 2010 72 % of the fresh water fish produced were Cyprinids, which equals an annual production of 24.2 million tons (FAO, 2012). The common carp (*Cyprinus carpio* Linnaeus 1758) belongs to the order *Cypriniformes* and is probably the best known teleost in the world. Carp and their cyprinid relatives have important roles as food fish, in the ornamental sector (koi *Cyrpinus carpio koi* and goldfish *Carassius auratus*) and as sport fish. The omnivorous carp is a very robust fish tolerating wide ranges of temperature and salinity (Billard 1999). Hence carp is one of the most valuable cultured species and this makes research on carp aquaculture scientifically and economically important.

An important factor that is significantly restricting aquaculture production is the presence of disease, which can spread rapidly between farmed fish and cause high animal mortalities and economical costs. In aquaculture fish are farmed at high densities, which not only allow pathogens to spread more easily from animal to animal but these high densities are also a major stressor in fish production systems (Iguchi *et al.*, 2003; Cabello, 2006). High densities together with other stress factors such as handling and temperature changes, which occur in a seasonal pattern, render the animals more susceptible to diseases

and facilitate disease outbreaks (Yin *et al.*, 1995; Saeij *et al.*, 2003c). Research into the prevention of such diseases is therefore urgently needed. In the past chemical and antibiotic treatments have been extensively used. However due to their potential to increase pathogen resistance and cause environmental problems their use is controversial. Hence the prevention rather than cure of disease is favoured and the possibility of enhancement of the immune response and the induction of immune protection has made significant advances over the last few decades through the increase in knowledge of the fish immune system. Until recently most of the research activities have concentrated on acquired immunity and the associated development of protective vaccines. However there is now a realisation that the use of immunostimulants to increase general resistance via the innate immune response may prove a promising weapon in our arsenal to protect fish against disease and thus improve fish production and economic profitability within the aquaculture and ornamental sectors. Hence the use of immunostimulants may be an alternative to the development of vaccines, which is time consuming and expensive.

One important aspect of the pathology induced by a pathogen, which is fundamental to the manifestation of the disease, is their influence on cell survival of both non-immune cells and immune cells (section 1.4). Such an association has important implications in the induction of immune responses by immunostimulants which themselves may induce cell death (section 1.5). This introduction will first focus on the innate immune system (section 1.1) and how it can be influenced by immunostimulating substances (section 1.2). In addition apoptosis (section 1.3) and how this process can be modulated by disease will be considered (section 1.4). The introduction concludes with the aims of this thesis.

1.1 The fish's immune system

In higher vertebrates the immune system comprises two components, a nonspecific (innate) and a specific (acquired) immune response. The innate immune response generally precedes the adaptive response, activates and determines the nature of the latter and co-operates in the maintenance of homeostasis. Invertebrates possess only the innate immune system whilst fish are the first vertebrate group that have both systems (Magnadóttir, 2006).

Plouffe *et al.*, (2005) suggested that the adaptive immune response of fish is less developed than that of higher vertebrates. This probably reflects the evolutionary status and the poikilothermic nature of the acquired immune response in fish which displays slow lymphocyte proliferation and a limited range of antibodies compared to mammals. The acquired immune response can take up to 12 weeks to be fully responsive whilst the innate system responds instantly to an invading pathogen (Magnadóttir, 2006). It is also known that the innate immune parameters are relatively temperature independent whilst the components of the acquired immune systems require comparably higher temperatures to function (Magnadóttir, 2006). Innate immunity hence plays a much more important role in resistance in fish than in homeothermic vertebrates (Jones, 2001).

1.1.1 Immune organs

Unlike mammals, teleosts do not possess bone marrow and lymph nodes, and therefore haematopoiesis must occur in another distinct organ. The anterior part of the kidney, also termed head kidney or pronephros, which has lost the excretory function and lacks nephrons, has been identified as the organ of haematopoiesis in teleosts (Zapata *et*

al., 1997) and is also the major organ for phagocytosis, antigen processing and the formation of immune memory (Tort *et al.,* 2003). Leucocytes such as lymphocytes develop, mature and reside in the pronephros (Figure 1.1), which with the spleen is involved in trapping antigens from the bloodstream (Press and Evensen, 1999). Mature lymphocytes migrate to antigen presenting sites in the posterior kidney and spleen (Zwollo *et al.,* 2005), where they are activated and differentiate into plasmablasts and plasma cells (i.e. antibody secreting B cells). These plasmablasts then migrate back into the pronephros and become long-lived plasma cells (Zwollo *et al.,* 2005). The spleen and gut-associated lymphoid tissue (GALT) act as the main secondary lymphoid organs. However, the pronephros has been shown to be a major site of antibody production, and hence has been suggested to play a major role in the immune response (Zapata *et al.,* 1997; Press and Evensen 1999). T lymphocytes, which are involved in the specific immune response are produced in the thymus, which is another lymphoid organ (Tort *et al.,* 2003).

In contrast to mammals, which possess well-organized lymphoid aggregates in the gut (i.e. Peyer's Patches), teleosts have isolated lymphoid cells in the lamina propria and the intestinal epithelium. This GALT appears along the gut and consists of non-encapsulated accumulations mainly of lymphocytes (T and B cells), macrophages, and plasma cells, and different types of granulocytes (Zapata *et al.,* 1997). The gut can be divided into three segments: the first segment (60 – 75 % of the gut length) is responsible for the uptake of food proteins, the second gut segment, i.e. 20-25 % of the gut length, is probably not involved in the nutritional functions but has the ability to endocytose macromolecules and to transport them to macrophages situated in the lamina propria and the circulation. The third segment (5 – 15 % of the gut) seems to have osmoregulatory rather than nutritional functions (Press and Evensen, 1999; Rombout *et al.,* 2011). Of these

three gut segments the second one is most important for the immune response since it is involved in the transport of antigens to immune cells, e.g. phagocytes. These phagocytic cells are mobile and resident macrophages, located in and under the intestinal epithelium. Such leucocytes are also found in the gills (Press and Evensen 1999) and phagocytosis of antigens can then evoke a local and systemic immune response (Rombout *et al.*, 2011).

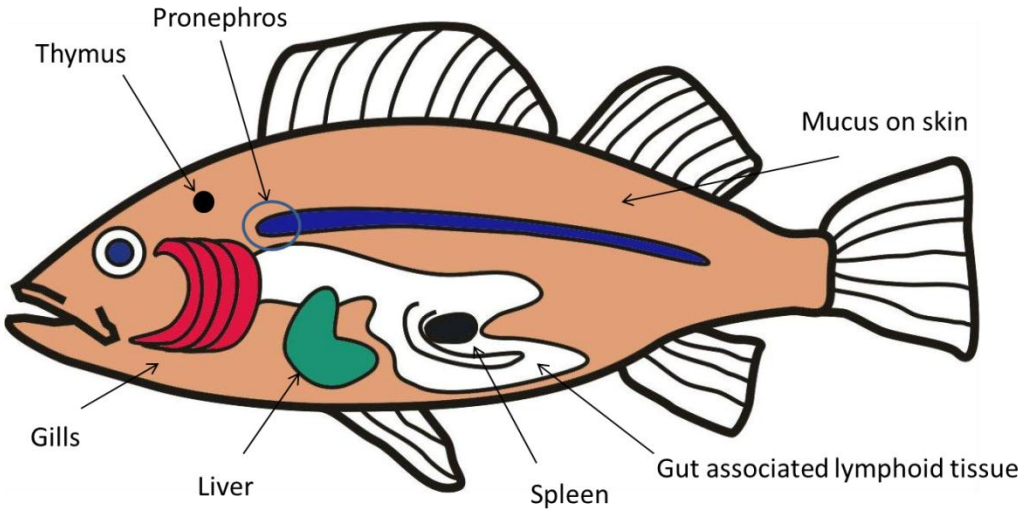

Figure 1.1: Immune organs of carp

Organs that show immunological functions in carp are the thymus, pronephros, spleen, liver, gut associated lympoid tissue (GALT) and also the gills and the mucus on skin. Adapted from Tort *et al.*, (2003).

1.1.2 Innate immunity

The innate immune system is responsible for the initial discrimination of pathogens from self and can be divided into physical barriers, cellular and humoral components. The physical barriers include mucus and epithelial tissues which cover the skin, gills, stomach, and gut. These are the first obstacles a pathogen has to cross prior to infection (Magnadóttir 2006). Once the pathogen breaches this defence and enters the host it encounters and activates the humoral and the cellular innate immune components. The former comprises amongst others anti-bacterial peptides, proteases, complement, transferrins and the antiviral myxovirus resistance-1 protein (Mx1). The role of this humoral response is to destroy or inhibit the growth of invading pathogens (Aoki *et al.*, 2008).

The cellular response mainly depends on the recognition, phagocytosis and intracellular killing of the pathogen. Various cell types are involved in this response and in teleosts this includes non-specific cytotoxic cells (NCCs), macrophages and neutrophil, eosinophil and basophil granulocytes (Iwama and Nakanishi 1997). NCCs are involved in the lysis of various target cells and in the immune response against protozoan parasites. In mammals these cells, which are found in the lymphoid tissue, blood and gut, are thought to be the precursors of natural killer cells. They have also been identified in fish, e.g. carp (*C. carpio*) and rainbow trout (*Oncorhynchus mykiss*) (Iwama and Nakanishi 1997; Plouffe *et al.*, 2005). Macrophages are phagocytes derived from blood monocytes and they are involved in all stages of the immune response. In general macrophages have two major roles: firstly to phagocytose and digest disease-associated material like bacteria and parasites and secondly the uptake and presentation of antigens to specific T cells (Benjamini *et al.*, 2000). In fish they can be found in blood, lymphoid organs (particularly

the kidney) and the peritoneal cavity (Iwama and Nakanishi, 1997). Granulocytes are present in these organs as well and similarly to macrophages they are phagocytic and are involved in the respiratory burst response, however compared to macrophages these properties are relatively limited.

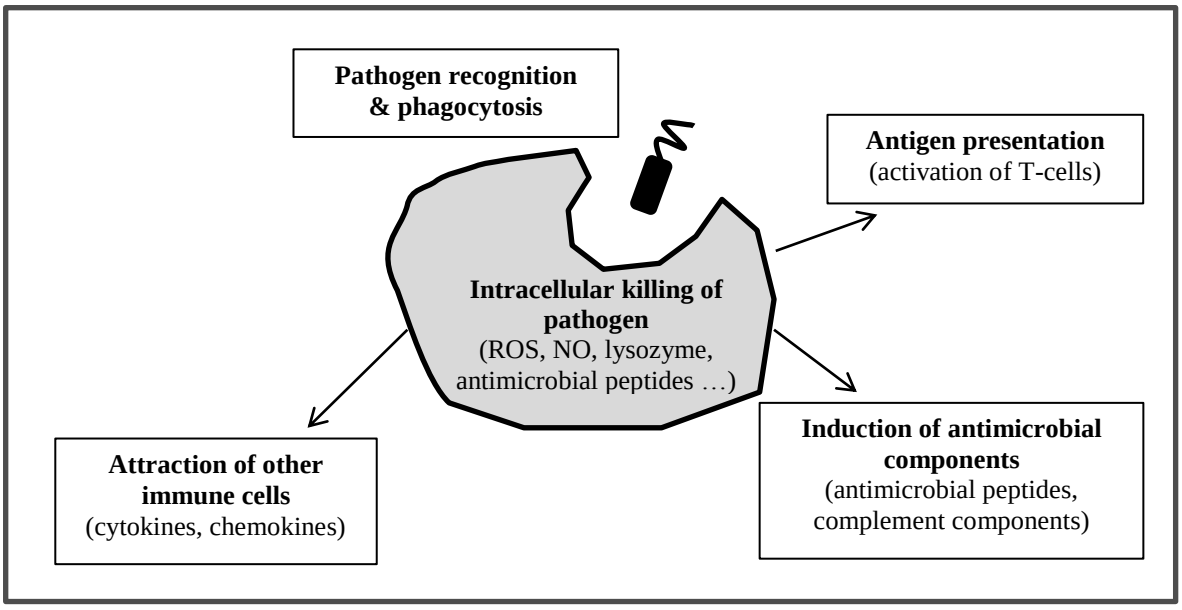

Figure 1.2: Innate immune response

Bacteria, viruses and other foreign material possess pathogen associated molecular patterns (PAMPs) on their surface, which are recognised by specific receptors (PRRs) on the surface of phagocytes. This recognition triggers phagocytosis of the pathogen and killing of it by exposure to reactive oxygen species (ROS), nitric oxide (NO), lysozyme and other microbicidal agents. Antigens are processed within the phagocyte and are presented on the cell surface to activate T cells. Phagocytosis also induces production of cytokines, which attracts other immune cells to the site of infection and it leads to the induction of antimicrobial components such as complement.

During the cellular response the pathogen is discriminated from the host's own cells by pattern recognition receptors (PRRs), which recognize pathogen associated molecular patterns (PAMPS) i.e. bacterial lipopolysaccharides (LPS) and peptidoglycans, viral double stranded RNA and β-(1,3)-glucans. A common characteristic of all these PAMPS is that they are not generally expressed in metazoans (Magnadóttir, 2006).

PRRs, which are found on macrophages and neutrophils, stimulate upon their activation a variety of innate immune responses. The PRRs C-type lectins, collectin family receptors and scavenger receptors stimulate phagocytosis and activate the complement pathway, whilst Toll-like receptors (TLRs) are involved in cell signalling and initiate anti-gen presentation to specific T-cells (Murphy *et al.,* 2008). Some TLRs are situated on the cell surface to detect extracellular pathogens and others act in the cytosol against intracellular pathogens. Various forms of TLRs have been identified in teleost fish of which some are teleost specific, whilst some mammalian TLRs were not found in fish. For example TLR 3, which is involved in viral recognition, has been identified in fish, but TLR 4, which recognizes LPS in mammals, is absent in most fish species and has only been identified in cyprinidae such as *Danio rerio* and *C. carpio.* In these fish species, however, TLR 4 appears not be involved in LPS recognition (Palti, 2011). The molecules of the TLR signalling pathway have been shown to be conserved in fish, which suggest a functional PAMP recognition and signalling mechanisms in this group of vertebrates (Aoki *et al.*, 2008). TLR signalling activates the transcription factor NF-κB, which initiates the expression of genes for cytokines, chemokines and co-stimulatory genes that initiate the adaptive immune system (Murphy *et al.,* 2008). Cytokines can be distinguished into different categories (Roitt *et al.,* 1998):

Interferons (IFNs) are especially important in limiting the spread of certain viral infections. IFNα and IFNβ are produced by virally infected cells whilst IFNγ is released by certain activated T cells. IFNs induce a state of antiviral resistance in uninfected cells. Their production occurs very early in infection and they serve as a first line of defence to a great number of viruses

Interleukins (ILs) are mainly produced by T cells. Most cytokines of this type are involved in stimulating other cells to divide and differentiate. Each IL acts on a specific, limited group of cells which express the specific interleukin receptor for that interleukin.

Tumor Necrosis Factors (TNF) are a group of cytokines that are involved in the immune response and apoptosis. The most important members of this family are TNF-α, TNF-β, Fas ligand, and CD40 ligand. TNF-α is produced by T-cells in membrane-associated and soluble forms and TNF-β is usually membrane associated and rarely secreted. The recognition of TNFs is mediated by so-called TNF receptors (TNFRs), which includes TNFR I and II, CD40, Fas (Apo 1), CD30, and CD27. Fas is especially expressed by activated lymphocytes and as discussed later Fas and TNF-α can induce apoptosis. In fish the best characterized cytokines are the pro-inflammatory TNF-α and IL-1β (Mackenzie *et al.,* 2003; Saeij *et al.,* 2003a; Zou *et al.,* 2003).

As mentioned above recognition of a PAMP by the host can lead to phagocytosis of a pathogen. During this process reactive oxygen species (ROS), i.e. the respiratory burst response, and nitric oxide (NO) are produced, both being potent antimicrobial agents, which help to kill the engulfed microorganism (Belosevic *et al.,* 2009). Under normal

physiological conditions the amount of ROS produced by mitochondria, ER, nucleus and cytoplasm is relatively low and is involved in the regulation of cell differentiation, proliferation and apoptosis. At high concentrations or after long-term exposure, ROS can lead to necrosis (Kamata and Hirata, 1999; Bironaite *et al.,* 2004). Production of ROS can result not only from phagocytosis but also from the extracellular exposure to inflammatory cytokines, chemical carcinogens, chemotherapeutic agents, UV light irradiation or from a decreased antioxidant capacity of the organism (Davies, 1999; Kamata and Hirata, 1999). In teleosts the respiratory burst response as well as the enzyme involved in ROS production (NADPH oxidase) have been characterized (Novoa *et al.,* 1996; Mulero and Meseguer, 1998; Neumann *et al.,* 2000).

As mentioned above another bactericidal event is the production of reactive nitrogen intermediates by inducible nitric oxide synthase (iNOS). Nitric oxide (NO) is an important mediator of various biological functions (i.e. vascular and muscle relaxation) and, in addition, it has microbiocidal and tumoricidal activity (Croen, 1993; Saura *et al.,* 1999; Campos-Pérez *et al.,* 2000; Saeij *et al.,* 2002; Murphy *et al.,* 2008). The response of iNOS is regulated through the activity of various cytokines (e.g. TNF, IFNγ, IL-1, IL-4, TGFβ) and in carp that had been infected with *Trypanoplasma borreli* the main producers of NO appeared to be the pronephric phagocytes. However some authors have reported a detrimental effect of NO in the host, probably due to its cytotoxicity, which has been observed *in vitro* (Saeij *et al.,* 2002) and nitric oxide has been related to apoptosis (Dimmeler and Zeiher, 1997).

Scientists have taken advantage of stimulation of the innate immune response following the exposure to pathogen associated molecular patterns in order to stimulate the

immune response and heighten disease resistance in animals and humans. This principal is known as immunostimulation or immunomodulation and the next section gives an introduction to this new and exciting area of research.

1.2 Introduction to immunostimulation

As mentioned above diseases are generally treated with antibiotics and other chemical medicinal products where appropriate. The use of such substances however is controversial. For example, increased antibiotic resistance has been observed in fish and the resistance factors of the bacteria can be transferred to terrestrial animal and human pathogens (Cabello, 2006). Other resulting problems include the presence of antibiotics in the fish meat, which leads to an unintended consumption of antibiotics which has consequences for the health of the consumer. The accumulation of antibiotics in the environment (i.e. in the sediment and water column) can lead to changes of the microbiota diversity, which in the long term can alter the ecological equilibrium and thus affect animal and human health through, for example, algal blooms or anoxic environments. Even though the use of antibiotics is now restricted in many countries, in some countries with growing aquacultures, such as Asia, antibiotics are subject to less restriction (Defoirdt *et al.*, 2011). It is therefore of great importance to develop means of consumer- and environment-friendly disease prevention.

A cost effective approach to disease prevention in farming of fish and other aquatic and land living animals is the administration of substances that enhance the immune system of the animal. Due to their effect on the immune system such substances are generally known as immunomodulators or immunostimulants and their immune stimulating properties can also be utilized to overcome the immunosuppressive effects of stress (Jeney *et al.*, 1997). Generally substances that have immunostimulatory functions act as pathogen associated molecular patterns (PAMPs) and induce an immune response by interaction with pattern recognition receptors (PRRs) (see section 1.1.2). One such PAMP

is β-glucan, a carbohydrate that is an essential cell wall component of fungi, bacteria, algae, oats and barley (Volman *et al.,* 2008). β-Glucan is an ideal target for the task of disease prevention since this substance stimulates various immune responses and enhances protection against viral and bacterial diseases (Sakai, 1999). It also occurs naturally in the environment and therefore raises less concern in regards to the environment and human health (Gannam and Schrock, 2001).

The structure and immunostimulatory activity of β-glucan depends on its source, solubility, molecular mass, tertiary structure and the degree of branching (Volman *et al.*, 2008). In this thesis particulate β-(1,3/1,6)-glucan from yeast (*Saccharomyces cerevisiae*) was used (Figure 1.3). This type of β-glucan is constructed of glucose and mannose with β-D-1,3 linkages on the backbone and β-D-glucosidic linkages at position 6 (Dalmo and Bøgwald 2008).

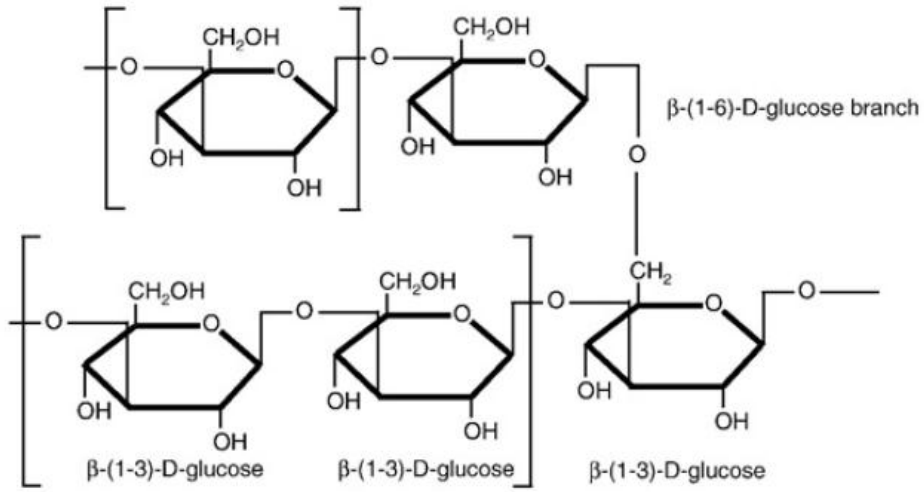

Figure 1.3: Chemical structure of β-(1,3/1,6)-glucan
Yeast β-glucan is a polymer of β-(1,3)-D-glycopyranosyl units with branching at β-(1,6)-glycopyranosyl units. Adapted from Volman *et al.,* (2008).

In humans β-glucans display various medicinal properties for example its application decreases cholesterol levels, enhances wound healing and inhibits cancer cell growth (Petravic-Tominac *et al.,* 2010). In mammals various receptors have been associated with the recognition of β-glucan such as complement receptor C3, dectin-1 and TLR1/6 (Dalmo and Bøgwald, 2008) although dectin 1 is considered as major β-glucan receptor (Brown *et al.,* 2003). Differential responses are elicited when β-glucan binds to dectin-1 alone or together with other receptors such as TLR 2 (Gantner *et al.,* 2003). β-Glucan recognition (Figure 1.4) results in the activation of macrophages, which induces phagocytosis, leukocyte migration and the production of cytokines (e.g. IL-1, TNFα), nitric oxide (NO) and reactive oxygen species, as well as the enhancement of complement activity (Hashimoto *et al.,* 1997; Vetvicka *et al.,* 2008).

β-Glucan can also affect programmed cell death (apoptosis) in cells, for example Kim *et al.,* (2009a) have shown that bacteria-derived β-glucan can induce apoptosis in human cancer cells (SNU-C4) and that apoptosis is involved in the tumouricidal effects of β-glucan (Kobayashi *et al.,* 2005; Zhang *et al.,* 2006).

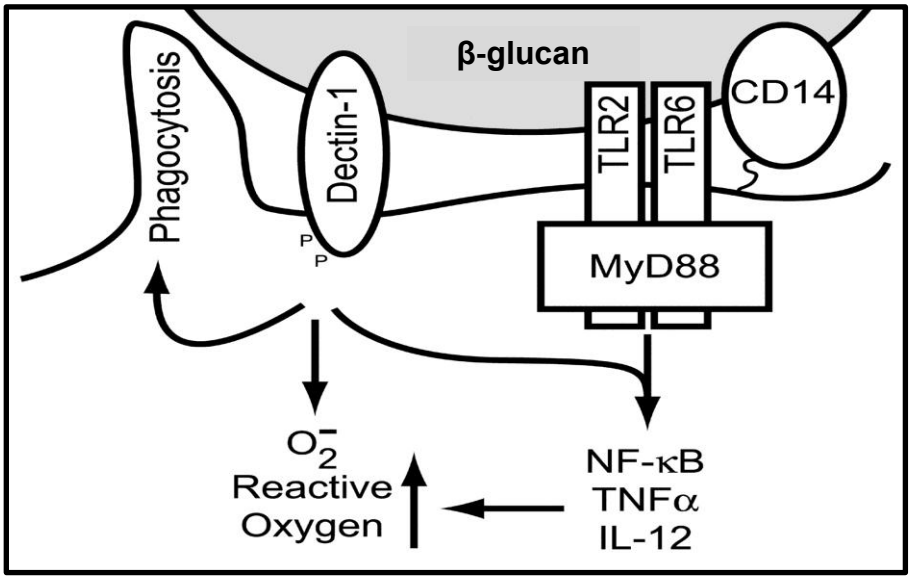

Figure 1.4: Cellular effects of β-glucan in mammals
β-glucan is detected by dectin 1 and TLR receptors, which trigger phagocytosis, production of ROS and cytokine production such as TNF-α and IL-12. Adapted from Gantner *et al.,* (2003).

The mammalian β-glucan receptors have not been identified in fish (Aoki *et al.*, 2008) however receptors that recognize β-glucan have been found in macrophages of Atlantic salmon (S*almo salar*) (Engstad and Robertsen 1994). The type of these receptors still needs to be characterized and currently studies are under way at Wageningen University to identify and characterize the β-glucan receptor in common carp.

In various fish species e.g. common carp (*C. carpio*), rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*S. salar*), channel catfish (*Ictalurus punctatus*) and sea bass (*Dicentrarchus labrax*) the observed effects of β-glucan administration are increased growth, stress reduction, enhancement of immune parameters and enhanced disease

resistance (Chen and Ainsworth 1992; Jørgensen and Robertsen 1995; Jeney *et al.*, 1997; Bagni *et al.*, 2000; Bagni *et al.*, 2005; Selvaraj *et al.*, 2005; Gopalakannan and Arul 2010). Yeast β-glucan has been shown to promote non-specific cellular and humoral immune responses against various viral, e.g. grass carp haemorrhage virus and infectious hematopoietic necrosis virus (LaPatra *et al.*, 1998; Kim *et al.*, 2009b), and bacterial pathogens including *Aeromonas hydrophila* (Selvaraj *et al.*, 2005; Russo *et al.*, 2006; Rodriguez *et al.*, 2009; Lin *et al.*, 2011). In addition it was shown that β-glucan can enhance the protective effect of vaccines. For example Russo and colleagues (2006) found that a combination of MacroGard® feeding and vaccination lowered the mortality of red-tail black shark (*Epalzeorhynchos bicolour*) when challenged with *Streptococcus iniae*. Hence there is effort to use β-glucan as an adjuvant during vaccination especially since it seems to cause less negative side effects as the currently used oil adjuvants (Dalmo and Bøgwald 2008).

However the immunostimulating effects of β-glucan have been shown to be dependent on dose, duration of administration, environmental temperature and the species (see Table 1.1). For example no effect of β-glucan on stress related parameters were observed in channel catfish (*I. punctatus*) (Welker *et al.*, 2007) whilst Jeney *et al.* (1997) observed stress reducing effects of dietary β-glucan in rainbow trout (*O. mykiss*). This effect was however dose dependent since stress reducing effects occurred at the low (i.e. 0.1 % β-glucan in feed) dose whilst a high dose (i.e. 2 % β-glucan in feed) induced adverse effects, which led to a suppressed immune response during and after the stress experience and seem to render the animals more susceptible to an infection. This raises the question of possible adverse effects of β-glucan. In crustaceans it has been demonstrated that such detrimental effects on immune cells occur. Hauton and Smith (2004) showed in a study

involving granulocytes from lobster that an increase in β-glucan concentration decreased the viability of the cells. In fish, studies have shown that β-glucan dosage in the feed affects the respiratory burst activity of macrophages leading to differences in time and height of the peak respiratory burst activity (Bonaldo *et al.,* 2007).

Overall the studies show that it is possible to use a nutritional supplement like β-(1,3/1,6)-glucan to manipulate the cellular and humoral defence mechanisms instead of simply providing sufficient vitamins, proteins and calories in the feed and this opens up new possibilities for intensive aquaculture (Siwicki *et al.,* 2009).

Throughout this thesis β-glucan in the form of a feed supplement, i.e. MacroGard®, was used. This supplement consists of approximately 60 % β-glucan and 40 % consists of other carbohydrates, lipids and organic salts and has been shown to enhance disease resistance and influence innate immune parameters (Ogier de Baulny *et al.,* 1996; Siwicki *et al.,* 2009; 2010).

Due to the observed enhancement of the respiratory burst, NO and cytokine production, which are all linked to apoptosis, it is possible that immunostimulation with β-glucan could lead to cell death via apoptosis. The next section will give an introduction to apoptosis and how it is linked to the immune response, immunostimulation and disease.

Table 1.1: Effects of β-glucan on fish immune response

Species	β-glucan specifications	Effects of β-glucan	Reference
Dietary			
Carp (*Cyprinus carpio*)	Dietary yeast β-glucan Concentration: 1, 2, 4 % Duration: 5 days	Antibody titre ↑ No change in complement pathway No change in leucocyte count No change in superoxide anion production Challenge with *A. hydrophila* No change in resistance	(Selvaraj *et al.*, 2005)
	Dietary yeast β-glucan Concentration: 0.15, 0.5 % Duration: 60 days	No change in weight gain or feed conversion Liver: Lysozyme activity ↓ Kidney: and gill: no change in lysozyme activity Challenge with *A. hydrophila*: Resistance ↓	(Ye *et al.*, 2011)
	Dietary yeast β-glucan Concentration: 1.0 % Duration: 60 days	Lysozyme activity ↑ (max at day 30 of feeding) Challenge with *A. hydrophila*: 30 and 60 d feeding: Resistance ↑	(Gopalakannan and Arul 2010)
Koi carp (*Cyprinus carpio koi*)	Dietary yeast β-glucan Concentration: 0.5 % Duration: 56 days	Lysozyme ↑ Superoxide dismutase ↑ Phagocytic activity ↑ Respiratory burst ↑ (Factors increase until 21d then decrease again) Total leucocyte counts ↑ No effect on alternative complement pathway Growth ↑ Protection against *Aeromonas veronii* ↑	(Lin *et al.*, 2011)

Species	**β-glucan specifications**	**Effects of β-glucan**	**Reference**
Yellow croaker (*Pseudosciaena crocea*)	Dietary yeast β-glucan Concentration: 0.09, 0.18%, Duration: 8 weeks	0.09 %: respiratory burst ↑, phagocytic activity ↑, lysozyme activity ↑ no effect on complement activity 0.18 %: no effect on respiratory burst, phagocytic activity, lysozyme activity ↑, no effect on complement activity	(Ai *et al.*, 2007)
Sea bass (*Dicentrarchus labrax*)	Dietary yeast β-glucan (MacroGard®) Concentration: 2 % Pulse feeding: 2 weeks every 3 month for 40 weeks	lysozyme activity ↑ complement activity ↑	(Bagni *et al.*, 2000)
	Dietary yeast β-glucan (MacroGard®) Concentration: 0.1 % Pulse feeding: 4 x 15 days feeding, 45 days control feed for	Day 15: Complement and lysozyme activity ↑ No change in lymphocyte count	(Bagni *et al.*, 2005)
Rainbow trout (*Oncorhynchys mykiss*)	Dietary fungal β-glucan Concentration: 0.1, 0.5, 1.0 % Duration: 4 weeks	β-Glucan feeding: % lymphocytes in blood ↓ % monocytes/neutrophils ↑ After stress recovery: 0.1 % diet: phagocytosis ↑ (higher than in control and other β-glucan concentrations 0.1, 0.5 % diet: ROS ↑ 1.0 % diet ROS ↓ Cortisol levels unchanged	(Jeney *et al.*, 1997; Volpatti *et al.*, 1998)

Species	β-glucan specifications	Effects of β-glucan	Reference
	dietary yeast β-glucan Concentration: 0.2 % Duration: 14 & 35 days feeding	Lysozyme activity ↑ Challenge with *Ichthyophthirius multifiliis*: Parasite count ↓ No difference between feeding periods	(Lauridsen and Buchmann 2010)
Rohu (*Labeo rohita*)	Dietary barley β-glucan Concentration: 0.01–0.05 % Duration: 56 days	Superoxide anion production ↑ Phagocytic activity ↑ Lysozyme activity ↑ Haemolytic complement activity ↑ Serum bactericidal activity ↑ (Factors increase until 42 d then decrease, max at 0.025 % dose) Growth rate ↑ at 0.025 – 0.05 % feed Mortality ↓	(Misra *et al.*, 2006a)
Red-Tail Black Shark (*Epalzeorhynchos bicolor*)	Dietary yeast β-glucan (MacroGard®) Concentration: 0.1, 0.2 % Duration: 24 d	Challenge with *Streptococcus iniae* Mortality ↓	(Russo *et al.*, 2006)
Atlantic salmon (*Salmon salar*)	3 dietary β-glucan diets Concentration: 0.3, 1 % Duration: 1 week	no effect on respiratory burst no effect on lysozyme no effect on mortality after challenge with *Neoparamoeba* spp.	(Bridle *et al.*, 2005)
Injections			
Rohu fingerlings (*Labeo rohita*)	Barley β-glucan Injections Concentration: 0, 5, 10, 15 mg/kg body weight Injections at 0, 14, 28 and Duration: 42 d	Phagocytic activity ↑ leucocyte count ↑ lysozyme activity ↑ injections (enhanced on d 42 after 3 x 10 mg/kg bodyweight) complement activity ↑ serum bactericidal activity ↑ *Aeromonas hydrophila* and *Edwardsiella tarda* Resistance ↑	(Misra *et al.*, 2006b)

Species	**β-glucan specifications**	**Effects of β-glucan**	**Reference**
Channel catfish (*Ictalurus punctatus*)	yeast β-glucan 2 x i.p. injection of Concentration: 5 and 75 µg/kg bodyweight of Duration: 3-14 days	No effect on bacterial killing No effect on H_2O_2 quantity (respiratory burst) Antibody concentration ↑ Challenge with *Edwardsiella ictaluri* Resistance ↑ Phagocytosis ↑	(Chen and Ainsworth 1992)
Hybrid Tilapia (*Oreochromis niloticus* X *O. mossambicus*) Japanese eel (*Anguilla japonica*)	Yeast β-glucan (MacroGard®) Concentration: 2x i.p. injection of 10 mg/kg analysis after 2d	Phagocytic activity ↑ Lysozyme activity ↑	(Wang *et al.*, 2007)
Rainbow trout (*Oncorhynchus mykiss*)	yeast β-glucan i.p. injection of 1 % (~29 µg/kg), Duration: 1-3 weeks	Bactericidal activity ↑at 2 weeks feeding O_2^- production ↑ at 2 and 3 weeks Lysozyme levels ↑ at 1-3 weeks	(Jørgensen *et al.*, 1993)
Atlantic salmon (*Salmon salar*)	I.p. injection yeast glucan 3- Duration: 6 weeks (~0.1 g/kg)	H_2O_2 ↑ Phagocytic activity ↑ at 3 weeks Lysozyme levels ↑ at 3 weeks	(Brattgjerd *et al.*, 1994)
Zebrafish (*Danio rerio*)	I.p. injection Concentration: 0.5, 2, 5 mg/ml	Challenge with *A. hydrophila*: 5 mg/ml mortality ↓ 5 mg/ml bactericidal activity ↑ No influence on TNF-α, IL-1β gene expression	(Rodriguez *et al.*, 2009)
Grass carp (*Ctenopharygodon idella*)	Fungal β-glucan, injection	Resistance against grass carp hemorrhage virus (GCHV) ↑ After viral infection Antiviral Mx gene expression ↑ than no β-glucan control	(Kim *et al.*, 2009b)

Species	**β-glucan specifications**	**Effects of β-glucan**	**Reference**
Carp (*Cyprinus carpio*)	Yeast β-glucan Concentration: 100, 500, 1000 μg/fish Injections on day 1, 3, 5	Leucocyte count ↑ (max at 1000 μg/fish) Superoxide production ↑ IL-1β gene expression ↑ Challenge with *A. hydrophila* 500 and 1000 μg/fish: resistance ↑ No change in complement activity Bactericidal activity ↑ 500 and 1000 μg/ml: antibody titre against *A. hydrophila* ↑	(Selvaraj *et al.*, 2005)

1.3 Apoptosis

Apoptosis, a type of programmed cell death, is a genetic program that allows the cell to remove itself from the cell population. This is an important process during development and leucocyte homeostasis and appears to have been conserved through evolution as there is evidence that programmed cell death occurs at all levels of cellular organisation including unicellular organisms such as Leishmania (Lee *et al.*, 2002; Raina and Kaur, 2012) and Trypanosoma (Smirlis *et al.*, 2010), plants (Ning *et al.*, 2002), fungi (Glass and Dementhon, 2006), invertebrates such as *Drosophila* and *Caenorhabditis elegans* (Dunn *et al.*, 2006; Terahara and Takahashi, 2008) and all vertebrates. The increasing complexity in organisms has however led to the development of large gene families, whose members offer a more complex control of cell death in higher organisms (Leist and Nicotera, 1997). Immunohistochemical studies have revealed that apoptosis occurs in teleost fish, where it has been observed *in vivo* during development (Abelli *et al.*, 1998), hypoxia (Poon *et al.*, 2007), stress (Engelsma *et al.*, 2003) and infection (do Vale *et al.*, 2003; Pirarat *et al.*, 2007). Additionally, because of the relationship between apoptosis and water pollution it has been suggested that apoptosis could be used as a biomarker for environmental quality (Bhaskaran *et al.*, 1999; Bervoets *et al.*, 2009).

The importance of apoptosis is highlighted by malfunctions in this process: overexpression of apoptosis leads to degenerative diseases like diabetes mellitus (Hayashi and Faustman, 2001) and Alzheimer's disease (Jacobson and Bergeron, 2002). Suppression of programmed cell death however can lead to tumour growth and cancer (Karp, 2005).

Apoptosis is distinguished from necrosis, which occurs after mechanical damage of the cells (Golstein and Kroemer, 2007). These two types of cell death can be discriminated on the morphological level (Figure 1.5). In necrosis, cellular damage leads to swelling of organelles and leakage of intracellular contents due to loss of membrane integrity, which results in damage and inflammation of the surrounding tissue. Apoptosis on the other hand has relatively limited negative effects on surrounding cells. The process of apoptosis is also associated with various morphological and genetic changes, many of which are used to detect or monitor apoptosis. Such morphological changes include membrane blebbing, chromatin condensation, nuclear fragmentation, and cell shrinkage without changes of the cytoplasmic organelles (Ziegler and Groscurth, 2004). In contrast to necrosis the membrane integrity is conserved until the final stages of the process. The shedding of apoptotic bodies, which are small rounded structures that are closely packed with cellular organelles and fragments of the chromatin, can be observed *in vitro* (Leist & Nicotera, 1997; Saraste & Pulkki, 2000; McCarthy, 2002). However, *in vivo* apoptotic cells are engulfed by phagocytes; mediated by phosphatidylserine exposure on the outer membrane. If phagocytosis is impaired, for example due to an infection (see section 1.4) or in cell culture, the apoptotic cell undergoes secondary necrosis (Silva *et al.*, 2008a).

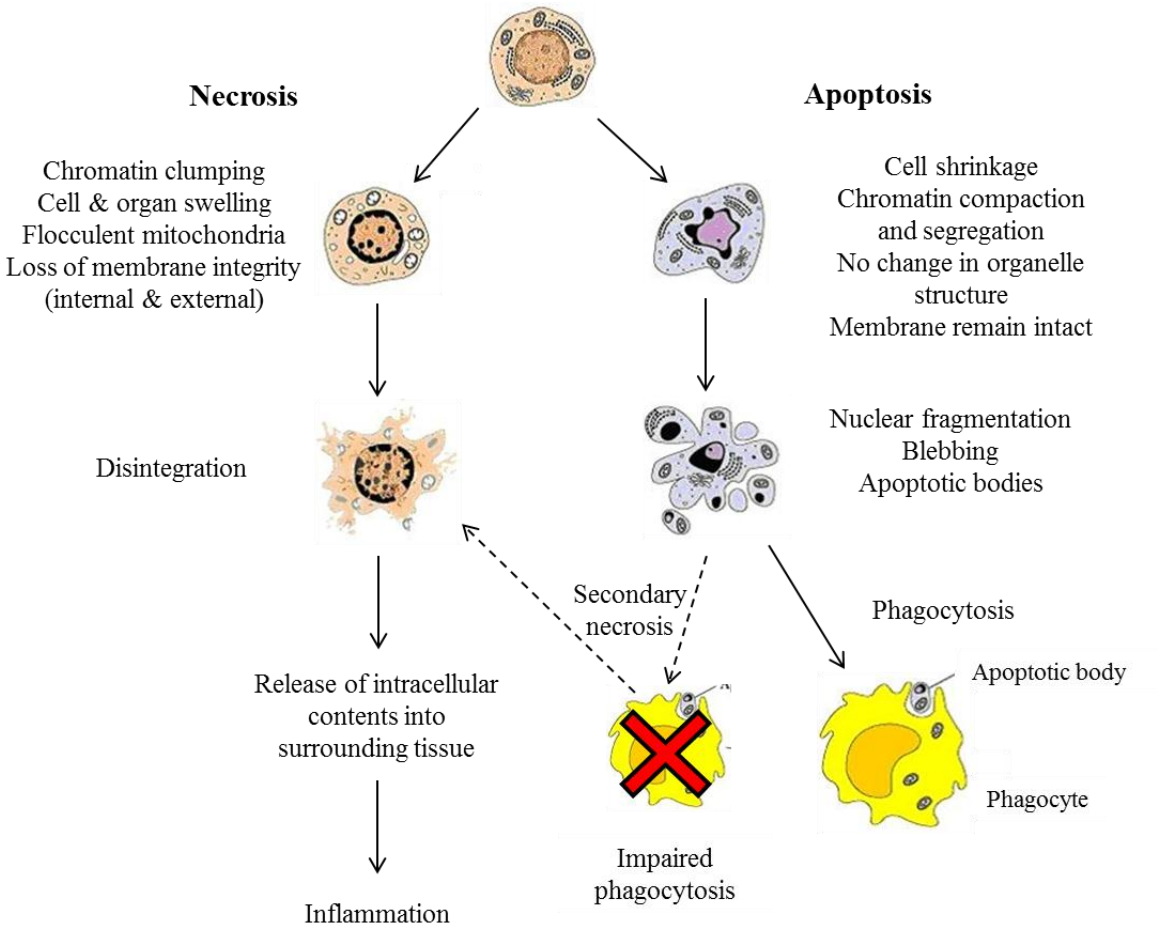

Figure 1.5: Morphological changes during necrosis and apoptosis

Cell death via necrosis results in swelling of the cytoplasm and organelles, chromatin clumping and loss of membrane integrity. The organelles disintegrate and the cell undergoes lysis. This results in inflammation of the surrounding tissue.

Apoptosis is characterized by cell shrinkage and condensation of the nucleus. The membrane integrity is intact and blebbing of the cell membrane occurs. The mitochondria become leaky and membrane bound vesicles are formed (apoptotic bodies), which results in the fragmentation of the cell into smaller bodies. These apoptotic bodies are phagocytosed by phagocytes and no immune response is elicited. If phagocytosis is impaired for example in cell culture or in disease the apoptotic cell undergoes secondary necrosis.

Adapted from American Institutes for Biological & Nutritional Sciences (2005)

1.3.1 Molecular pathways involved in apoptosis

The genetic pathways in apoptosis are very complex involving numerous genes, and the function of many is still not fully understood. This introduction will therefore only focus on the key processes of the apoptotic pathways which have a direct relevance to the aim of the study.

Apoptosis has been mainly studied in mammals and knowledge within fish is limited. To date, apoptosis in teleosts has been best characterized in *Danio rerio* (Eimon and Ashkenazi, 2010) but key players of the apoptotic pathways have also been identified in other fish species such as *C. carpio* (Cols Vidal 2006; Cols Vidal *et al.,* 2008) and *Gadus morhua* (Feng and Rise, 2010). In contrast to the classical apoptosis models *C. elegans* and *Drosophila*, fish exhibit all core components of the mammalian apoptosis system and the central mammalian pathways seem to be highly conserved in teleosts (Krumschnabel and Podrabsky, 2009; Eimon and Ashkenazi, 2010).

In mammals and fish apoptosis is mediated via two pathways: the intrinsic and extrinsic (Eimon and Ashkenazi, 2010). The intrinsic pathway developed with the evolution of multicellular organisms and genes associated with this pathway have been identified in all metazoans (Youle and Strasser 2008). Internal stimuli such as irreparable DNA damage (Rich *et al.,* 2000) or immense oxidative stress caused by large amounts of reactive oxygen species (ROS) (Simon *et al.,* 2000) or nitric oxide (NO) (Dimmeler and Zeiher, 1997) induce apoptosis via this mitochondria mediated pathway. A key player of this pathway is tumour suppressor protein p53, which induces apoptosis upon irreparable DNA damage and cellular stress (Figure 1.6).

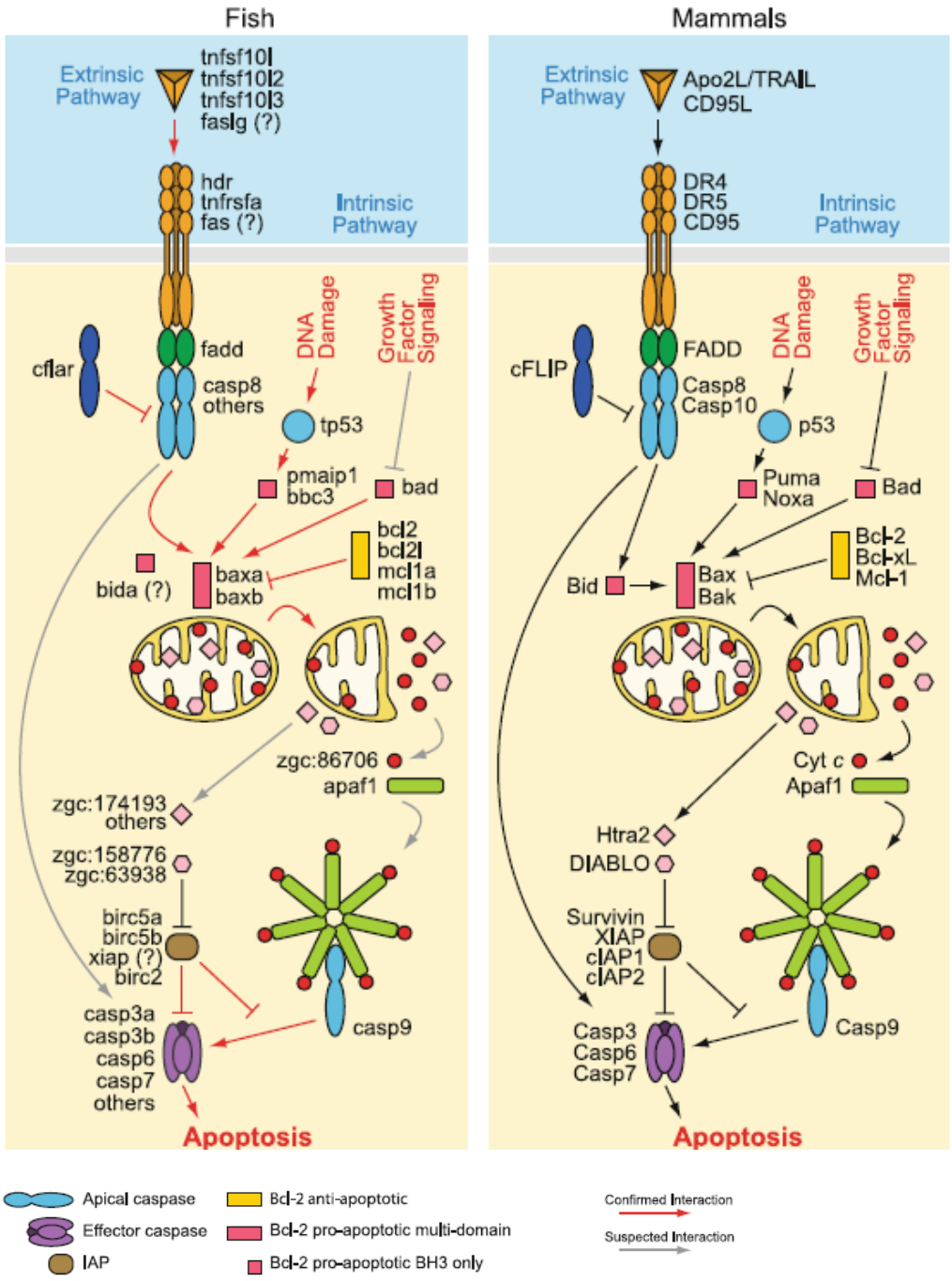

Figure 1.6: Apoptosis pathways in fish and mammals.

Key components of the intrinsic and extrinsic pathways are compared in fish and mammals. From Eimon and Ashkenazi (2010).

p53 has been identified in various fish species including carp (*C. carpio*) (Cols Vidal, 2006), zebrafish (*D. rerio*) (Cheng *et al.*, 1997b), medaka (*Oryzias latipes*) (Chen *et al.*, 2001), and rainbow trout (*O. mykiss*) (de Fromentel *et al.*, 1992). p53 initiates apoptosis by acting on pro-apoptotic members of the Bcl-2 family and Apaf-1. In mammals the Bcl-2 protein family consists of at least 12 members, which can either promote or inhibit apoptosis and most members of this family have been identified in zebrafish (*D. rerio*) (Kratz *et al.*, 2006), Atlantic cod (*G. morhua*) (Feng and Rise, 2010), and common carp (*C. carpio*) (Cols Vidal *et al.*, 2008). The Bcl-2 family strictly controls the intrinsic apoptosis pathway and apoptosis is induced by activation of pro-apoptotic members (Bid, Bad, Bax and Bak) and repression of the anti-apoptotic ones (Bcl-2, Bcl-xl, Mcl-1). In mammals this results in the permeabilization of the outer mitochondrial membrane, which leads to release of apoptogenic molecules like cytochrome c into the cytosol. Cytochrome c together with Apaf-1 then forms a protein complex called apoptosome, which is responsible for the activation of pro-caspase-9 (Youle and Strasser, 2008). Caspase-9 and Apaf-1 have also been identified in fish but little is known about their function and control (Reis *et al.*, 2007; Cols Vidal *et al.*, 2008; Mu *et al.*, 2010). It has been shown that in fish Caspase-9 is involved in apoptosis and that in sea bass (*D. labrax*) its expression is enhanced in the pronephros during bacterial infection (Reis *et al.*, 2007). However evidence for the complete mechanism of cytochrome c-induced apoptosome formation and Caspase-9 activation is lacking in fish (Krumschnabel and Podrabsky, 2009).

The other possible pathway leading to apoptosis is the extrinsic pathway (Figure 1.6), which is initiated by extracellular signals, so called death factors. These factors are members of the tumor necrosis factor (TNF) family (see section 1.1.2) like the pro-inflammatory cytokine TNF-α or the stress-induced Fas ligand (FasL) (Ashkenazi and Dixit, 2009). The signals are transmitted into the cell via transmembrane death receptors, which belong to the tumour necrosis factor receptor (TNFR) superfamily. Of this family the Fas and the TNFR-1 receptor are the most widely studied (Nagata, 1997). The Fas receptor is activated upon binding of its ligand FasL, which then causes clustering of the receptors and the recruitment of its death domain FADD. FADD then recruits pro-caspase 8 and facilitates its splicing it into the active form. Eimon *et al.,* (2006) and Sakata *et al.,* (2007) confirmed that the extrinsic apoptosis pathway in teleosts resembles the mammalian pathway and homologues to FasL, Apo2/TRAIL, FADD, TRADD and several homologues for the TNF receptor superfamily have been identified in zebrafish (*D. rerio*) (Eimon *et al.,* 2006), medaka (*O. latipes*) (Sakamaki *et al.,* 2007) rock bream (*Oplegnathus fasciatus*) (Jeong *et al.,* 2011), black rockfish (*Sebastes schlegelii*) (Kim *et al.,* 2011) and channel catfish (*I. punctatus*) (Long *et al.,* 2004). Caspase 8 has been identified in zebrafish (*D. rerio*) (Sakata *et al.,* 2007), sea bass (*D. labrax*) (Reis *et al.,* 2010), channel catfish (*I. punctatus*) (Long *et al.,* 2004) and common carp (*C. carpio*) (Cols Vidal *et al.,* 2008).

In mammals TNF-α acts via TNFR-1 to induce apoptosis but also to activate the transcription factor NF-κB (Nagata, 1999). Similar to the Fas induced pathway TNFR-1 recruits its death domain TRADD after ligand binding. If TRADD binds FADD this leads to apoptosis (Murphy *et al.,* 2008).

Both the extrinsic and intrinsic pathway converge in the activation of the same effector Caspases 3, 6, and 7 which then splice the same cellular target molecules (Karp,

2005). These targets include proteins that have functions in the cytoskeleton, cell cycle and replication, and transcription factors such as NF-κB, protein kinases and members of the Bcl-2 family (Nagata, 1999). Endonucleases which cleave chromatin DNA into internucleosomal fragments of multiples of 180 bp are also activated, which results in DNA fragmentation (Zhang & Xu, 2000). Effector caspases have been identified in yellow croaker (*Pseudosciaena crocea*) (Li *et al.,* 2011) and zebrafish (*D. rerio*) (Yabu *et al.*, 2001).

These two pathways are independent from each other but there is evidence that the Bcl-2 family member Bid contributes in the linkage of the two pathways. After activation by Caspase 8, Bid translocates to the mitochondria and stimulates the release of cytochrome c, which results in apoptosis involving intrinsic apoptosis genes. In this way the induced caspase cascade is amplified and cross-talk between the two pathways is possible (Kaufmann *et al.,* 2012).

Apoptosis can be provoked by various conditions, for example cortisol (Weyts *et al.,* 1998; Cols Vidal *et al.,* 2008), LPS (Xiang *et al.,* 2008), UV (Lesser *et al.,* 2001; Cols Vidal, 2006) and after exposure to pathogens (Shao *et al.*, 2004). *Aeromonas hydrophila*, for example is associated with the induction of apoptosis in immune cells (Shao *et al.*, 2004). This relationship between pathogens and apoptosis will be examined in more detail in the next section (section 1.4).

1.4 Infection and apoptosis

In a normal functioning and healthy immune system apoptosis is used to regulate the number of immune cells before and after the immune response (Elmore, 2007; Murphy *et al.,* 2008), and to secure homeostasis and a correct development of the immune cells (Rathmell and Thompson, 2002). More than 95 % of T cells are eliminated during their development because they are self-reactive or useless to the organism (Nagata, 1997). For this purpose lymphocytes are subjected to checkpoints at various stages of their development and programmed cell death is induced if they, for example, fail to obtain cytokines or enough extracellular survival factors, if they do not properly rearrange and express an antigen receptor or if they are not selected for memory (Rathmell and Thompson, 2002).

In addition to the development of immune cells apoptosis is also important in the termination of the immune response by eliminating activated immune cells. Neutrophils for example have to be removed after the immune response since they would continue to excrete cytotoxic compounds, which damage the surrounding cells (Labbe and Saleh, 2008). Additionally, activated T cells have to be removed, which can be facilitated via the extrinsic or the intrinsic apoptosis pathway. Activated cytotoxic T cells express Fas and FasL and can therefore induce extrinsic apoptosis in other lymphocytes via Fas-Fas ligand interaction (Murphy *et al.,* 2008). Decreased inflammation leads to a reduced cytokine production, which can initiate intrinsic apoptosis (Maniati *et al.,* 2008).

Apoptosis is not only related to the homeostasis of the immune system but it is also involved in the immune response. In the case of an infection the host can induce cell death

in infected cells thereby destroying the pathogen and additionally limiting its replication and dissemination (Labbe and Saleh, 2008). In mammals this is mediated via cytotoxic T cells and this principle has also been established in fish (Nakanishi *et al.,* 2002; Shen *et al.,* 2002; Somamoto *et al.,* 2002). Moreover the components of the apoptotic process can cause death of the pathogen itself. The nucleases that are responsible for degrading the cellular DNA during the apoptotic process can destroy viral DNA and thus prevent the assembly of virions and their dissemination (Murphy *et al.,* 2008). Other enzymes that are also activated during the apoptotic process may be able to kill non-viral intracellular pathogens. The killing of the host cell via apoptosis is preferable to necrosis because this prevents the release of the intact pathogen, which could infect other cells (Murphy *et al.,* 2008). The dying cells are rapidly phagocytosed and because no immune response is stimulated this process is seen as immunologically silent (Murphy *et al.,* 2008).

However, viruses, bacteria and parasites have developed a variety of strategies to avoid the host's immune system. One such strategy is to induce apoptosis in immune cells such as lymphocytes, monocytes and neutrophils which impairs the host's immune response and therefore secure the pathogen's own survival and reproduction (DeLeo, 2004; Shao *et al.,* 2004). For this purpose bacteria have developed an array of toxins which induce apoptosis via different mechanisms. For example members of the *Aeromonas* family express exotoxins (i.e. LPS), which induces apoptosis via the intrinsic pathway in fish lymphocytes (Xiang *et al.,* 2008). Other examples are the alpha toxin of *Staphylococcus aureus* which inserts pores into the host's cell membrane and thus triggers apoptosis in T lymphocytes, while *Corynebacterium diphtheria*'s Dtx toxin induces apoptosis by inhibiting an essential part of the protein synthesis process (Weinrauch and

Zychlinsky, 1999). This strategy of impairing the host's immune response is also known to be utilised by protozoan parasites which can manipulate apoptosis by the extrinsic pathway (Hoole and Williams, 2004; Sitjà-Bobadilla, 2008).

Another strategy adopted by pathogens is to inhibit apoptosis in the host and thus impair the immune response. Several viruses are known to interfere with the cell death machinery. For example, the herpes simplex virus inhibits apoptosis by targeting Bax and preventing cytochrome c release (Aubert *et al.*, 2007), while Epstein-Barr virus (another herpes virus) expresses a viral homologue of Bcl-2, which leads to apoptosis inhibition (Henderson *et al.*, 1993). The inhibition of apoptosis is not exclusively a strategy of viruses. Intracellular bacteria *Rickettsia rickettsii* and the protozoan parasite *Toxoplasma gondii* activate the NF-κB pathway in the infected cell, which causes an inhibition of apoptosis and protects the pathogen from the immune response (Weinrauch and Zychlinsky, 1999; Kim *et al.*, 2006). The protozoan parasite *Trypanosoma cruzi* has been shown to inhibit the Fas mediated apoptosis pathway in mammalian cells (Nakajima-Shimada *et al.*, 2000). This strategy allows the pathogen to secure their proliferation inside the cell (Hoole and Williams, 2004).

1.5 Hypothesis and aim of thesis

This introduction elaborated the hypothesis that the innate immune system responds to pathogen associated molecular patterns (PAMPs) with phagocytosis and the production of radical oxygen species (ROS, NO) and cytokines (section 1.1.2). This mechanism is exploited during immunostimulation with β-glucan (section 1.2) however ROS/NO and cytokines are also connected to apoptosis (section 1.3). In addition β-glucan was linked by several authors to the induction of the intrinsic apoptosis pathway (Kobayashi *et al.*, 2005; Zhang *et al.*, 2006; Kim *et al.*, 2009a).

I therefore hypothesize that the observed adverse effects of β-glucan at high concentrations/doses are due to the induction of apoptosis in immune cells. I further hypothesize that β-glucan leads to apoptosis via the intrinsic apoptosis pathway due to oxidative stress (Figure 1.7) and that apoptosis can be affected by bacterial and viral diseases as well. A direct induction of apoptosis via the β-glucan receptors was excluded from the hypothesis since Battle *et al.*, (1998) demonstrated that in a murine cell line β-glucan signals via NF-κB but does not induce apoptosis via this pathway.

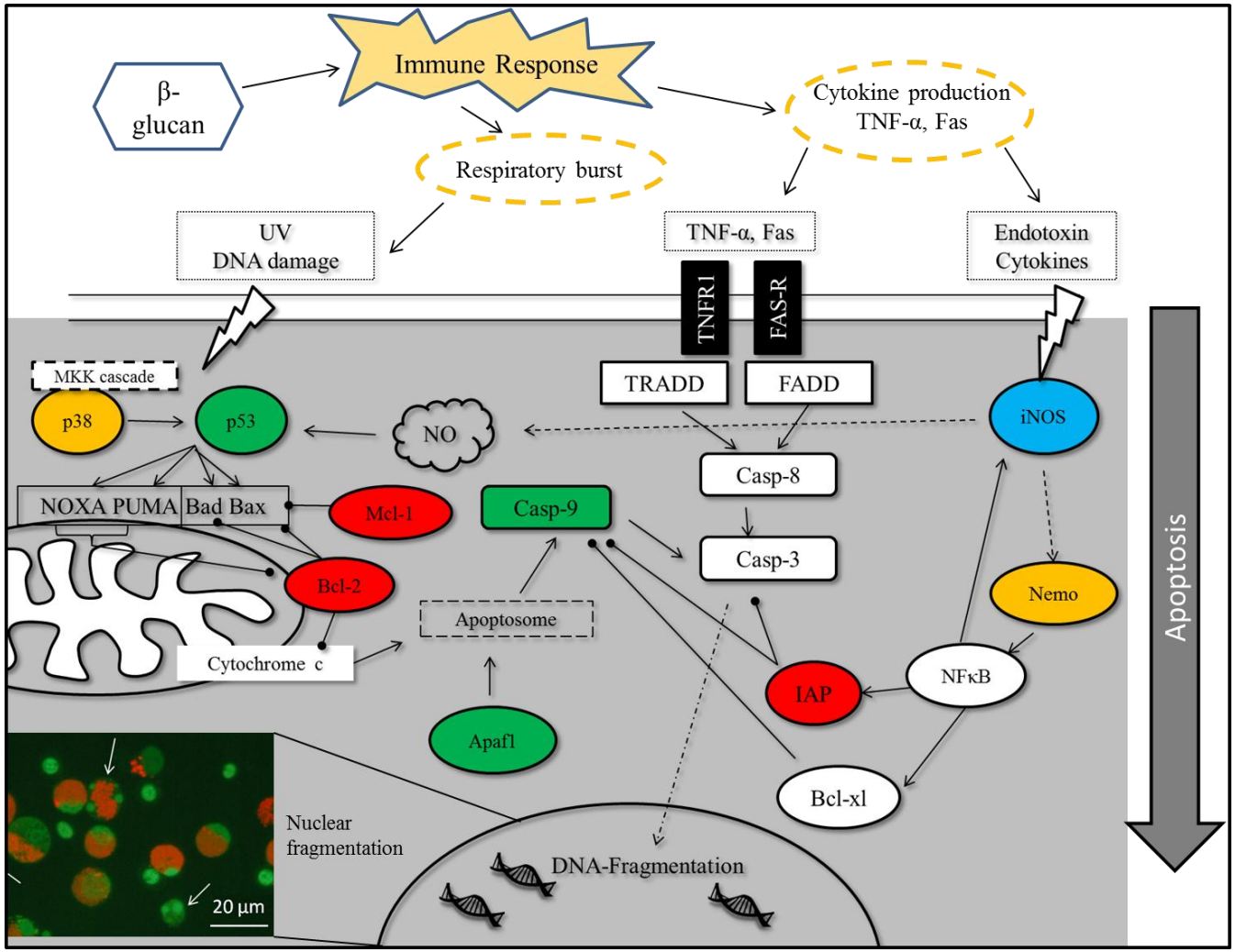

Figure 1.7 (See legend next page)

Figure 1.7: Possible apoptosis induction pathways by β-glucan.

In this thesis it is hypothesized that β-glucan can induce apoptosis via the intrinsic apoptosis pathway due to induction of reactive oxygen species and nitric oxide (NO). This induces oxidative stress and cell damage and induces the intrinsic apoptosis pathway which leads to morphological changes such as nuclear fragmentations as seen in the staining photo (staining: acridine orange).

Genes targeted within this thesis are marked by a coloured background. Red marks anti-apoptosis genes, green are pro-apoptotic, orange are genes involved in cell signalling and blue is the inducible NO synthase (iNOS) which is involved in NO production. Genes marked white, grey or black were not targeted. Pointed arrows indicate activating effects on gene expression whilst blunt lines indicate inhibition.

Note that bacterial exotoxin can induce apoptosis as well via induction of NO production.

To test the hypothesis indicated above an array of genes were chosen to evaluate the effect of infection and β-glucan on the apoptotic process and possible mechanism of induction. As established in the previous section (section 1.5, Figure 1.7) it was hypothesized that apoptosis is induced via the intrinsic apoptosis pathway and hence genes encoding proteins involved in this pathway were targeted, i.e. p53, Apaf-1, Caspase 9, Bcl-2, IAP, Mcl-1b. In addition genes encoding iNOS, p38 and Nemo were analysed to evaluate the effect on nitric oxide production and the p38 MAPKK and NF-κB pathway.

The inducible NO Synthase (iNOS) enzymatically produces nitric oxide (NO) from L-arginine (Aktan 2004). Its family members eNOS (endothelial NOS) and nNOS (neuronal NOS), are constitutively expressed in the cell and produce low concentrations of nitric oxide (NO). These low NO concentrations have physiological functions such as smooth muscle relaxation. In contrast iNOS is not constitutively expressed in the cell and is induced by binding of cytokines and PAMPS to toll-like receptors (Aktan, 2004). iNOS is the only NO synthase that is involved in the immune response (Rieger and Barreda, 2011). This induction of iNOS is mediated by activation of nuclear factors including NF-κB and therefore iNOS is mainly regulated at the transcriptional level (Saeij *et al.*, 2000; Aktan, 2004). Various studies have demonstrated the anti- as well as the pro-apoptotic properties of iNOS depending on the amount of NO produced (Dimmeler and Zeiher, 1997). High levels of NO can lead to the production of $ONOO^-$ and other reactive oxidizing compounds. These substances can cause oxidative damage to proteins, DNA, carbohydrates and lipids and can even inhibit the activity of antioxidant enzymes (Aktan, 2004). In fish iNOS has been sequenced for example in common carp (Saeij *et al.*, 2000),

goldfish (Laing *et al.,* 1996), and rainbow trout (Laing *et al.,* 1999) and its expression and activity demonstrated in teleost phagocytes (Barroso *et al.,* 2000; Saeij *et al.,* 2000).

The p38 mitogen-activated protein kinase (MAPK) pathway is a cascade of protein kinases that link extracellular signals to transcription in the nucleus. Such extracellular stimuli include UV light, heat, inflammatory cytokines (TNF-α and IL-1), lipopolysaccharide (LPS) and growth factors (Zarubin and Han, 2005). Upon activation via the cascade p38 MAPK activates various transcription factors including p53 and regulates genes of cytokines, transcription factors, iNOS and cell surface receptors (Bulavin *et al.,* 1999; Zarubin and Han, 2005). However there is evidence that the activation of this pathway is dependent on the cell type and the type of stimulus. In addition p38 is linked to the modulation of apoptosis by acting up- and downstream of caspases. Hence the p38 pathway has been linked to both infection and apoptosis (Zarubin and Han, 2005). In teleosts p38 MAPK has been identified in common carp (Hashimoto *et al.,* 2000) and orange-spotted grouper and has been shown to have a similar function as in mammals (Cai *et al.,* 2011).

NF-κB essential modulator (Nemo) also known as IKKγ is involved in the NF-κB cell signalling pathway. This pathway signals in response to genotoxic agents as well as inflammatory and immunological signals such as cytokines, bacterial and viral products and oxidative stress and is involved in lymphocyte development and apoptosis (Miyamoto, 2011). Nemo is involved in the transduction of signals from toll-like receptors (TLR) to the nucleus. In mammals the NF-κB pathway is involved in β-glucan signalling after dectin-1 activation (Tada *et al.,* 2009). Members of the NF-κB pathway have been identified in

other fish species but this is the first study investigating this pathway in carp (Schlezinger *et al.,* 2000; Serfling *et al.,* 2004).

B-cell lymphoma 2 (Bcl-2) is an anti-apoptotic member of the Bcl-2 superfamily. This family is involved in regulating cell fate and the balance between the pro- and anti-apoptotic Bcl-2 family members determines if apoptosis is promoted or inhibited (Borner, 2003). In this thesis the anti-apoptotic family member Bcl-2 is studied. This protein suppresses intrinsic apoptosis by inhibiting its pro-apoptotic family members (Figure 1.6) and amongst others it has been characterized in common carp (Cols Vidal *et al.,* 2008) and zebrafish (Langenau *et al.,* 2005; Porreca *et al.,* 2012) and ESTs similar to Bcl-2 have also been identified in other fish species (dos Santos *et al.,* 2008). Its anti-apoptotic role as been confirmed in over-expression studies involving zebrafish larvae. In these studies it was shown that Bcl-2 inhibits apoptosis induced by irradiation or dexamethasone exposure (Langenau *et al.,* 2005).

Mcl-1 protein is also a member of the Bcl-2 family and has always been regarded to be anti-apoptotic. However Bae *et al.,* (2000) reported that depending on the splicing region it can have pro- as well as anti-apoptotic properties. Mcl-1 was identified in common carp (*C. carpio*) (Cols Vidal *et al.,* 2008), cod (*Gadus morhua*) (Feng and Rise, 2010) and Atlantic salmon (*Salmo salar*) (Matejusova *et al.,* 2006). In zebrafish two copies were found: Mcl-1a and Mcl-1b (Kratz *et al.,* 2006). Similarly to Bcl-2, overexpression of Mcl-1 inhibits irradiation-induced apoptosis in zebrafish, and hence has been shown to have anti-apoptotic properties and to be involved in the intrinsic apoptosis pathway in fish (Kratz *et al.,* 2006).

Inhibitor of Apoptosis IAP are a family of anti-apoptotic proteins. To date little is known about their specific mechanism of action but it has been demonstrated that some of the human IAP members can inhibit Caspases 3, 7, and 9 directly by binding to them (Deveraux and Reed, 1999). Apoptosis inhibition or induction is regulated by alterations in the IAP gene expression levels, which set the threshold for levels of caspase activity required to trigger apoptosis (Salvesen and Duckett, 2002). In fish IAP has for example been identified in zebrafish (Inohara and Nuñez 2000) and carp (Cols Vidal, 2006). In carp IAP gene expression has been shown to be down-regulated during *A. hydrophila* infection (Cols Vidal, 2006). In catfish (*I. punctatus*) cell lines exposed to Poly(I:C), a mimic of viral infection, IAP gene expression was dependent on the cell line tested, resulting either in up- or down-regulation (Milev-Milovanovic *et al.*, 2009).

Caspases are cysteine proteases, which are activated in the early processes of apoptosis and that are responsible for most of the changes during apoptotic cell death (Karp, 2005). Caspases exist in the cell as inactive zymogens (pro-caspases) and are activated by proteolysis (Stennicke and Salvesen 2000). Two subgroups of caspases can be distinguished: the initiator and the effector caspases. The initiator caspases i.e. Caspases 2, 8, 9, and 10, are activated by recruitment to specific adapter molecules and cleave the effector caspases, i.e. Caspase 3, 6, and 7, into their active form (Chowdhury *et al.*, 2008). Caspase 9, which is analysed in this thesis, is involved in the execution of the intrinsic apoptosis pathway. Pro-caspase 9 together with Cytochrome c and *Apaf-1* forms the apoptosome, which activates Caspase 9. The latter then cleaves the effector Pro-Caspase 3 into the active form. It is not known to date if intrinsic apoptosis in fish involves the

apoptosome however it has been established that all involved genes exist in fish (Krumschnabel and Podrabsky, 2009). Caspase 9 has been sequenced in sea bass (Reis *et al.*, 2007) and yellow croaker (Mu *et al.*, 2010).

Apaf-1 has been identified and characterized in common carp and it was shown to contain a caspase 9-activation motif. Constitutive expression of Apaf-1 was detected in liver, spleen and pronephros (Cols Vidal *et al.*, 2008). Cols Vidal (2006) also demonstrated that Apaf-1 gene expression is enhanced in carp pronephric lymphocytes during cortisol treatment and in carp during *A. hydrophila* infection. Up to date, Apaf-1 has not been characterised in any other fish species however EST sequences with similarity to murine Apaf-1 have been identified (dos Santos *et al.*, 2008).

The tumour suppressor *p53* has pro-apoptotic properties and is involved in the mediation of the intrinsic and extrinsic apoptosis pathway. In mammals p53 is involved in the cell cycle control and is induced by oxidative stress, DNA damage, and UV-light (Almog and Rotter, 1997). p53 homologues have been sequenced in a variety of fish including medaka (*Oryzias latipes*) (Chen *et al.*, 2001), rainbow trout (*O. mykiss*), zebrafish (*D. rerio*), puffer fish (*Tetraodon miurus*) (Bhaskaran *et al.*, 1999) and common carp (*C. carpio*) (Cols Vidal *et al.*, 2008). It was shown that p53 is expressed in most organs for example liver, gut, heart, skin, pronephros and spleen and in the CLC (carp leucocyte cell line) cell culture model (Bhaskaran *et al.*, 1999; Cols Vidal, 2006). Even though there is high conservation in the gene sequence there seem to be functional differences in fish (Chen *et al.*, 2001; Embry *et al.*, 2006; Krumschnabel and Podrabsky, 2009). For example, p53 protein expression was increased after UV exposure in Atlantic

cod larvae (*G. morhua*) (Lesser *et al.*, 2001) but p53 gene expression was not modulated after UV exposure in medaka fry or cell culture (Chen *et al.*, 2001).

This thesis therefore aims to establish if the β-glucan containing feed supplement MacroGard® has the potential to induce apoptosis (chapter 3) and if dietary MacroGard® induces apoptosis in carp at the cellular and gene expression level (chapter 4). In addition it aims to elucidate the apoptotic effects of bacterial (*Aeromonas salmonicida*) and viral pathogens (koi herpes virus and spring viremia of carp virus) and how pathogens modulate apoptosis at the gene expression level (chapter 4 – 7). Last but not least it aims to establish the combined effects of immunostimulation and pathogens on apoptosis and to elucidate the effects of these combined treatments on apoptosis-related gene expression (chapter 4 – 6).

2 Materials & Methods

In this chapter all the materials and methods described are the general procedures used throughout this thesis. In the following experimental chapters study-specific alterations or additional methods used will be described.

2.1 Fish

For the studies carried out at Keele University, common carp (*Cyprinus carpio*) were obtained from Fair Fisheries, Shropshire, England and reared in black 1 m x 0.5 m tanks with 225 litres of 15 °C dechlorinated water at pH 7. The water in each tank was circulated and cleaned by a temperature regulating biological pond filter (Eheim). Fish were fed daily with commercial dry pelleted food (Tetra Pond feed) and kept on a 12 hour light/dark cycle. The head - tail length of the fish ranged from 7.9 to 18.1 cm (mean length 10.6 cm) and weight ranged from 8.2 to 89.7 g (mean weight 36.3 g). The condition factor K was calculated as (weight/length^3) x 100.

For organ sampling fish were removed from the tank by netting and sacrificed with a lethal dose (~ 0.2 %) of 2-Phenoxyethanol (Sigma Aldrich, P1126) in aquarium water. The dissection was carried out in a laminar flow cabinet (Gelaire®, BSB 4A) under sterile conditions, and organs were removed and processed as required for the study undertaken.

For studies carried out at other laboratories the method of fish husbandry will be described in the materials and method section of the appropriate chapters.

2.2 Primary pronephric cell cultures

2.2.1 Isolation of cells

Fish were sacrificed as described section 2.1, dissected using a ventral median incision and the head kidney (pronephros) isolated under sterile conditions in a laminar flow cabinet (Gelaire®, BSB 4A). A pronephric cell suspension was prepared using a modification of the procedure described by Verburg-van Kemenade (1994). In brief, the organ was gently disrupted through a sterile cell strainer with 100 µm pore diameter (BD Falcon Cell strainer, Scientific Laboratory Supply, 352360) in 1 ml of modified RPMI medium. The modified cell culture medium (hereafter referred to as RPMI+) consisted of RPMI with 0.3 g/L L-glutamine (Sigma Aldrich, R7388) with 0.5 % sterile water, 0.05 % pooled carp serum, 0.05 mM β-mercaptoethanol (Sigma Aldrich, M-3148), penicillin (50 U/ml), and streptomycin (50 µg/ml) (Sigma Aldrich, P4458). The pooled carp serum had been previously obtained by exsanguination of numerous *C. carpio* with sterile syringes and needles. The blood was stored in sterile tubes at 4 °C overnight and serum and blood cells were separated by centrifugation (5 min at 1400 g; Boeco Germany U32-R). The serum of the fish was pooled and stored at -20 °C.

2.2.2 Cell concentration and viability

Cell viability (unstained cells/total number of cells counted) and concentration (cells/ml) were assessed using the trypan blue exclusion test (Howard and Pesch, 1968; Hauton and Smith, 2004). In this test the cytoplasm of damaged cells is stained by trypan blue as a result of increased membrane permeability; vital cells, however, will exclude the

dye. Trypan blue solution (0.4 %, Sigma Aldrich, T8154) was mixed 1:1 with cell solution, the total numbers of vital and stained cells in 5 squares of a haemocytometer (Improved Neubauer, depth 0.1 mm, 1/400 mm^2) were determined under the microscope (Olympus CH2) and cell viability and concentration were calculated. Cell suspensions were diluted with RPMI+ to a working concentration of 1 x 10^7 cells/ml and only cell suspensions with at least 95 % viability were used for experimentation. Samples were distributed to flat bottom, plastic 96 well plates (Sarstedt, 83.1835.500) with 100 µl cell suspension/well or 24 well plates (Greiner bio-one, 662160) with 500 µl cell suspension/well depending on the experimental requirements.

2.3 Cell line culture

2.3.1 Culturing

If not stated otherwise the culture medium for the carp leukocyte cell line (CLC) consisted of L-glutamine free RPMI (Sigma; R-0883) modified with 5 % FBS (Fetal clone I, Hyclone, SH30080.03, not heat inactivated), 2.5 % pooled carp serum (heat inactivated, 30 min at 56 ºC in water bath), 50 IU/ml penicillin-G, and 50 mg/ml streptomycin (Sigma P4458). In the following chapters this modified medium is referred to as clcRPMI+. Cells were fed every 3 days and subcultured every 4 – 5 days.

The cultured cells were ascertained to be mycoplasma free using the EZ-PCR Mycoplasma Test Kit (Gene Flow, 20-700-20). The principle of this test is the detection of mycoplasma-specific 16S rRNA by PCR and gel electrophoresis. For this purpose 1 ml cell culture supernatants from three culture flasks were centrifuged (250 g for 1 min) to sediment any cellular debris and the supernatant was transferred into new tubes. This cell-free supernatant was centrifuged at 20.000 g for 10 min to pellet any mycoplasma. The supernatant was carefully decanted and the pellet re-suspended in 50 µl of the provided Buffer Solution and mixed by pipetting. Using a heat block (Techne, Dri-Block® DB 2H) the samples were heated to 95°C for 3 min. For the PCR reaction 35 µl of water were mixed with 10 µl Reaction mix (provided) and 5 µl sample. A positive control sample, provided in the diagnostic kit, was run in parallel. For this reaction 1 µl of provided positive control template was added to 10 µl Reaction mix and 39 µl of water. The following program was run for the PCR (Applied Biosystems, GeneAmp® PCR System 9700): 1 cycle: 94 °C – 30 sec; 35 cycles of: 94 °C – 30 sec; 60 °C – 120 sec and 72 °C – 60 sec; 1 cycle: 94 °C – 30 sec; 60 °C – 120 sec and 72 °C – 5 min. Gel electrophoresis

was performed at 100 V with 20 µl of the PCR product on a 2 % agarose gel. In case of a mycoplasma contamination a PCR product of 270 bp was expected.

2.3.2 Thawing

Cells from the CLC cell line were cryopreserved in 2005 by Montserrat Cols Vidal and stored in liquid nitrogen at Keele University, UK. Cells from this stock were thawed in 2010 to revive the cell line. A vial was removed from the liquid nitrogen and immediately placed in a 37 ºC water bath. As soon as the solution was thawed it was transferred to 9 ml Hanks Balanced Salt Solution (HBSS) and centrifuged at 1500 g at room temperature for 8 min. The pellet was resuspended in 1 ml fresh modified clcRPMI+ and the cell viability determined with trypan blue. The cell solution was transferred to a T25 flask (Sarstedt, Corning, Nunc) containing 4 ml of fresh clcRPMI+. The cells were left to adhere for at least 3 days before subculturing.

2.3.3 Subculturing

Cells were checked daily for their confluency. Flasks that displayed 70 – 80 % confluency were split 1:4. Three different methods to remove cells adhered to the plastic culture vessels were tested.

Scraping

Approximately three quarters of the spent medium were removed from the flask, stored in a sterile 15 ml tube (Sarstedt). In each new flask 1 – 3 ml of the spent medium was transferred. The cells were detached from the bottom of the flask by scraping with a disposable sterile plastic scraper (Greiner Bio-one, 541070) and the resulting cell

suspension was transferred to the tube with spent medium. The cells were centrifuged at 1000 g for 3 min at room temperature (Heraeus Megafuge 1.0R) and the pellet re-suspended in 1 ml of fresh clcRPMI+.

Trypsinisation

The spent medium was removed from the flask and kept for later use in a sterile 15 ml tube (Greiner). From this spent medium approximately 1 – 3 ml were given into a new flask. The cell monolayer was gently washed with Hanks Balanced Salt Solution (HBSS, Sigma, H9262) and the HBSS subsequently removed. Enough 0.1x Trypsin-EDTA (Sigma) was added to cover the bottom of the flask and the monolayer was observed continually for detachment of the cells. When 60 – 70 % of the cells had become detached the spent medium was added to neutralize the Trypsin. The cells were centrifuged at 1000 g for 3 minutes at room temperature (Heraeus Megafuge 1.0R) and the supernatant was discarded. The cells were resuspended in 1 ml of fresh clcRPMI+ medium and placed into the new flasks.

Magnesium-free PBS

This method was similar to the subculturing with Trypsin (described above) except the washing step was carried out with magnesium-free PBS, which was then removed. The cells were then incubated with 1 – 3 ml of magnesium-free PBS and the cells were checked for detachment. When 60 – 70 % of the cells were detached spent medium was added and the cells were further processed as described for the trypsinisation procedure (see above).

Independent of the procedure used for cell detachment the viability and cell concentration was assessed by trypan blue staining before splitting the cells into the new flasks. The cell solution was split 1:4 and given to new flasks containing 1 – 3 ml of spent medium and fresh clcRPMI+ medium was added. The amount of added medium was dependent on the flask volume; for T25 flasks 4 ml of fresh medium were added while for T75 flasks 9 ml were added. After 24 hours the amount of medium was doubled (i.e. total volume in T25 flasks: 10 ml, T75 flasks: 20 ml) to provide enough nutrients for the cells.

2.3.4 Cryopreservation

Cell lines can be stored in liquid nitrogen for an unlimited period of time and can be resurrected for further use. Storage of the CLCs was carried out by diluting them to 1 – 2 x 10^6/ml fresh clcRPMI+ and 0.5 ml transferred to a cryovial and the same volume of 2x freezing medium (clcRPMI+ with 10 % DMSO (Sigma)) added. The cell suspension was gradually frozen in a cell freezer (Nalgene, Cryo 1 °C freezing container) at -80 ºC overnight and then transferred to liquid nitrogen.

2.3.5 Identification

There has been a debate if the CLC cell line is a genuine carp derived cell line. Therefore molecular characterisation of the cell line identity was conducted using PCR using primers specific for carp cytochrome oxidase.

The primers used were designed by Mikolaj Adamek (Veterinary University Hanover, Germany) against the sequence of cytochrome oxidase of *Cyprinus carpio*:

Forward primer: CTTGTATTTGGTGCCTGA

Reverse primer: GTGTTTGGTATTGGGAGATG

The master mix contained 2.5 µl of a 25 mM $MgCl_2$ solution 5x Go Taq® Flexi Buffer (4 µl) (Promega), dNTPs (0.5 µl of 10 mM solution) (Invitrogen), 0.5 µl of each primer (of 10 µM stock), 0.1 µl Go Taq® Flexi Polymerase (Promega), 3 ng cDNA and topped up to 20 µl with DEPC treated water (Invitrogen, Ultrapure).

The PCR was run in a thermocycler (Applied Biosystems, GeneAmp PCR System 9700) for 95 ºC – 2 min; 95 ºC – 30 s; 40 cycles of 95 ºC – 30 s, 57 ºC – 30 s, 72 ºC; ∞ 4 ºC. The PCR product was analysed on a 2 % agarose gel at 100 V (Bio-RAD Mini-Sub® Cell GT with Bio-RAD PowerPac 300) and resulting bands visualised using a UV camera (Syngene, Gene Genius) (see section 2.5.4). For results see chapter 6.2.1.

2.4 MacroGard® preparation

MacroGard® was provided by Biorigin (Brazil) and for feeding studies it was integrated into pelleted fish food provided by Tetra GmbH (Germany) in a concentration of 0.1 %. Table 2.1 shows the consistency of the basic feed provided by Tetra which was then supplemented with 0.1 % MacroGard® and was used in the feeding studies throughout this thesis (chapter 4 – 7). According to the certificate of analysis the MacroGard® batch (batch 250813) consisted of 67.8 % carbohydrates, 5.1 % protein (dry matter), 14.2 % lipids, 6.5 % ash and 4.6 % moisture. The microbiological analysis was negative for *Salmonella* sp., *Escherichia coli* and Coliforms (analysed by Biotech Pharmacon). MacroGard® from this batch was incorporated into the feed and was also used for *in vitro* studies.

Table 2.1: Experimental feed composition (courtesy of Tetra GmbH)

Feed Formula	%
Fish Protein Concentrate	45.000
Wheat starch	41.000
Cellulose	2.565
Soybean oil	4.500
Fish oil	4.500
Ethoxyquin	0.015
Vitamin-Premix	0.250
Stabilized Vitamin C	0.110
Mineral-Premix	2.060
TOTAL	100.00

Due to the production process the particle size of MacroGard® can be up to 99 µm (personal communication Rolf Nordmø, Biorigin). In this form most of the β-glucans are unavailable to the cells and therefore the particles have to be broken down prior to use (see Figure 2.1). For this purpose a stock solution of MacroGard® in deionized and autoclaved water (sH_2O) was sonicated (2 x 30 s at power 6, Sonics, vibra-cell). Sterility was ensured by pasteurisation of the solution in a water bath (Grant) at 80 ºC for 20 min and left at room temperature to cool down. Concentrations of MacroGard® were prepared from the stock solution in sH_2O as required. This protocol had been recommended by the manufacturer (Biorigin) and Castro *et al.,* (1999) had shown that sonication leads to reduced particle size of MacroGard®.

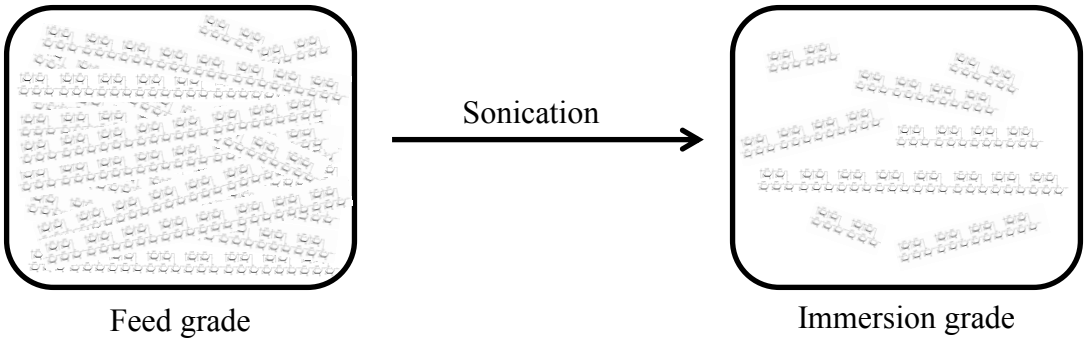

Figure 2.1: Effect of sonication on MacroGard® particles
The feed grade of MacroGard® consists of long chained β-glucan, which is insoluble. Sonication breaks up these long chains and makes it more available to the cells.

2.5 Molecular analysis of apoptosis

The process of apoptosis is reflected in altered gene expression patterns, which can be detected by PCR (Polymerase Chain Reaction). This method allows the exponential amplification of a specific DNA fragment and therefore the analysis of gene expression (Saiki *et al.,* 1985; 1988).

General principle

The polymerase chain reaction uses a thermostable DNA polymerase and sense- and anti-sense primers to amplify a short DNA sequence. The process consists of three steps. During the first step the double strands of the DNA template are separated (denaturation), prior to the following step where the sense and anti-sense primers anneal to the complementary sequences of the template. During the last step the DNA-polymerase uses the annealed primers as starting points and extends the strand by adding dNTPs complementary to the template strand (elongation). By repetition of this process it is possible to amplify the DNA fragment exponentially.

If mRNA is used as a template it has to be reverse-transcribed into complementary DNA (cDNA) first since PCR requires DNA not RNA and the structure of the 3' untranslated region impedes the reaction (Raymaekers *et al.,* 2009). This process mimics the method that a RNA virus uses to cause a host cell to produce viral DNA from the virus' RNA (Baltimore 1970). The reverse-transcriptase enzyme uses the mRNA strand as a template to synthesize a complementary DNA strand elongating random hexamer primers. This is known as reverse-transcription PCR (RT-PCR) and is a powerful tool to analyse mRNA expression.

Once the template is prepared different types of PCR can be conducted. Primarily two alternatives can be distinguished: semi-quantitative and quantitative PCR. In the semi-quantitative PCR the amount of synthesised DNA is analysed at the end of the process via gel-electrophoresis. Due to variations in efficiency during the later stages this type of analysis is at best semi-quantitative. A much more accurate and sensitive method is the analysis by real-time quantitative PCR (qPCR) in which the PCR product is detected during the exponential phase. It is this more accurate qPCR that was utilised in this thesis (see section 2.5.5).

2.5.1 RNA extraction

From organ

Organs were excised from the killed fish and stored in RNA later (for recipe see Appendix) at -80 ºC. RNA extraction was carried out with the RNeasy kit (Qiagen) following the manufacturer's instructions. Briefly a small amount of an organ (≤ 30 mg) was placed in 300 µl lysis buffer, sonicated on ice at power 4 (Sonics, vibra-cell) and centrifuged at 17900 g for 10 min at 4 ºC (centrifuge: Sigma 1-15 PK) to sediment any cell debris. The supernatant was mixed with 350 µl of 80 % ethanol and different washing steps and centrifugation steps were carried out on the supplied columns. First the column with the supernatant-ethanol mixture was centrifuged at ≥ 8.000 g for 20 seconds and the flow through was discarded. The column was then washed with 700 µl of the supplied RW1 buffer and centrifuged at ≥ 8.000 g for 20 seconds. 2 washing steps with 500 µl of RPE buffer were carried out. For the first washing step the sample was centrifuged at ≥ 8000 g for 20 seconds while for the second step the centrifugation was prolonged to 2 minutes to ensure removal of ethanol. After this step the collection tube was removed and

the column placed in a sterile 1.5 ml tube. The RNA was eluted in 30 – 50 µl DEPC-treated water by centrifugation at ≥ 8000 g for 1 min. All steps were carried out at room temperature. The concentration of RNA was determined spectrophotometrically (Thermo Scientific, Nanodrop 1000) utilising the corresponding software (nanodrop 1000, version 3.7.1.). RNA samples were diluted to a common concentration ranging from 50 to 1000 ng/µl depending on experiment.

From cell suspension

RNA extraction was carried out as described above with modifications to suit cell suspensions. In brief cells were removed from 24 well plates and concentrated by centrifugation at 300 g (Sigma 1-15 PK). The cell pellet was then re-suspended in the lysis buffer of the RNeasy kit. No sonication was carried out and samples can be stored in lysis buffer at -80 ºC for up to 6 weeks. The extraction steps were carried out as described for organs. Since cell suspensions normally give a low RNA yield the RNA was eluted in 30 µl of DEPC water. RNA concentration was analysed as described above, adjusted to 30 ng/µl unless otherwise stated, stored at -80 ºC and transcribed to cDNA within one week.

2.5.2 Reverse transcription

500 ng RNA were added to 4 µl 25 mM $MgCl_2$, 2 µl 10x PCR Buffer II, 1 µl 10 mM dNTPs, 0.5 µl 50 µM random hexamers, 0.5 µl 40 U/µl RNase inhibitor, and 0.5 µl of 50 U/µl MuLV reverse transcriptase. The samples were adjusted to 20 µl with DEPC treated water. Samples were mixed and centrifuged briefly before incubating at 25 ºC for 10 min followed by 30 min at 42 ºC. Reactions were inactivated by incubation at 95 ºC for

5 min (Thermocycler: Applied Biosystems, GeneAmp PCR System 9700). Samples were diluted 1:10 with DEPC treated water and stored at -20 ºC.

2.5.3 Design and verification of qPCR primers

Genes encoding the following proteins were targeted for the analysis of apoptosis and cell signalling (See section 1.5): iNOS, p53, Apaf-1, Caspase 9, Mcl-1b, IAP, Bcl-2, Nemo and p38. Primers for p53 and Bcl-2 were obtained from Cols Vidal (2006) and primers for Nemo were provided by Mikolaj Adamek. cDNA sequences were obtained from NCBI Genebank® for p38, Apaf-1, iNOS and Mcl-1b (Table 2.3) and from Cols Vidal (2006) for IAP.

A search with NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for sequences similar to Caspase 9 of *Danio rerio* in *C. carpio* revealed and expressed sequence tag (EST) within the carp genome with 64 % similarity (see Appendix). This EST sequence was used to design primers against carp Caspase 9.

The sequences of targeted genes were translated into their amino acid sequence to check for the correct reading frame (http://web.expasy.org/translate/). This ensured that chosen primers occurred within the translated region of the gene. Primers were then designed with the Primer 3 software using the following parameters (Table 2.2).

Table 2.2: Primer properties

	Max	Min	Comments
Melting temperature of primer [ºC]	59	61	Maximum difference between primers: 1ºC
GC content [%]	45	55	Maximum primer 3' GC: 3%
Primer length [bp]	15	25	Optimal length: 20bp
Primer G repeats	3		

From the obtained list of primers the primer pairs with optimal properties were checked for similarity with other genes. For this purpose the primer sequences were inserted into NCBI BLAST and checked against *C. carpio* ESTs and the nucleotide collection. Primers that did not show similarities were tested for their ability to form primer-dimers or hairpins with the oligoanalyser from idtdna (www.idtdna.com). The selected primers were supplied by Eurofins MWG Operon (Germany). A quantitative real-time PCR was run with these primers (for real-time PCR see section 2.5.5) and the product analysed for size and single product on a 2 % agarose gel (see 2.5.4). In case of a single product the DNA was cleaned up and concentrated with the Bioline Isolate PCR and Gel kit (Bioline, BIO-52029). For this purpose 500 µl Binding Buffer A and 50 µl of the PCR mixture were added to the spin column and mixed well by pipetting. The column was spun at 10,000 g for 2 min, the collection tube was discarded and the spin column placed in a new 1.5 ml tube. 20 µl Elution Buffer was pipetted straight onto the membrane of the column and incubated for 5 min at room temperature. Afterwards the column was centrifuged at 6,000 g for 1 min and the concentration of the obtained DNA solution was measured spectrophotometrically (Thermo scientific, Nanodrop 1000) utilising the corresponding software (nanodrop 1000, version 3.7.1.) The PCR product was then sequenced by BioScience (UK) to test if the primers amplified the correct fragment.

Primer efficiencies were determined by measuring the amplification over a range of cDNA template dilutions (1:1 to 1:100) in duplicate. A standard curve of log dilution vs. the threshold cycle was plotted and the efficiency was calculated from the slope of the linear regression. Primer efficiency is defined as $E = 10^{(-1/slope)}$ (Pfaffl 2001; Karlen *et al.*, 2007).

2.5.4 Gel electrophoresis

Small agarose gels were cast at densities of 0.5 – 2 %. Gels with lower densities are required for the analysis of heavier molecules like plasmids or large products. 2 % gels are useful for the analysis of small/light molecules like real-time PCR products. The corresponding amount of agarose (Hi pure low EEO Bio Gene, 300-300) was added to 50 ml of TAE 1x buffer and heated in a microwave (medium setting, 1 min, Proline, SM18) to dissolve. When the agarose was fully dissolved the solution was allowed to cool before 5 µl (i.e. 2.5 µg) of ethidium bromide (Sigma, E1385) were added. The solution was poured into a small gel chamber with comb and left to solidify.

The gel chamber with the gel was placed into a gel tank (Mini Sub cell GT, BioRad) filled with TAE 1x buffer and the comb was removed. PCR product was mixed with 6x orange blue loading buffer (5 µl PCR product to 1 µl loading buffer, Promega G190A) and loaded into the well in the gel. A 100 bp DNA ladder (Promega, G210A) was mixed with loading buffer and placed into one of the wells to give an indication of the product size. The electrophoresis was run at 100 V (BioRad, Power Pack 300) and analysed with a UV camera (Syngene, Gene genius) and corresponding software (Syngene, Gene Snap 7.12).

Table 2.3 List of used qPCR primers

Function	Gene name	Genebank ID	Primer type	Sequence	E ($10^{-1/slope}$)	Primer Source
House-keeping	40S	AB012087	FW	CCGTGGGTGACATCGTTACA	2.00	(Huttenhuis *et al.*, 2006b)
			RV	TCAGGACATTGAACCTCACTGTCT		
NO production	iNOS	AJ242906	FW	TGGTCTCGGGTCTCGAATGT	2.09	
			RV	CAGCGCTGCAAACCTATCATC		
Pro-apoptotic	p53	(Cols Vidal 2006)	FW	CCAAACGCAGCATGACTAAAGA	2.26	(Cols Vidal 2006)
			RV	CGTGCTCAGTTTGGCCTTCT		
	Caspase 9	EC394517.1 (EST)	FW	CGAGAGGGAGTCAGGCTTTC	2.09	
			RV	TCAGAAGGGATTGGCAGAGG		
	Apaf-1	EU490407	FW	CGCTCACAGGTCACACTAGAACTG	2.12	
			RV	AGATACTCACCGGTCCTCCACTT		
Anti-apoptotic	Bcl-2	EU490408	FW	TGTCCCACCAGATGACATTCAG	2.06	(Cols Vidal 2006)
			RV	TCCCACCAAACTCAAAGAAAGG		
	IAP	(Cols Vidal 2006)	FW	CGTGGAGTGGAGGATATGTCTCA	2.04	
			RV	TCCTGTTCCCGACGCATACT		
	Mcl-1b	EU490409.1	FW	TGGAGCAGAAAGGAGAAGATGTG	2.05	
			RV	GCATACCATTGCCCCAAATG		
NF-κB pathway	Nemo	JQ639082	FW	CGCTGAAGAACGAGAGG	2.08	M. Adamek, personal communi-cation
			RV	CTCCTGTGATTGGCTTG		
P38 MAPK pathway	p38	AB023481	FW	CGGCTGACTGATGATGAAATGA	2.11	
			RV	GCATCCAGTTGAGCATGATCTCT		

FW: Forward primer, RV: reverse primer

2.5.5 Real-time PCR

Principle

In real-time PCR a fluorescent dye is used to measure the amount of product during the exponential phase of the PCR reaction. Throughout the process the fluorescent signal is measured and the cycle at which it rises above a background threshold is recorded. This value is denoted as "cycles to threshold" or Ct. It is assumed that the detected fluorescence is directly related to the amount of product and that during the exponential phase this is directly proportional to the quantity of initial template DNA (Higuchi *et al.,* 1992; Karlen *et al.,* 2007). This means that Ct is inversely proportional to the amount of template DNA and that high Ct values represent a low amount while a low Ct equals a high amount of template.

The results can be quantified in different ways depending on the aim of the analysis. Two frequently used methods are the absolute quantification and the relative quantification threshold method. The former is used to establish the number of gene copies in a sample, while the latter is used to compare the difference in expression between samples. In this thesis the relative quantification approach was utilised as it allowed the determination of the change of expression levels between treatments.

Method

Real-time PCR primers specific for apoptotic genes in carp were used and are listed in Table 2.3. The ribosomal 40S gene served as reference gene for the analysis (Huttenhuis *et al.,* 2006b). For the RT-PCR 2 µl of cDNA were added to 10 µl of 2x Power SYBR® Green Master Mix (Applied Biosystems), 1.8 µl of 10 µM (final concentration: 900 nM) forward and reverse primer each and adjusted to 20 µl with DEPC treated water

(Invitrogen). Samples were set up in a 96 well PCR plate (Applied Biosystems, MicroAmp®) and centrifuged at room temperature for 4 min at 440 g (Boeco, U-32R). The qPCR was carried out in an ABI 7000 real-time cycler (Applied Biosystems) with 2 min at 50 ºC, 10 min at 95 ºC and 40 cycles of 15 sec at 95 ºC and 1 min at 60 ºC. After each run melting curves of PCR products were obtained between 60 and 90 ºC with 1 ºC intervals.

Data analysis

Analysis of gene expression was carried out according to the ΔΔCt method (Livak & Schmittgen 2001). In this method the relative change of gene expression is examined. Therefore the gene expression of a treatment group is related to the expression of an untreated control. During the process of cDNA preparation the concentration of cDNA can vary between samples due to differences in preparation such as different amounts or varying quality of starting material (Radonic *et al.*, 2004). Therefore Ct values of the target genes have to be normalized against a reference gene which accounts for inter-sample variability. Suitable reference genes are generally housekeeping genes, e.g. β-actin and ribosomal RNAs, which are not altered during the experimental treatment.

ΔΔCt describes the difference of corrected Ct values between the control and treatment.

$$\text{Relative gene expression} = 2^{-\Delta\Delta Ct}$$

$$\text{With } \Delta\Delta Ct = (C_{T,\,Target} - C_{T,\,Ref})_{Treatment} - (C_{T,\,Target} - C_{T,\,Ref})_{Control}$$

As shown in Table 2.3 the efficiency of all primers was similar and showed a doubling of product with each cycle (i.e. 100 % efficiency). This fulfils the assumption of

the ΔΔCt method that the efficiencies of all amplifications are approximately equal to 2 and equal between reactions (Livak and Schmittgen 2001; Karlen *et al.,* 2007). It was also established that results from the ΔΔCt method are comparable to results from the Pfaffl method. This is another standard method, which takes the primer efficiencies into account (Pfaffl 2001).

40S was determined as the most stable reference gene, i.e. it displayed least up- or down-regulation under the experimental treatments, in comparison with other housekeeping genes (18S, β-actin) using the BestKeeper software (Pfaffl *et al.,* 2004). For gene expression analysis the target genes were normalized against the reference gene 40S and x-fold gene expression was calculated in relation to the control group of each time point.

2.6 Statistics

All statistical analysis was carried out using GraphPad Prism 12 and SPSS 19 (IBM) and all data are presented as mean ± SEM. Data were tested for normality and, if appropriate, for equal distribution of variances. In case of unequal distribution of variances data were normalized with log-transformation or, in case of percentage data, arc-sin transformed, if not stated otherwise. Significance was defined as $p \leq 0.05$. Statistics specific for the individual experiments will be described in the corresponding chapters.

3 Characterisation of the association between immunostimulant and apoptosis *in vitro*

3.1 Introduction

In the introduction to this thesis it was hypothesized that the process of immunostimulation can lead to apoptosis due to the production of cytokines and nitric oxide during the immune response (Figure 1.7).

The aim of this first experimental chapter was therefore to establish if MacroGard® induces apoptosis *in vitro*. To facilitate this aim, a suitable positive control for apoptosis was developed, and various methods of visualizing apoptosis compared and assessed for effectiveness and practicality. For this purpose three different apoptosis inducing factors were tested for their suitability as positive controls: UV light, cortisol and dexamethasone. All three apoptosis inducers were tested in primary cell cultures of freshly isolated pronephric leucocytes from carp (see section 2.2) at a concentration of 1×10^6 cells/ml. The apoptosis inducing potential was then compared in order to ascertain which of these treatments exhibits ideal (i.e. high level of apoptosis vs. length of time needed for treatment) apoptosis inducing properties.

In the introduction to this thesis (chapter 1.3) it was also noted that apoptosis is a process that can be detected not only at the gene expression level but also at the protein and morphological levels. This chapter therefore focuses on the visualisation of apoptosis

on the cellular level using various staining techniques for caspases, DNA strand breaks and nuclear morphology. Numerous methods exist to detect the hallmarks of apoptosis; however these methods have generally been optimized for use in mammals, and hence their suitability for use in fish had to be established prior to their application in carp. Various methods used to ascertain the proportion of apoptotic cells in a cell population were hence tested in order to establish the best method (i.e. least time needed to execute in combination with reliable results). This method was then adopted throughout this thesis.

In previous *in vitro* studies β-glucan displayed a concentration dependent effect with lower concentrations inducing immunostimulatory effects whilst higher concentrations often led to a suppression of the cellular response (Castro *et al.,* 1999). A range of MacroGard® concentrations (low to high) commonly used in animal *in vitro* studies were thus tested for their ability to induce apoptosis in carp pronephric leucocytes. The concentrations of MacroGard® correspond to concentrations used in other investigations involving different experimental animals for example 1 – 200 µg/ml for mouse macrophages (Hetland and Sandven 2002), 100 – 800 µg/ml for porcine leucocytes (Sonck *et al.,* 2010), 1 µg/ml for macrophages of rainbow trout (*Oncorhynchus mykiss*) (Novoa *et al.,* 1996), and 0.5 – 500 µg/ml for the phagocytes of turbot (*Psetta maxima*) and gilthead sea bream (*Sparus aurata*) (Castro *et al.,* 1999).

3.2 Material & Methods

3.2.1 Apoptosis inducing treatments

To ascertain the effectiveness of each of the detection techniques of apoptosis, known induction of this form of cell deaths had to be verified. Two apoptosis inducers were utilised; exposure to UV and treatment with cortisol. The exposure to UV light through its ability to cause DNA damage is known to induce apoptosis in mammalian cells (Bulavin *et al.,* 1999; Kulms and Schwarz 2000) and in carp leucocytes (Cols Vidal 2006; Cols Vidal *et al.,* 2008). Two different UV dosages within the range utilised by Cols Vidal (i.e. 324 J/m^2 and 1080 J/m^2), were tested for their potential to induce apoptosis. For this purpose a UV-C light source (UVP, UVG-54) was used and its intensity ascertained with a UV meter (UVP, Blak-Ray J225) to be 1.8 W/m^2 at a 25 cm distance. UV dosage was calculated as:

UV intensity [W/m^2] x time [s] = dose [J/m^2] (Chen *et al.,* 2006).

Isolated pronephric leucocytes from carp (see section 2.2) were exposed to either 324 J/m^2 (i.e. 3 minute exposure) or 1080 J/m^2 (i.e. 10 minute exposure). Cortisol, a short-acting stress-related glucocorticoid (biological half-life of 8 – 12 hours), has also been shown to induce apoptosis in carp cells (Weyts *et al.,* 1997b; 1998; Verburg-Van Kemenade 1999; Saha *et al.,* 2004; Cols Vidal *et al.,* 2008) at concentrations between 5 – 100 ng/ml. Freshly isolated pronephric cells were therefore incubated for 24 hours with 100 ng/ml water-soluble hydrocortisol (Sigma, H0396).

In comparison to cortisol, dexamethasone, a synthetic corticosteroid analogue, has a longer biological half-life and is active for up to 54 hours (Pavlaki *et al.,* 2011).

Dexamethasone was hence also tested for its apoptosis inducing ability and for this purpose pronephric carp leucocytes were incubated with 100 ng/ml of dexamethasone.

In all three treatments i.e. UV, cortisol and dexamethasone, a range of cytochemical and staining tests were utilised to analyse the levels of apoptosis. Percentage of apoptosis data were acrsin transformed and analysed for significant differences using a 1-way ANOVA with a Tukey post-hoc test.

3.2.2 Assessment of suitability of cytochemical methods in the detection of apoptosis

Live/Dead staining

The live/dead assay distinguishes between live and dead cells by the simultaneous detection of intracellular esterase activity and plasma membrane status. Whilst this assay does not identify apoptotic cells *per se*, it can be used as an alternative to trypan blue (see chapter 2.2.2) when using fluorescence microscopy, fluorescence multi-well plate readers and flow cytometers. The LIVE/DEAD® Viability/Cytotoxicity Assay Kit (Invitrogen, L-3224) utilises calcein AM and ethidium homodimer-1 (EthD-1) dyes. Live cells show an intracellular esterase activity which converts the non-fluorescent cell-permeable calcein AM to the green fluorescing calcein (excitation/emission ~ 495 nm/~ 515 nm). EthD-1 permeates damaged cell membranes and undergoes an enhancement of fluorescence by binding to nucleic acids and thereby produces a bright red fluorescence (ex/em ~ 495 nm/~ 635 nm) in dead cells. Like trypan blue, EthD-1 is excluded by intact plasma membranes of live cells.

Isolated pronephric cells (see section 2.2) at a concentration of 1×10^6 cells/ml were incubated with 50 ng/ml hydrocortisol (Sigma, H0396) for 24 hours to induce cell death and the Live/Dead assay was conducted according to the manufacturer's instructions. In brief, cells were washed with PBS (600 g for 10 min), incubated with 250 µl EthD-1/Calcein AM for 15 min, washed three times with PBS (Sigma Aldrich, P4417), fixed with 200 µl of 4 % paraformaldehyde (Sigma Aldrich, 16005) for 20 min and washed again three times with PBS. The cell pellet was re-suspended in a small amount of the supernatant and stained cells were examined under the fluorescence microscope (Nikon Eclipse E400) with an FITC-Rhodamin filter (excitation band pass of 490 nm and emission at 520 nm). Pictures were taken with a digital camera (Nikon D70s).

CaspaTag staining

One central component of the apoptotic process is the caspase cascade during which inactive caspases are cleaved and thereby activated (Takle and Andersen, 2007). The CaspaTag™ Pan-Caspase *In Situ* Assay kit (Millipore, APT420) detects active caspases through FLICA (fluorochrome inhibitors of caspases) (Bedner *et al.*, 2000). The caspase inhibitor used is a carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor (FAM-VAD-FMK), which binds to a reactive cysteine residue of the active caspase heterodimer and which inhibits further enzymatic activity of the caspase. This treatment leads to a green fluorescence of the apoptotic cells.

In order to induce apoptosis 100 µl of isolated pronephric leucocytes (1×10^7 cells/ml) were incubated with 100 ng/ml hydrocortisol (Sigma, H0396) for 6 and 24 hours. The CaspaTag assay was then carried out according to the supplier's instructions:

30 µl of the cell suspension were incubated with 1 µl FLICA for 1 hour at 20 °C and swirled every 20 min. After the incubation period approximately 1 ml of supplied wash buffer was added to the cells followed by a centrifugation step (600 g for 5 min). The pellet was subjected to another washing step before the cells were re-suspended in 30 µl wash buffer. The labelled cells were then analysed under the fluorescence microscope (Nikon Eclipse E400) with an excitation band pass of 490 nm and emission at 520 nm (FITC–Rhodamin filter). Apoptotic cells were characterized by a green fluorescence. Pictures were taken with a digital camera (Nikon D70s).

TUNEL staining

DNA fragmentation is one of the key characteristics of apoptosis (see section 1.3) and is produced by endonucleolytic degradation of higher-order chromatin structures. As a consequence of these DNA strand breaks apoptotic cells contain a large amount of free 3'-OH termini. The APO-BrdU^{TM} TUNEL (terminal deoxynucleotide transferase dUTP nick end labelling) assay kit (BD Biosciences, 556405) uses terminal deoxynucleotidyl transferase (TdT) to add bromo-deoxyuridine triphosphate nucleotides (Br-dUTP) to the free 3'-OH termini of the DNA strand breaks. A fluorescein labelled antiBrdU monoclonal antibody is then used to identify these added Br-dUTP sites. Non-apoptotic cells contain very few DNA 3'-OH ends and will therefore not be significantly stained by this assay (Phoenix Flow Systems 2009). This method can be used for quantitative studies by fluorescence microscopy and flow cytometry, and has also been used for visualizing apoptosis in histological preparations (Negoescu *et al.,* 1998; Vermes *et al.,* 2000; Kim *et al.,* 2009a).

The TUNEL assay, which was conducted according to the supplier's instructions (Phoenix Flow Systems, AU1001), comprised two steps: fixation and staining.

A pronephric cell suspension (see section 2.2) in which apoptosis had been induced by incubation with 50 ng/ml hydrocortisol (Sigma, H0396) for 24 hours was concentrated by centrifugation (1500 g for 9 min with a Sanyo MSE, Micro Centaur centrifuge) and the supernatant discarded. 1 – 2 x 10^6 cells were incubated on ice for 15 min with 5 ml of 1 % paraformaldehyde in PBS (1 ml paraformaldehyde in 3 ml PBS). The cells were then centrifuged at 300 g for 5 min and the supernatant discarded. The cell pellet was washed twice with PBS (centrifugation at 300 g for 5 min), resuspended in 500 µl PBS and stored in 5 ml of 70 % ethanol at -20 °C until further processing.

For the TUNEL staining process positive and negative control cells supplied by the manufacturer were treated in parallel. The ethanol was removed from the cells by centrifuging the cells at 300 g for 5 minutes and cells were washed twice with 1 ml of the provided wash buffer (centrifugation at 600 g for 5 min). Cells were then incubated for 60 min in 50 µl freshly prepared DNA Labeling Solution containing 10 µl TdT reaction buffer, 0.75 µl TdT enzyme, 8 µl Br-dUTP and 32.25 µl H_2O. The incubation was carried out at 37 °C and the cells were shaken every 15 min, after which 1 ml of the provided Rinse Buffer was added and the cells centrifuged (600 g for 5 min) and the supernatant removed. After another washing step, cells were incubated at room temperature for 30 min in the dark in a freshly prepared fluorescein~PRB-1 containing antibody solution (5 µl fluorescein~PRB-1 plus 95 µl Rinse Buffer).

The labelled cells were then analysed with a UV-microscope (Nikon Eclipse E400) using a FITC filter for the green TUNEL staining. Under these conditions a green

fluorescence characterized labelled apoptotic cells. Pictures were taken with an attached digital camera (Leica, DC 200).

Acridine orange

The nuclear morphological changes during the process of apoptosis can be visualized by the green luminescence which occurs when acridine orange intercalates into double-stranded DNA (excitation/emission 502 nm/525 nm) (Traganos *et al.,* 1977; Daryznkiewicz, 1990).

In order to induce apoptosis in the cells a primary cell culture of isolated pronephric leucocytes from carp (see section 2.2) was incubated with 5 ng/ml hydrocortisol (Sigma, H0396) for 24 hours. If not otherwise stated 100 µl of this cell suspension was concentrated by centrifugation (670 g; 4 min) and an equal volume (10 µl) of cell suspension and acridine orange solution (10 µg/ml in sterile H_2O, Sigma Aldrich, A 6014) was mixed on the slide and viewed with an UV-microscope (Nikon Eclipse E400-excitation 490 nm; emission 520 nm -FITC-filter). The percentage of apoptotic cells was determined by counting a minimum of 200 cells at x400 magnification. Pictures were taken with a digital camera (Nikon D70s) and Nikon Capture 4.2 (Nikon) software.

3.2.3 Time and concentration dependency of MacroGard® induced apoptosis

Carp were killed and the pronephric cells isolated and cultured in RPMI+ medium as described in section 2.2. The viability of the cell population was ascertained with trypan blue staining and only cell suspensions with a viability of at least 95 % were used. The concentration of the cell suspensions was measured by counting the cells with a haemocytometer (section 2.2.2) and the cells were then diluted to 1 x 10^7 cells/ml with RPMI+ medium. Cells were set up in 96 well plates (Sarstedt, UK) with 100 µl per well. A stock solution of MacroGard® was prepared as described (chapter 2.4) and dilutions were prepared with sterile water to give the following end-concentrations in the cell culture: 1, 50, 100, 250, 500, and 1000 µg/ml after addition of 5 µl of the MacroGard® solution. In addition three controls were set up: an untreated control (i.e. no additives), a control with H_2O as additive and a positive UV exposed (324 J/m^2) control. Cells where then incubated for 6 h (concentration 50 and 1000 µg/ml were not analysed at this time points), 24 h (50 µg/ml was not analysed at this time point) or 48 h (1000 µg/ml was not analysed at this time point) at 20°C. Apoptosis was visualized using the acridine orange staining described above.

Prior to statistical analysis the data were arcsin transformed to meet the assumption of normal distribution. Missing data points (n = 3) were calculated using the Trend function in Excel. The data were analysed for the influence of exposure time (i.e. 6, 24, and 48 hours) and the various treatments (i.e. controls and MacroGard® concentrations) with 2-way repeated ANOVA. For the analysis of the distinct effects the treatments had on apoptosis a 1-way ANOVA was performed for the individual time points with a

subsequent Turkey's post-hoc test. Analyses were performed using Minitab Release 14 and GraphPad Prism 4.

3.3 Results

3.3.1 Apoptosis inducing treatments

In the UV treatment (Figure 3.1) the proportion of apoptotic cells in the cell population was significantly different between treatments ($F = 16.7$, $p < 0.0001$). Both doses, i.e. 324 J/m^2 and 1080 J/m^2, caused apoptosis, which was significantly elevated (23.7 ± 4.3 and 30 ± 3 % respectively) compared to 5.7 ± 0.7 % apoptosis occurring in the untreated control (both $p < 0.001$). Cortisol and dexamethasone exposure only led to slightly elevated apoptosis (9.3 ± 0.8 % and 10.6 ± 0.5 % respectively) but this was not significantly different to the untreated control. Apoptosis caused by the UV treatments was hence significantly higher than apoptosis caused by the cortisol and dexamethasone treatments ($p \leq 0.05$).

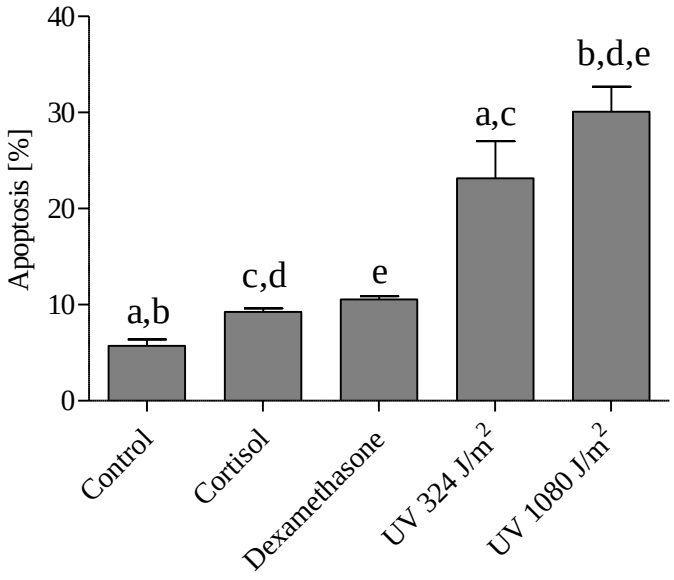

Figure 3.1: Apoptosis inducing potential of UV light, cortisol and dexamethasone

Percentage of apoptotic cells in pronephric leucocytes after exposure to cell culture medium (= control, n = 6), 100 ng/ml cortisol (n = 2), 100 ng/ml dexamethasone (n = 2) or UV light in doses of 324 J/m^2 (n = 5) or 1080 J/m^2 (n = 3). Apoptosis was recorded after 24 hours by analysing the proportion of cells with fragmented nuclei in the cell suspension with acridine orange staining. Data are displayed as mean ± SEM and significance was defined as $p \leq 0.05$. Same letters indicate differences between samples. a, b: $p \leq 0.001$, d: $p \leq 0.01$ c, e: $p \leq 0.05$.

3.3.2 Assessment of suitability of cytochemical methods in the detection of apoptosis

Live/Dead staining

Red and green fluorescent cells were observed under the microscope (Figure 3.2) which reflect dead and live cells. Hence it can be concluded that dead and live cells were distinguished utilizing the Live/Dead assay in pronephric cells, which had been treated with 50 ng/ml cortisol for 24 hours.

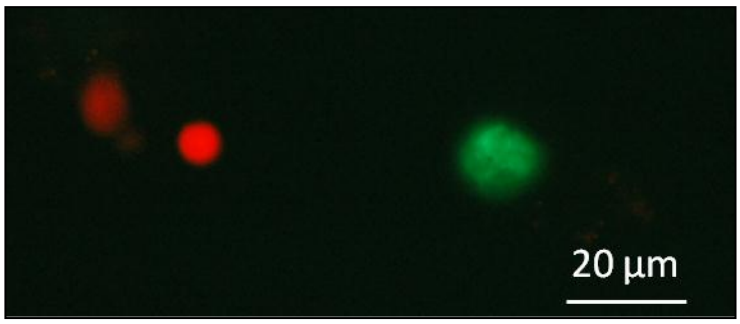

Figure 3.2: Live/Dead staining
Isolated pronephric cells were exposed to 50 ng/ml cortisol and cultured for 24 h and then stained with the Live/Dead staining to distinguish live (green) and dead (red) cells.

CaspaTag staining

In the analysis no active caspases were detected after 24 hours however after 6 hours apoptotic and non-apoptotic cells could be differentiated with the CaspaTag assay (Figure 3.3).

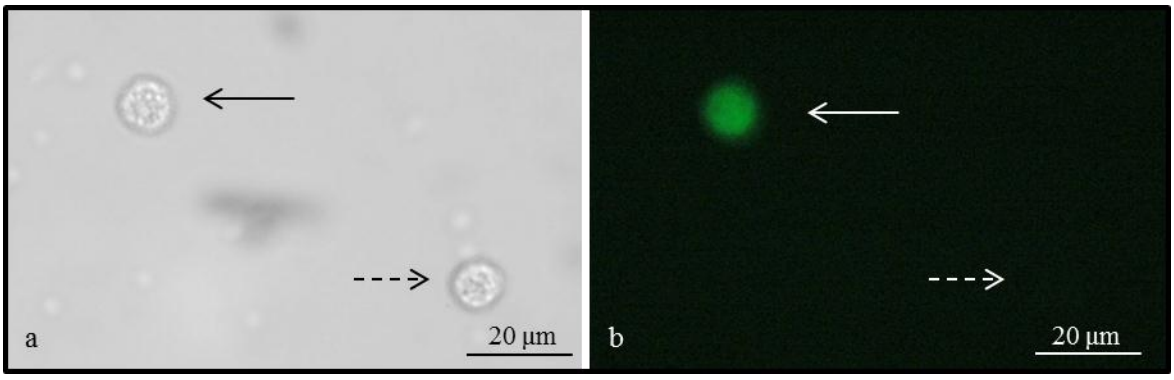

Figure 3.3: CaspaTag staining

Isolated pronephric cells were exposed to 100 ng/ml cortisol, cultured for 6 hours and apoptotic cells were visualized with CaspaTag. In the figure cells are displayed under white light (a) and UV light (fluorescence excitation) (b). The continuous arrows indicate the same cell, which is CaspaTag positive, under the two light conditions. The other cell (disconnected arrow) in a) is CaspaTag negative and thus does not fluoresce in b).

TUNEL

In mammals TUNEL is a well established apoptosis detection system, e.g. in mice (Majumdar *et al.*, 2009) and humans (Saraste and Pulkki, 2000; Kim *et al.*, 2009a), but it has also been used to detect apoptosis in the teleost system (Nolan *et al.*, 2002; Cols Vidal, 2006; Monteiro *et al.*, 2009) and in fish cell lines, e.g. brown bullhead fibroblast cell line (Busch *et al.*, 2004). In the present study clear images of apoptotic cells could be observed (Figure 3.4) after treatment of pronephric cells with cortisol (50 ng/ml) for 24 hours.

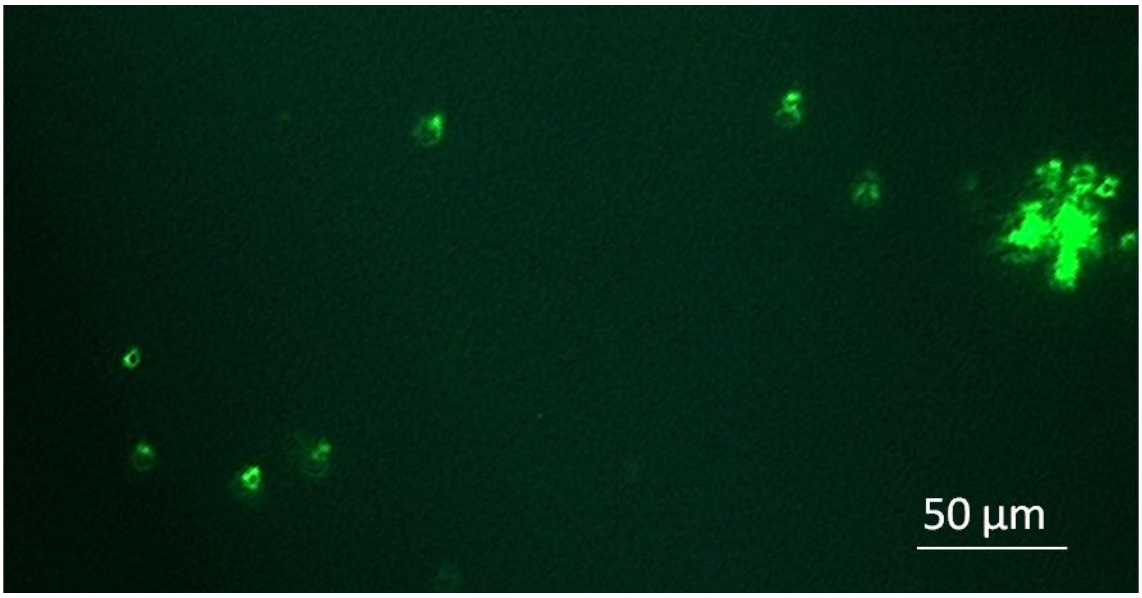

Figure 3.4: TUNEL staining

Cells from the pronephros of carp were isolated, exposed to 50 ng/ml cortisol, cultured for 24 hours and then stained with the TUNEL staining. The green fluorescence indicates free 3' OH-ends, as they occur during the DNA strand breaks caused by the apoptosis process.

Acridine Orange

In cell cultures, which had been treated with cortisol, cells with fragmented nuclei were observed (Figure 3.5). Such morphological changes of the nucleus are a hallmark of apoptosis and therefore these cells were defined as apoptotic.

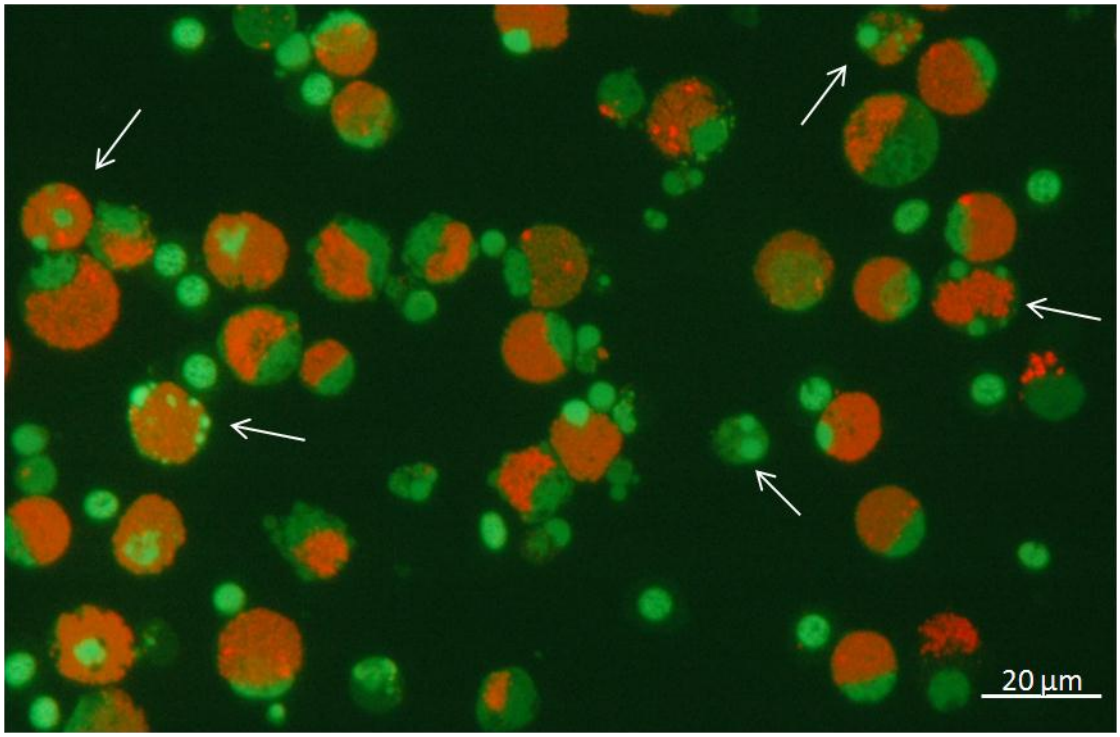

Figure 3.5: Acridine orange staining
Cells from the carp pronephros were isolated, exposed to 5 ng/ml cortisol and cultured for 24 hours. Staining was carried out with acridine orange. The arrows indicate cells with fragmented nuclei, which is a hallmark of apoptosis.

3.3.3 Time and concentration dependency of MacroGard® induced apoptosis

The concentration of MacroGard® ($F = 9.426$, $p < 0.0001$) and the exposure time ($F = 15.81$, $p < 0.0001$) had a significant effect on apoptosis levels. No interaction between these two factors was found, which means that the effect of treatment was independent of the exposure duration (Figure 3.6).

At all time points there was no significant difference detected in apoptosis levels of the two negative controls (i.e. untreated and with added water). By running a positive control (i.e. UV exposure) alongside the samples it was established that apoptosis could be induced in the tested cell population. This UV exposure caused higher percentages of apoptosis positive cells after 6 hours (15.8 ± 1.54 %) and 24 hours (32.3 ± 2.8 %) when compared to the non-treated control (both $p \leq 0.001$). When cells were exposed to MacroGard® for 6 hours none of the tested concentrations (1 – 500 µg/ml) induced apoptosis in the pronephric carp leucocytes. Most effects were observed after 24 hours of MacroGard® exposure. At this time point 500 and 1000 µg/ml induced significantly higher apoptosis in the cell culture (i.e. 14.5 ± 2.4 % and 32.2 ± 5.0 % respectively) compared to the non-treated control (5.1 ± 0.7 % apoptosis) ($p \leq 0.01$ and 0.001 respectively) and the 1 µg/ml MacroGard® concentration (7.0 ± 1.1 % apoptosis) ($p \leq 0.05$ and 0.001 respectively).

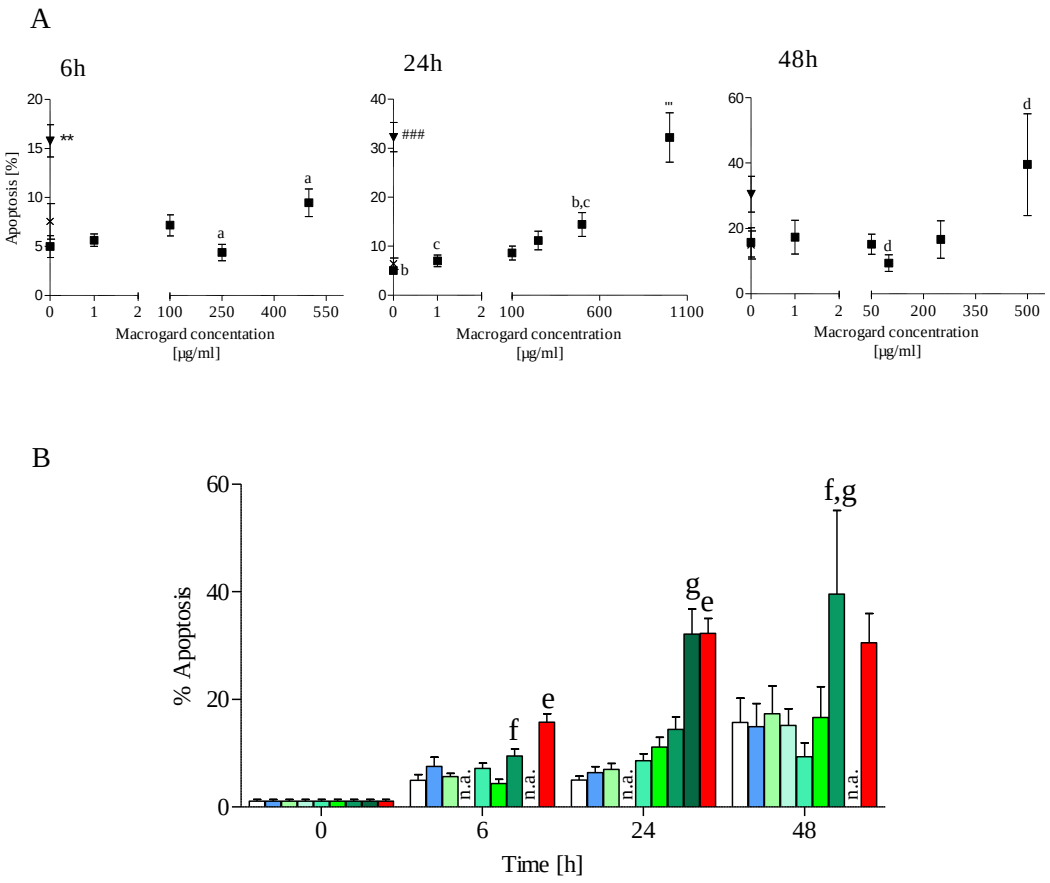

Figure 3.6: Time and concentration dependency of MacroGard® influence on apoptosis

Percentage of apoptotic cells in the cell suspension was analysed with acridine orange. Cell suspensions were treated with different concentrations of MacroGard® and incubated over 3 different time periods. Significance was defined as $p \leq 0.05$. Symbols for significant differences: * = significantly different to all other samples with ** = $p \leq 0.01$ and *** = $p \leq 0.001$; ### = significantly different to all other samples except 1000 µg/ml with $p \leq 0.001$; ''' = significantly different to all other samples except UV with $p \leq 0.001$, same letters indicate differences between samples (with a, c, d, f = $p \leq 0.05$, b = $p \leq 0.01$, f, g = $p \leq 0.001$). If one sample is significantly different to a range of other samples this is indicated by arrows, n.a. = not analysed. Data are shown as mean ± SEM with n = 8. A) Concentration dependency of MacroGard® effects at different time points. X = water control, ▼ = UV control, n = 6 – 8. B). Time dependency of various MacroGard® concentrations. = UV, = 1000 µg/ml, = 500 µg/ml, = 250 µg/ml, = 100 µg/ml, = 50 µg/ml, = 1 µg/ml, = water, = 0 µg/ml.

3.4 Discussion

Apoptosis inducing treatments

Neither of the two tested glucocorticoids induced significant apoptosis in the cell culture, which is not consistent with results from studies by Saha *et al.*, (2004) and Cols Vidal (2006), who reported approximately 20 % apoptosis in cortisol treated cell cultures. This contradiction could be due to the slightly lower cortisol concentrations used by Cols Vidal (2006) and the different apoptosis detection system utilized (acridine orange in the present study vs. flow cytometry used by Saha *et al.* (2004)).

The only treatment that significantly increased apoptosis was the UV exposure and hence UV exposure was adopted as a positive control throughout this thesis. In the study conducted by Cols Vidal (2006) only the dose of 1560 J/m^2 significantly increased apoptosis and the proportion of apoptotic cells detected after UV treatment (1560 and 376.2 J/m^2) were reduced by 30 – 50 % compared to the result in the present study. This difference in susceptibility to UV radiation may be due to the origin of the target cells utilised. Cols Vidal (2006) studied apoptosis in a carp leucocyte cell line whilst a heterogeneous pronephric cell suspension was used in the present investigation. This may suggest that in the pronephric cell suspension some cell types may be more vulnerable to UV light than the homogeneous macrophage-like cells used by Cols Vidal (2006). Since there was no significant difference between levels of apoptosis induced by the two UV treatments utilised, the shorter exposure time (i.e. UV dose of 324 J/m^2) was used as a positive control for apoptosis. Such a level of UV exposure, i.e. 324 J/m^2, has also been used by other authors to induce apoptosis, for example 200 – 800 J/m^2 by Leverkus *et*

al., (1997), 150 – 300 J/m^2 by Chaturvedi *et al.*, (2005), and 300 J/m^2 by Narine *et al.*, (2010).

Assessment of suitability of cytochemical methods in the detection of apoptosis

In this chapter various methods have been tested for their ability to detect apoptosis in pronephric cells. The Live/Dead staining distinguished between live and dead cells. This corresponds to previous studies since its effectiveness has also been shown in studies involving mammalian cells (Nakagawa *et al.*, 2000) and cell lines (Monack *et al.*, 1996), insects (Hunter and Birkhead, 2002) and fish (Winzer *et al.*, 2001; Schönleber and Anken, 2004). As mentioned the Live/Dead staining can be utilized as an alternative to the trypan blue staining and Hu *et al.*, (2006) demonstrated for a mammalian cell line that these two viability tests correspond in the detected cellular viability. However trypan blue is, compared to the Live/Dead assay, less time consuming and therefore preferable for the purpose of a quick viability assessment.

The CaspaTag assay is utilized in a variety of animal models, cell types and cell lines to detect caspase activity (e.g. human spermatozoa (Paasch *et al.*, 2003), chick cochlea (Kaiser *et al.*, 2008), *Plasmodium berghei* (Al-Olayan *et al.*, 2002), carp leucocytes (Cols Vidal 2006), EoL-1 eosinophilic cell line (Al-rabia *et al.*, 2004), CEM-C7 leukemic human T-cell line (Mourtada-Maarabouni *et al.*, 2008), carp leucocyte cell line (Cols Vidal *et al.*, 2009)). In the present study the CaspaTag assay detected apoptotic cells in the cell culture after 6 hours of incubation with cortisol. This corroborates the findings by Cols Vidal (2006) that this staining is suitable for visualizing apoptosis in carp leucocytes within a few hours after the treatment with cortisol. CaspaTag detects activated caspases, and since no apoptotic cells were detected in the samples taken after 24 hours of

cortisol exposure, it is possible that the cortisol exposure used only induced apoptosis immediately after treatment. The biological half-life of cortisol is 8 – 12 hours (Pavlaki *et al.,* 2011) and therefore after 24h only the very late stages of apoptosis may be present, which would have to be visualized using the TUNEL and acridine orange staining. Hence it was confirmed in this chapter that TUNEL can be used to detect apoptosis in a carp leucocyte culture after 24 hours exposure to cortisol as previously seen by other authors (Weyts et al. 1997; Cols Vidal, 2006).

The acridine orange staining is a popular technique for apoptosis assessment and has been used in various organisms, such as *Drosophila melanogaster* (Cashio *et al.,* 2005; Arama and Steller 2006), cell lines from humans and mice (Longthorne and Williams, 1997; Krzyminska *et al.,* 2009), and fish (Cols Vidal, 2006). This staining method is the least time consuming assay (10 min from start to analysis) of the techniques tested in this chapter (i.e. TUNEL: 3 hours, CaspaTag: 1.5 hours) and was therefore the method of choice for the quick assessment of apoptosis in cells throughout this thesis. However, as fragmentation of the nucleus is a late stage characteristic of apoptosis, early apoptosis is not detected with this method, which must be taken into account with respect to analysis and interpretation of results.

In summary it was concluded that all the staining methods used are suitable to visualize apoptosis in carp cells. It appears that TUNEL and acridine orange are suitable to assess late stages of apoptosis whilst the CaspaTag assay may only be suitable for early apoptosis detection. Some of the assays were more time consuming than others and some involved a more complex protocol and hence acridine orange was chosen as the standard staining method for apoptosis, as it provided the best compromise between staining clarity and ease of use.

Table 3.1: Overview of utilized methods to visualize apoptosis

Method	Time required to execute staining	Apoptosis stage detected
CaspaTag	1.5 hours	Early stage
TUNEL	3 hours	Late stage
Acridine orange	10 minutes	Late stage

Time and concentration dependency of MacroGard® induced apoptosis

The *in vitro* effects of MacroGard® have not been widely studied and only few authors have used cell cultures to ascertain effects of this feed supplement in fish (Jørgensen and Robertsen 1995; Novoa *et al.,* 1996; Castro *et al.,* 1999). Even though the MacroGard® concentrations tested in the present chapter correspond to concentrations used previously by other authors (Novoa *et al.,* 1996; Castro *et al.,* 1999) the β-glucan effects on cell death have not been previously evaluated in fish.

In humans fungal β-glucan has been tested for its ability to cause apoptosis (Kim *et al.,* 2009a) since this might be responsible for the observed β-glucan induced tumour reduction during cancer treatment (Kobayashi *et al.,* 2005; Yamamoto *et al.,* 2009). In the study conducted by Kim *et al.,* (2009a) it was shown that pure fungal β-glucan can induce apoptosis in human tumour cells at a concentration of 100 µg/ml after 24 hours. According to the supplier (personal communication R. Nordmø, Biorigin) MacroGard® contains approximately 60 % β-glucan. Therefore a dose of 100 µg/ml pure β-glucan as used by

Kim *et al.*, (2009a) is equal to circa 170 µg/ml of MacroGard®. This is the range in which increased levels of apoptosis were observed in the present study but the increase was not significant below 500 µg/ml MacroGard® (= 300 µg/ml pure β-glucan). It can therefore be concluded that apoptosis can be induced by MacroGard® when used at high concentrations and that the apoptosis inducing effects are consistent with those expected from the β-glucan component of this feed supplement.

In the present study as well as in several other studies it has been demonstrated that the effects of β-glucan are dose dependent (Castro *et al.*, 1999; Hauton and Smith 2004; Ai *et al.*, 2007). For instance the cell viability in lobster granulocytes decreased with an increase from 50 to 250 µg/ml in MacroGard® concentration (Hauton and Smith 2007). The respiratory burst activity in response to MacroGard® exposure is also dependent on the applied concentration as demonstrated by Castro *et al.*, (1999). In their report concentrations ranging from 0.5 to 5 µg/ml had no effect on the production of ROS while concentrations of 7.5 to 500 µg/ml significantly induced the respiratory burst. In addition the authors investigated the stimulatory effects of MacroGard® and found that low concentrations (i.e. 1 – 2.5 µg/ml) led to higher ROS production after secondary stimulation with another ROS inducing agent compared to cell cultures that were not pre-treated with MacroGard®. In the same study higher concentrations of MacroGard® (i.e. 10 – 500 µg/ml) inhibited the ROS response to the secondary stimulation, which was interpreted by the authors as a sign of exhaustion of the cells. However in regard to the findings described in this chapter it is possible that the lower ROS production could be due to the onset of apoptosis and thus lower cell numbers in the culture. Reactive oxygen species are known for their cytotoxicity in fish (Risso-de Faverney *et al.*, 2001; Xiang and Shao 2003) and hence it is possible that the higher β-glucan concentrations induce ROS,

causing apoptosis which then results in lower respiratory burst activity due to the reduced cell numbers.

This *in vitro* concentration dependency is also reflected *in vivo* when the immunostimulant is administered orally or by injection. For instance the respiratory burst activity of the pronephric cells of sea bass (*Dicentrarchus labrax*) depended on the administered oral MacroGard® concentration (0.025 - 0.1 %) (Bonaldo *et al.*, 2007). In this study concentrations of 0.05 and 0.1 % MacroGard® in the feed caused high levels of ROS production at day 10 and 14 of the trial but the 0.1 % concentration caused a strong reduction of respiratory burst activity after day 14. This *in vivo* concentration and time dependency will be further discussed in chapter 4.

Within this chapter it has therefore been concluded that staining with acridine orange, because of its easy of use and reliability, is the detection method of choice for apoptosis. However fragmentation of the nucleus can also occur during late stages of necrosis (Fairbairn and Oneill 1995), hence in the next chapters the morphological changes related to apoptosis detected with acridine orange are supported by analysis of the expression of pro- and anti-apoptotic genes.

In addition, MacroGard® induced morphological changes related to apoptosis in pronephric leucocyte cultures of carp when administered at high concentrations (≥ 500 µg/ml) over 24 hours but has no such effect at low concentrations (≤ 250 µg/ml). This can be expected since most substances are toxic above a certain threshold (Turner 1996). This concentration dependency has to be considered during dietary administration of the immunostimulant. In the setting of fish farms it is important to feed MacroGard® both at a

concentration high enough to stimulate the immune system but also low enough to avoid adverse effects. The latter is especially important since it can lead to immunosuppression and thus render the animals more vulnerable to pathogens. In order to avoid such negative effects it has to be established that the MacroGard® concentrations used *in vivo* do not induce apoptosis. The aim of the next chapter (chapter 4) is therefore to investigate the apoptotic effects of the dietary MacroGard® concentration used by the aquaculture industry.

4 Apoptosis after MacroGard® feeding and PAMP exposure

In the previous chapter (chapter 3) the *in vitro* capacity of MacroGard® to induce apoptosis was investigated. This chapter will extend these observations and will focus on the *in vivo* effect of MacroGard® on apoptosis. In doing so it reflects the situation in aquaculture conditions in which MacroGard® is fed during periods of increased vulnerability to diseases. Additionally the association between this feeding protocol and bacterial and viral products on the apoptosis process is investigated.

4.1 Introduction

The dietary administration of various forms of β-glucan supplemented feed has shown contradictory effects on the fish's immune response. Many studies have reported a dependency on the administered dose as well as the feeding duration (see Table 1.1). These effects have either stimulating or suppressing effects on the immune system and can modulate the immune response towards an intruding pathogen. In this chapter so called pathogen associated molecular patterns (PAMPs) were used to mimic bacterial (LPS) and viral diseases (Poly(I:C)).

Lipopolysaccharide (LPS), also known as endotoxin, is an outer-membrane component of gram-negative bacteria and as a PAMP (see chapter 1.1.2) it induces an immune response in mammals and fish (Nayak *et al.,* 2008; Swain *et al.,* 2008; Nayak *et al.,* 2011). LPS has therefore been used to mimic bacterial infections in animals (Boltaña *et al.,* 2009; Chettri *et al.,* 2011), and in mammals it can induce an often fatal septic shock. Lower vertebrates such as fish and amphibians on the other hand have a lower sensitivity to this PAMP and do not experience septic shock. Nevertheless LPS is a major virulence factor and responsible for pathogenicity of several bacterial infections in fish (Swain *et al.,* 2008). Studies revealed that LPS can induce NO production and increase iNOS gene expression in primary fish cell cultures of rainbow trout (*Oncorhynchus mykiss*) and goldfish (*Carassius auratus*) (Laing *et al.,* 1999; Saeij *et al.,* 2000). LPS has also been associated with apoptosis in the thymus of mice (Zhang *et al.,* 1993), in goldfish lymphocytes *in vitro* (Xiang *et al.,* 2008) and in brook trout ovary *in vivo* (MacKenzie *et al.,* 2006).

Poly(I:C) (Polyinosinic:polycytidylic acid) is a synthetic double stranded RNA, so called dsRNA, which is produced by many viruses during their replication. Like LPS, dsRNA acts as a PAMP and provokes an anti-viral immune response (Fortier *et al.,* 2004). Various studies in mammals and fish have therefore used Poly(I:C) to mimic viral infections (Fortier *et al.,* 2004; Reimer *et al.,* 2008; Boltaña *et al.,* 2009). The immune response in fish after exposure to this PAMP has been associated with cytokine production, induction of the respiratory burst and enhanced levels of iNOS (Falco *et al.,* 2007; Boltaña *et al.,* 2009; Chettri *et al.,* 2011). The anti-viral response also led to apoptosis of rainbow trout macrophages *in vitro*, which is interpreted as a defense mechanism to limit virus replication in the host cells (DeWitte-Orr *et al.,* 2005).

The aim of this chapter was to analyze the possible apoptotic effects of dietary MacroGard® and to investigate the mechanisms by which the immunostimulant influences the gene expression pattern in experimental systems modeling bacterial and viral infections. For this purpose the following genes were chosen for analysis:

- Nemo & p38 for exploration of signaling pathways
- iNOS for the expression of inducible NO synthase
- Caspase 9, Apaf-1, p53 as pro-apoptotic genes
- Bcl-2 & IAP as anti-apoptotic genes

The analysis was conducted in four organs, which are related to the immune response: Pronephros, spleen, mid-gut and liver.

4.2 Materials & Methods

4.2.1 Oral MacroGard® administration and PAMP injections

Common carp (*Cyprinus carpio*) with an average weight of 40 g were kept in individual tanks with circulated water at 16 ºC at Keele University as described in chapter 2.1. The fish were divided into two experimental groups, distributed to 2 tanks and fed a MacroGard®-free control diet for two weeks. Following this acclimatization phase one group of fish was kept on the control diet (1 % bodyweight per day) while the other group were fed 1 % bodyweight of a 0.1 % MacroGard® containing feed, which corresponds to 10 mg/kg bodyweight MacroGard® per day (recommended dose by commercial suppliers Biorigin, Brazil). The fish were then dissected as described in chapter 2.1 and organ samples of liver, mid-gut, pronephros and spleen were taken at 7 and 25 days of MacroGard® feeding. A small amount of pronephros tissue was used for morphological analysis of apoptosis utilising acridine orange staining (chapter 3.3.4) and the remaining organs were stored in RNAlater (Invitrogen, UK) at - 80 ºC for analysis of gene expression. Samples for real-time PCR were prepared as described in chapter 2.5.

At day 25 of the feeding experiment fish from both feeding regimes were intraperitoneally (i.p.) injected with 100 µl PBS (Sigma, P4417) containing either LPS (4 mg/kg: from E. coli 0111:B4, tlrl-eblps, Invivogen, UK) or Poly(I:C) (5 mg/kg: tlrl-picw, Invivogen, UK), or just PBS alone. These doses of LPS and Poly(I:C) correspond to the concentrations used by Xu *et al.*, (2010) where they led to a modulation of immune-relevant chemokine receptors in grass carp (*Ctenopharyngodon idella*). The fish of the 6 treatments (i.e. Control fed plus either PBS, LPS or Poly(I:C) injection and MacroGard® fed plus PBS, LPS or Poly(I:C) injection) were distributed to 6 tanks. Tissue samples of

spleen, pronephros, liver and mid-gut were then taken at 1 and 7 days post injection (p.i.) as described in chapter 2.1 and tissue samples of pronephros were analysed for apoptosis with acridine orange (as shown in chapter 3.3.4). Figure 4.1 shows a graphical description of the experimental design.

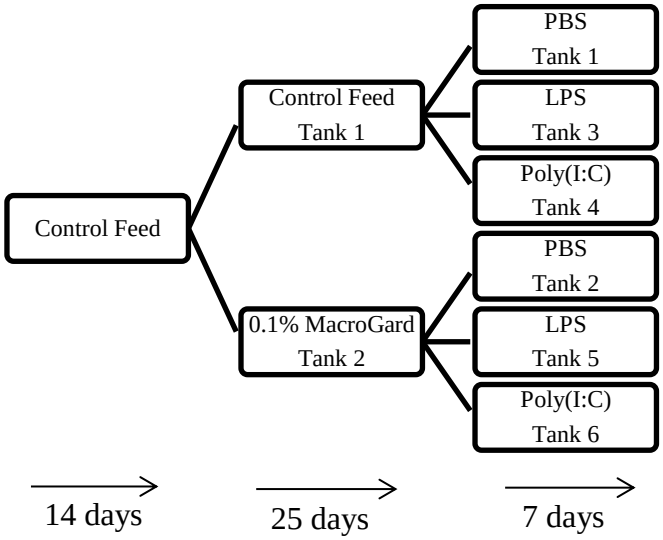

Figure 4.1: Graphical depiction of experimental design.

The experimental design consisted of a 14 day acclimatisation period in which fish were fed a MacroGard® free control diet. Fish were then introduced to two feeding regimes: Control feed (0 % MacroGard®) and feed containing 0.1 % MacroGard®. Fish were sampled at 7 and 25 days of feeding. At day 25 fish were injected with 100 µl PBS, PBS + 4 mg/kg LPS or PBS + 5 mg/kg Poly(I:C). Fish were sampled at day 1 and day 7 p.i.

4.2.2 Statistics

Before statistical analysis the data were tested for equal distribution of variances and were transformed to meet this assumption. For this purpose percentage data were arcsin transformed and gene expression data (x-fold data) were log-transformed.

For the data analysis results from the feeding, LPS, and Poly(I:C) study were analysed separately. For this purpose a 2-way ANOVA with “Treatment” and “Experimental duration” as the variables was used. A Bonferroni post-hoc test analysed the statistical differences between all treatment groups for each time point. All data are displayed as mean ± SEM and significance was defined as $p \leq 0.05$.

4.3 Results

4.3.1 MacroGard® feeding

Feeding of MacroGard® over a period of 25 days had no influence on the percentage of pronephric cells displaying fragmented nuclei (Figure 4.2). In both feeding regimes a maximum of 2 % of the pronephric cells were apoptotic, which can be considered as baseline apoptosis.

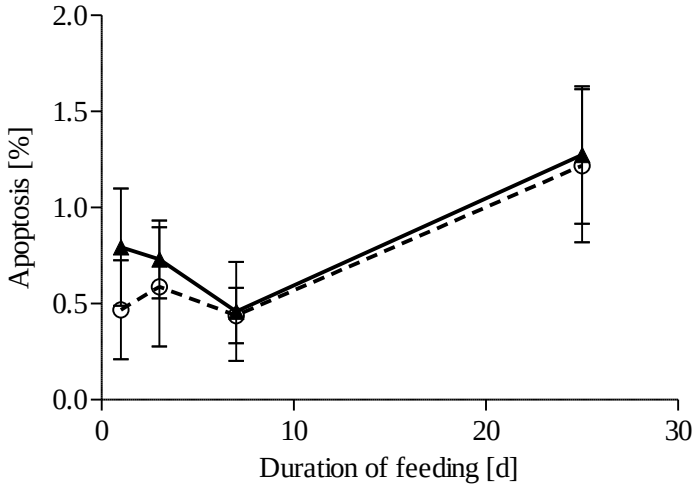

Figure 4.2: Percentage of apoptotic cells during MacroGard® feeding
Percentage of apoptotic cells, defined as cells with fragmented nuclei in the pronephros of *C. carpio* either fed with a 0 % (○) or a 0.1 % (▲) MacroGard® supplemented diet. Graph shows mean ± SEM. No significant differences ($p \leq 0.05$) were detected.

At the gene expression level the MacroGard® effect was dependent on the organ examined and varied with the feeding duration (Table 4.1). Of all the analysed organs liver was least affected by the feeding treatment, since in this organ only the expression of iNOS is significantly different between treatments. Spleen on the other hand displayed the most response with four genes (i.e. iNOS, Caspase 9, Bcl-2, Nemo) being differently regulated between treatments. The analysis also revealed an influence of the experimental duration which was evident in the fact that most gene up-regulation only occurred at 25 days of feeding and not at day 7. This is also reflected in the significant interaction between the treatment and the time factor of some genes. For example in pronephros and spleen the treatment induced change in iNOS gene expression was also significantly different at the two sampling points.

Table 4.1: 2-way ANOVA of x-fold gene expression during feeding treatment

		Interaction		Treatment		Time	
Pronephros		**F**	**p**	**F**	**p**	**F**	**p**
I	iNOS	10.76	0.006	12.94	0.003	10.42	0.006
+	p53	2.82	0.11	2.43	0.14	3.37	0.085
	Caspase 9	0.96	0.34	0.0007	0.98	1.26	0.28
	Apaf-1	0.015	0.91	8.44	0.01	0.04	0.84
-	IAP	2.83	0.11	1.15	0.30	1.76	0.20
	Bcl-2	8.65	0.01	3.74	0.07	10.1	0.006
S	p38	2.88	0.11	0.007	0.93	3.09	0.10
	Nemo	1.15	0.30	0.97	0.34	1.59	0.23
Spleen							
I	iNOS	11.78	0.003	9.19	0.008	11.11	0.004
+	p53	8.84	0.009	3.02	0.10	8.66	0.010
	Caspase 9	2.05	0.17	11.15	0.004	2.44	0.14
	Apaf-1	0.005	0.54	0.03	0.13	0.004	0.57
-	IAP	0.003	0.96	0.018	0.90	0.034	0.86
	Bcl-2	7.20	0.02	4.85	0.04	7.02	0.018
S	p38	12.61	0.003	1.08	0.32	12.44	0.003
	Nemo	2.98	0.10	11.05	0.004	2.59	0.13
Gut							
I	iNOS	2.49	0.14	10.47	0.006	2.46	0.14
+	p53	0.54	0.48	6.71	0.02	0.57	0.46
	Caspase 9	3.62	0.08	1.33	0.27	3.97	0.07
	Apaf-1	0.45	0.51	0.002	0.97	1.37	0.26
-	IAP	10.46	0.005	6.10	0.03	11.12	0.004
	Bcl-2	10.06	0.007	3.82	0.07	9.37	0.009
S	p38	0.0004	0.98	1.12	0.31	1.84	0.19
	Nemo	6.84	0.02	2.35	0.15	7.66	0.01
Liver							
I	iNOS	1.33	0.27	41.98	<0.0001	0.14	0.72
+	p53	0.03	0.87	0.94	0.35	0.02	0.89
	Caspase 9	0.35	0.56	0.17	0.69	0.24	0.63
	Apaf-1	0.38	0.55	0.14	0.71	0.70	0.42
-	IAP	0.26	0.62	1.98	0.18	0.13	0.72
	Bcl-2	0.33	0.57	1.58	0.23	0.47	0.50
S	p38	1.39	0.26	1.74	0.21	1.56	0.23
	Nemo	0.06	0.81	0.08	0.79	0.35	0.57

Grey background indicates significance, Gene function coding: I: involved in the immune response, +: pro-apoptotic, -: anti-apoptotic, S: involved in cell signalling.

In spleen (Figure 4.3) pro- as well as anti-apoptotic genes were significantly increased ($p \leq 0.01$). Caspase 9 and Bcl-2 experienced a 2 – 3 fold increase in gene expression at 25 days of feeding, whilst pro-apoptotic p53 was the only gene up-regulated on day 7 likewise displaying a 2 – 3 fold enhancement. iNOS and the members of key cell signalling pathways p38 and Nemo were also enhanced by day 25 of MacroGard® feeding ($p \leq 0.01$). In the other main immune organ, the pronephros, 25 day of MacroGard® administration enhanced iNOS expression approximately 4 times compared to the control ($p \leq 0.001$). The expression of pro-apoptotic genes was not significantly influenced under this treatment but mRNA levels of anti-apoptotic Bcl-2 were approximately 4 times higher ($p \leq 0.01$) than in the control (Figure 4.3). At 25 days of feeding the induced iNOS gene expression (~ 4 fold) in gut (Figure 4.4) was accompanied by enhanced levels of anti-apoptotic genes (Bcl-2, IAP) ($p \leq 0.01$). Additionally Nemo expression was induced as a response to MacroGard® feeding ($p \leq 0.05$). As mentioned earlier the liver (Figure 4.4) was influenced least by the MacroGard® treatment, which only enhanced iNOS gene expression at both 7 and 25 days of feeding ($p \leq 0.01$).

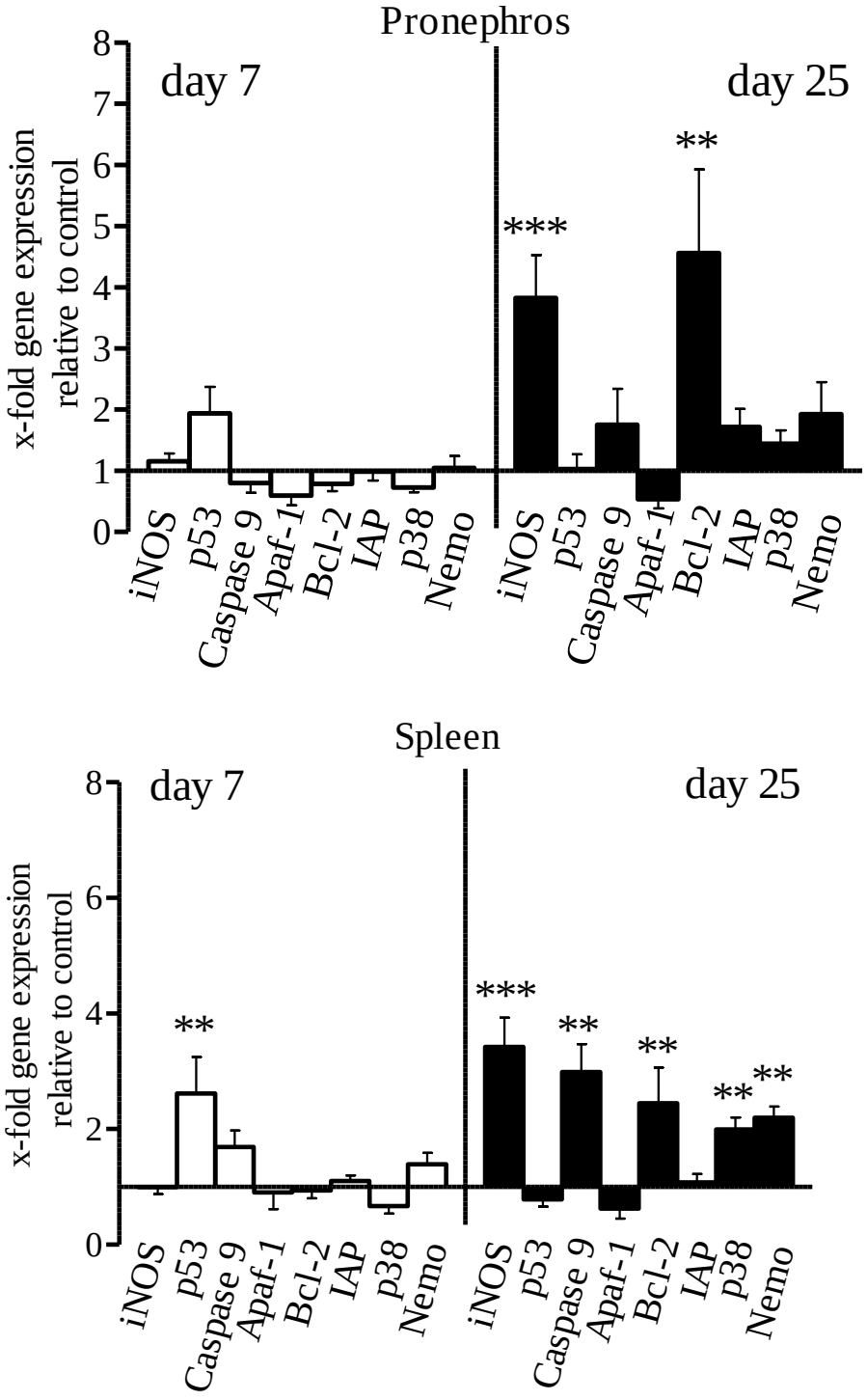

Figure 4.3: Gene expression in pronephros and spleen during feeding

Carp were fed with control (results not shown since equal to 1) or 0.1 % MacroGard® supplemented diet. Samples were taken at 7 (white bars) and 25 days (black bars) of feeding. Gene expression was analysed and displayed as x-fold gene expression relative to control. Graphs show mean ± SEM with n = 5. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

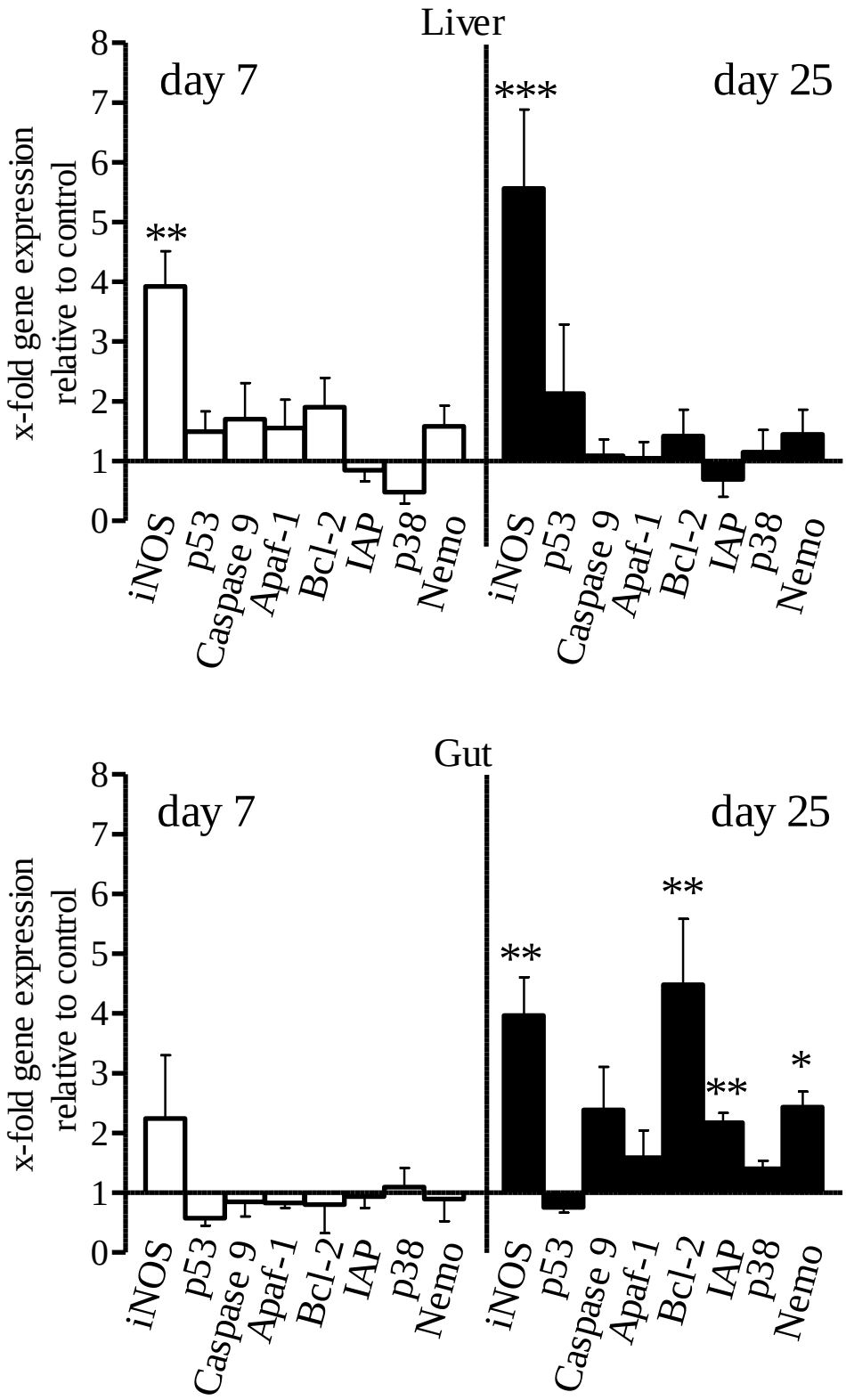

Figure 4.4: Gene expression in liver and gut during feeding

Carp were fed with control (results not shown since equal to 1) or MacroGard® containing diet. Samples were taken at 7 (white bars) and 25 days (black bars) of feeding. Gene expression was analysed and shown as x-fold gene expression relative to control. Graphs show mean ± SEM with n = 5. **: $p \leq 0.01$, ***: $p \leq 0.001$.

4.3.2 Treatment with PAMPs

After the injection of LPS and Poly(I:C) no significant changes in apoptosis levels were observed in any of the treatments. The maximum percentage of apoptotic cells was 1.4 %, which implies that apoptosis was not elevated from baseline levels (Figure 4.5).

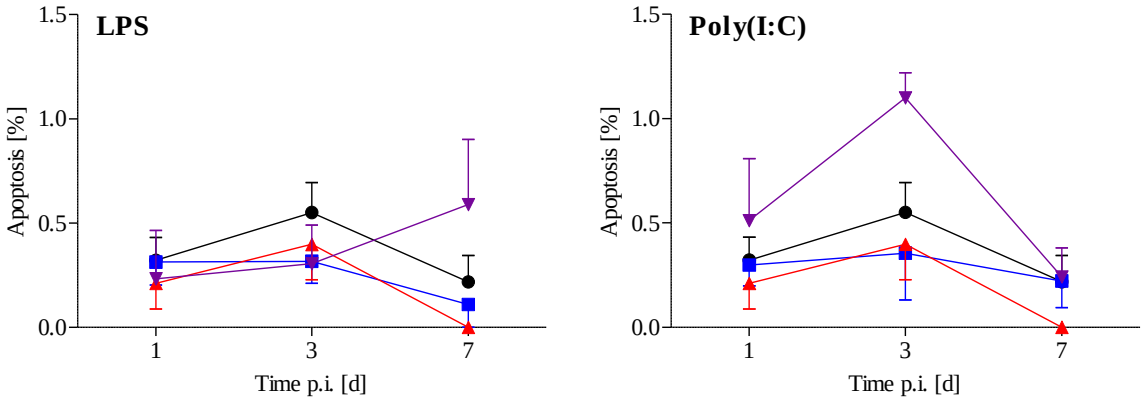

Figure 4.5: Percentage of apoptotic cells after PAMP injection
Apoptosis was defined as cells with fragmented nucleus as detected by acridine orange staining. Fish that had been fed a control or a MacroGard® containing diet were injected with LPS or Poly(I:C). ●: control feed + PBS injection, ■: control feed + LPS/Poly(I:C) injection, ▲: MacroGard feed + PBS injection, ▼: MacroGard® feed + LPS/Poly(I:C) injection n = 4, graphs show mean ± SEM.

Poly(I:C) injection

This sub-section focusses on the influence of Poly(I:C) on the animals fed with or without MacroGard®. The effect of LPS is discussed later and also the influence of MacroGard® on the PBS injected fish is described later in this results section.

MacroGard® and Poly(I:C) most influenced the gene expression in gut of treated fish (Table 4.2). In this organ Bcl-2 was the only gene not affected by any of the applied conditions while all other genes were differentially expressed due to these treatments ($F = 3.0 – 27.1$, $p \leq 0.05$). Most gene expression profiles, except for iNOS and Caspase 9, were also influenced by the time course of the experiment ($F = 18.6 – 50.6$, $p \leq 0.0002$) and an additional interaction between this time factor and the treatment factor was also detected for most genes ($F = 3.3 – 10.6$, $p \leq 0.04$).

Table 4.2: 2-way ANOVA analysis of x-fold gene expression in gut samples during Poly(I:C) study

		Interaction		Treatment		Time	
		F	p	F	p	F	p
I	iNOS	1.69	0.20	7.36	0.001	2.63	0.12
+	p53	10.57	0.0001	16.98	< 0.0001	45.17	< 0.0001
	Caspase	3.52	0.03	3.03	0.05	0.01	0.91
	Apaf-1	4.63	0.01	27.07	< 0.0001	32.87	< 0.0001
-	Bcl-2	3.37	0.04	1.06	0.39	22.69	< 0.0001
	IAP	4.89	0.009	7.93	0.0008	33.64	< 0.0001
S	p38	4.53	0.01	5.47	0.005	18.61	0.0002
	Nemo	7.10	0.001	9.24	0.0003	50.64	< 0.0001

Grey background indicates significance, Gene function coding: I: involved in the immune response, +: pro-apoptotic, -: anti-apoptotic, S: involved in cell signalling.

In the fish that received control feed the gut is the only organ where an effect on iNOS gene expression was detected in response to Poly(I:C) injection, which caused a reduction of mRNA levels by 95 % ($p \leq 0.01$) on day 7 p.i. (Figure 4.6). An additional down-regulation occurred in the expression of IAP at day 1 p.i., which resulted in a decrease to approximately 20 % of the control levels ($p \leq 0.05$). However MacroGard® feeding altered the Poly(I:C) induced expression profile of pro- and anti-apoptotic genes, which is summarized in Table 4.5 at the end of this result section. In this group of fish IAP gene expression was doubled ($p \leq 0.05$) and Caspase 9 gene expression was significantly, approximately 5-fold, elevated ($p \leq 0.01$) 1 day after the injection when compared to the MacroGard® fed control. Furthermore p38 expression levels were increased after the combined MacroGard® and Poly(I:C) treatment ($p \leq 0.05$). The influence of MacroGard® becomes apparent when both Poly(I:C) treatment groups (control and MacroGard® fed) were compared. The supplemented feed led to a significant elevation of pro-apoptotic (i.e. Caspase 9, Apaf-1, p53) and anti-apoptotic (IAP) genes ($p \leq 0.05 - 0.001$). In addition it caused elevated levels of the cell signalling genes p38 and Nemo ($p \leq 0.05$ and 0.001 respectively).

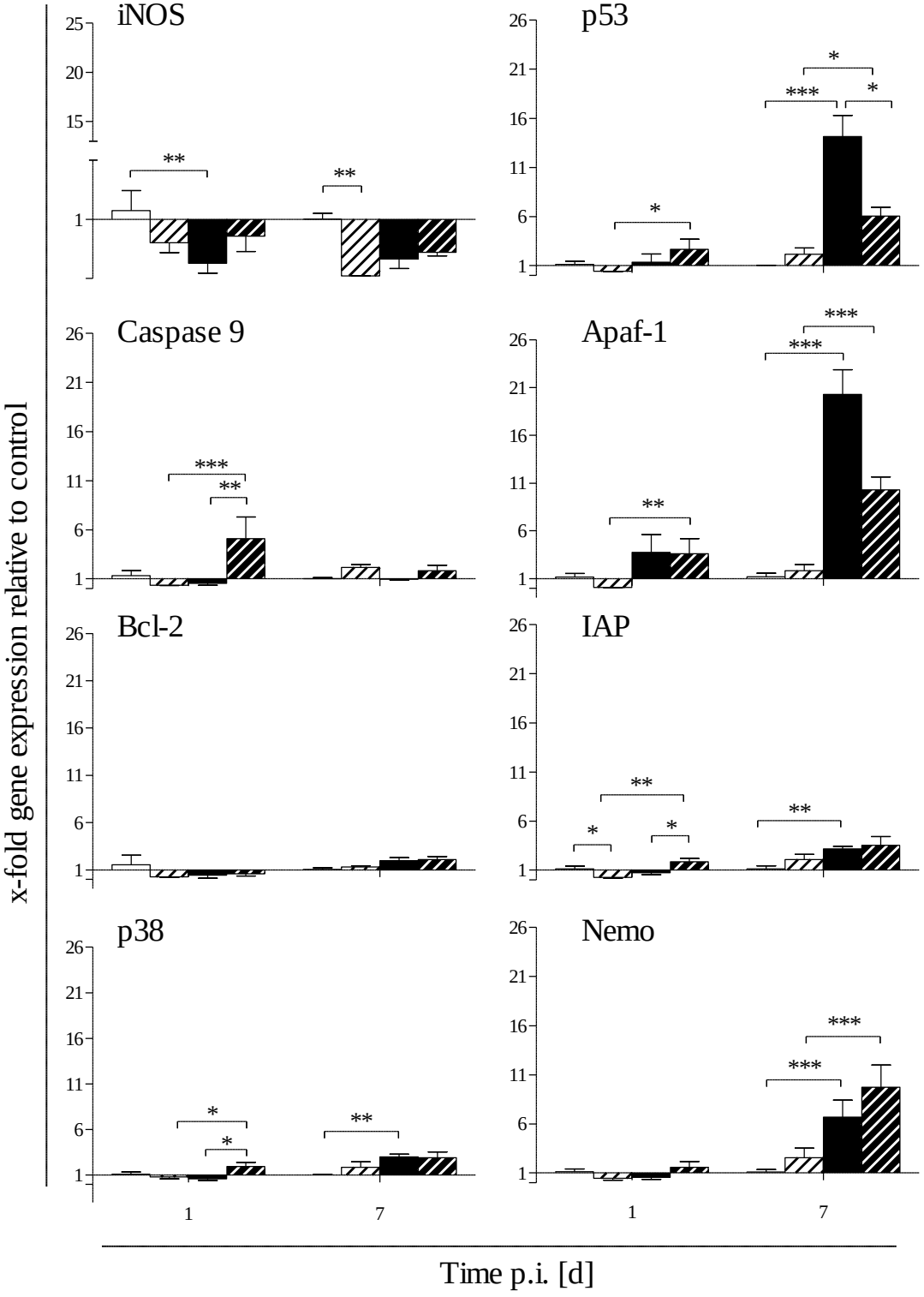

Figure 4.6: Gene expression in gut during Poly(I:C) study

Carp were fed with control or MacroGard® containing feed prior to injection with Poly(I:C). X-fold gene expression was analysed relative to control. Bars: white: control feed + PBS, white striped: control feed + Poly(I:C) injection, black: MacroGard® feed + PBS, black striped: MacroGard® feed + Poly(I:C). Graph shows mean ± SEM with n = 4. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$

Even though the ANOVA (Table 4.3) detected an influence of the different treatments on expression of all genes in liver, except the pro-apoptotic genes Caspase 9 and Apaf-1 ($F = 5.8 – 16.3$, $p \leq 0.005$), post-hoc analysis indicates that these changes are mostly due to the feeding of MacroGard® and not due to the Poly(I:C) injection (Table 4.5). The expression pattern of most genes changed with the time course of the experiment ($F = 8.2 – 27.5$, $p \leq 0.009$) but an interaction between the two analysed factors was only detected in p53 and IAP ($F = 12.6$ and 3.5 respectively, $p \leq 0.03$).

Table 4.3: 2-way ANOVA analysis of x-fold gene expression for liver samples during Poly(I:C) study

		Interaction		Treatment		Time	
		F	p	F	p	F	p
I	iNOS	1.346	0.2865	5.761	0.0049	13.96	0.0012
+	p53	12.6	< 0.0001	13.29	< 0.0001	24.48	< 0.0001
	Caspase	2.96	0.052	0.13	0.94	11.2	0.003
	Apaf-1	0.85	0.48	0.81	0.50	2.10	0.16
-	Bcl-2	2.14	0.12	7.17	0.001	8.20	0.009
	IAP	3.52	0.03	16.25	< 0.0001	27.49	< 0.0001
S	p38	0.49	0.69	6.87	0.002	2.78	0.11
	Nemo	2.72	0.08	10.82	0.0001	19.07	0.0002

Grey backgrounds display significance. Gene function coding: I: involved in the immune response, +: pro-apoptotic, -: anti-apoptotic, S: involved in cell signalling.

The post-hoc analysis (Figure 4.7) revealed that the liver displayed the least response to the Poly(I:C) injections when compared to the other organs analysed, since only IAP gene expression was up-regulated due to this treatment ($p \leq 0.01$). Also no influence on gene regulation was detected when fish from the two MacroGard® treatment groups (PBS and Poly(I:C) injected) were compared. On day 1 p.i. Poly(I:C) injected carp from the MacroGard® fed group showed elevated levels of iNOS ($p \leq 0.05$) and anti-apoptotic genes, i.e. Bcl-2 ($p \leq 0.01$) and IAP ($p \leq 0.05$), when compared to PBS treated fish from the same feeding regime. This expression profile was not apparent at the later sampling day, instead gene levels of IAP ($p \leq 0.05$), p53 and Nemo (both $p \leq 0.01$) were enhanced.

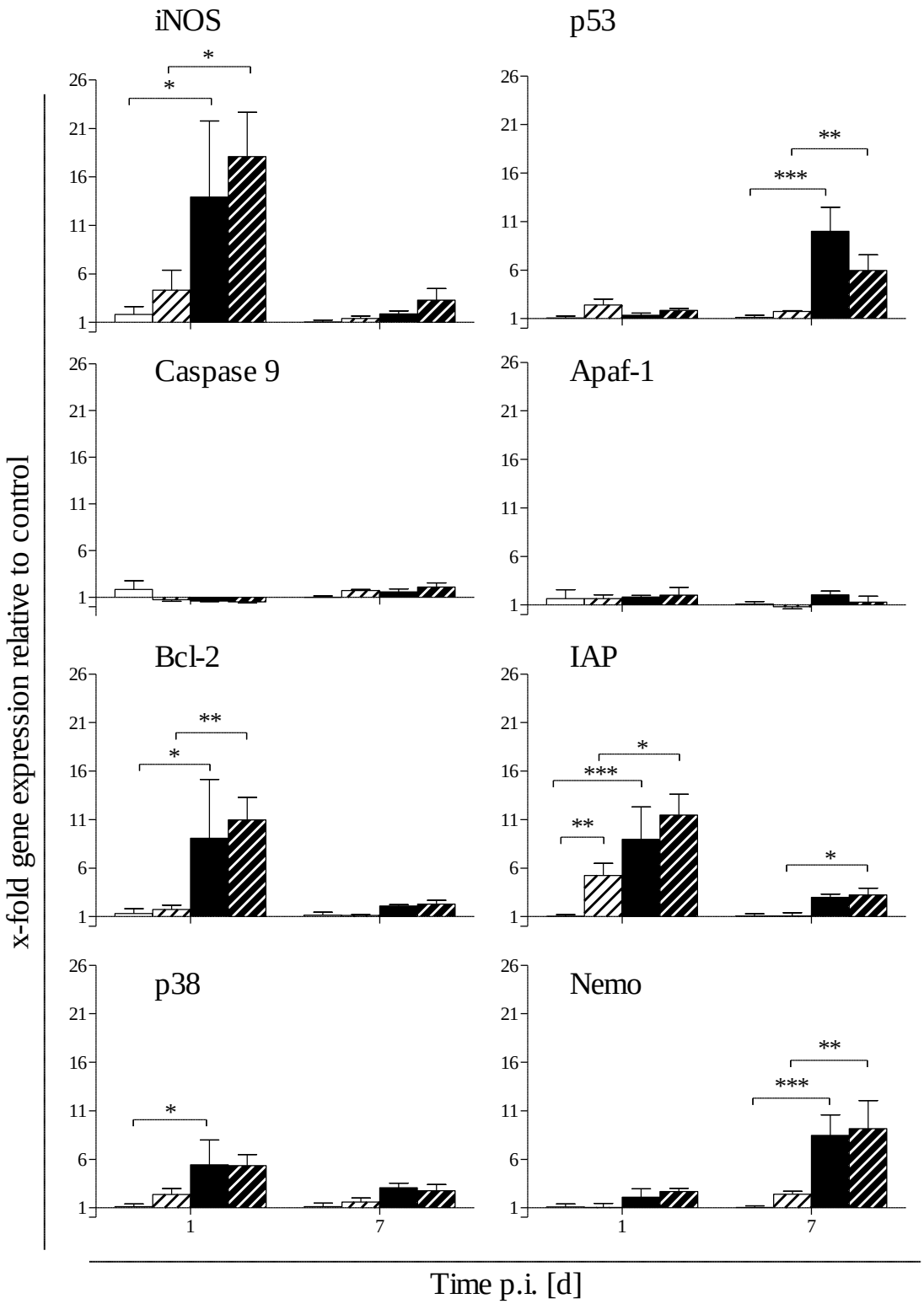

Figure 4.7: Gene expression in liver during Poly(I:C) study

Carp were fed with control or MacroGard® containing feed prior to injection with Poly(I:C). X-fold gene expression was analysed relative to control. Bars: white: control feed + PBS, white striped: control feed + Poly(I:C) injection, black: MacroGard® feed + PBS, black striped: MacroGard® feed + Poly(I:C). Graph shows mean ± SEM with n = 4. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$

The pronephros and spleen were similarly affected by the treatments (Table 4.4). In both cases anti-apoptotic gene expression was not significantly different between treatments whereas genes involved in the induction of apoptosis, e.g. Apaf-1 and p53, as well as iNOS and cell signalling genes were influenced by the treatments ($F = 5.8 – 24.3$, $p \leq 0.004$). The time course influenced the expression profile differently in these two organs. Whilst in spleen only iNOS, Caspase 9 and Nemo were influenced by the experimental duration ($F = 4.5 – 18.8$, $p \leq 0.04$), the pronephros showed an influence on all genes analysed except Bcl-2 ($F = 5.1 – 33.9$, $p \leq 0.03$).

Table 4.4: 2-way ANOVA analysis of x-fold gene expression in pronephros and spleen samples during Poly(I:C) study

		Interaction		Treatment		Time	
Pronephros		F	p	F	p	F	p
I	iNOS	2.20	0.12	5.97	0.004	5.14	0.03
+	p53	6.02	0.003	15.11	< 0.0001	7.33	0.01
	Caspase	2.67	0.07	1.86	0.16	7.25	0.01
	Apaf-1	1.88	0.16	14.15	< 0.0001	6.46	0.02
-	Bcl-2	1.51	0.24	0.37	0.78	2.71	0.11
	IAP	6.58	0.002	2.46	0.09	33.91	< 0.0001
S	p38	3.39	0.03	1.92	0.15	10.93	0.003
	Nemo	2.64	0.07	12.8	< 0.0001	12.01	0.002
Spleen							
I	iNOS	1.34	0.29	5.85	0.004	4.54	0.05
+	p53	3.1	0.05	10.53	0.0001	2.57	0.12
	Caspase	2.4	0.09	2.27	0.11	15.81	0.0006
	Apaf-1	1.70	0.19	16.31	< 0.0001	0.11	0.75
-	Bcl-2	1.40	0.27	1.61	0.21	0.36	0.56
	IAP	1.36	0.28	2.64	0.07	3.20	0.09
S	p38	8.81	0.0005	13.51	< 0.0001	0.00000	1.00
	Nemo	1.67	0.20	24.32	< 0.0001	8.17	0.009

Grey backgrounds display significance. Gene function coding: I: involved in the immune response, +: pro-apoptotic, -: anti-apoptotic, S: involved in cell signalling.

The injection of Poly(I:C) had no influence on the gene expression pattern in pronephros (Figure 4.8) in control fed fish but in combination with MacroGard® feeding an up-regulation of cell signalling genes occurred on day 1 p.i., i.e. p38 expression was doubled ($p \leq 0.05$) and Nemo gene levels were increased 4 times ($p \leq 0.01$) when compared to the MacroGard® control group. Additionally mRNA levels of p53 (4-fold) and iNOS (5-fold) were elevated on day 1 and day 7 respectively ($p \leq 0.01$). As with most of the studied organs the influence of MacroGard® feeding was greatest when the two Poly(I:C) injection groups were compared (Table 4.5). In this case all pro-apoptotic genes were affected by the MacroGard® treatment, while no changes in anti-apoptotic genes were observed. For example Apaf-1 gene expression was enhanced approximately 4-fold on day 1 and 6-fold on day 7 p.i. ($p \leq 0.01$). Furthermore iNOS and Nemo gene expression was significantly enhanced in the MacroGard® fed Poly(I:C) injected fish, when compared to the Poly(I:C) injected control fed fish ($p \leq 0.01$).

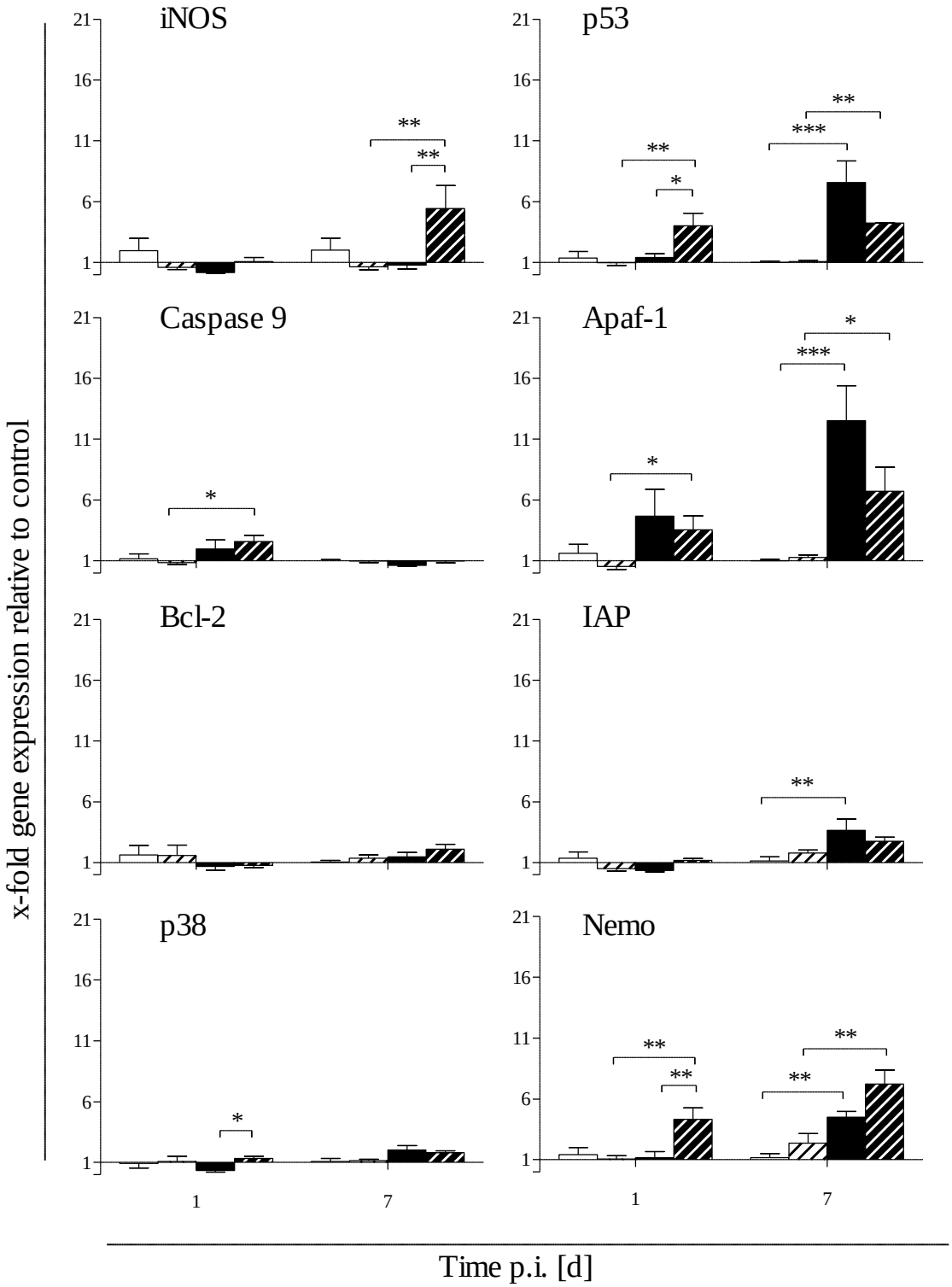

Figure 4.8: Gene expression in pronephros during Poly(I:C) study

Carp were fed with control or MacroGard® containing feed prior to injection with Poly(I:C). X-fold gene expression was analysed relative to control. Bars: white: control feed + PBS, white striped: control feed + Poly(I:C) injection, black: MacroGard® feed + PBS, black striped: MacroGard® feed + Poly(I:C). Graph shows mean ± SEM with n = 4. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$

The injection of control fed fish with Poly(I:C) significantly ($p \leq 0.05$) increased gene expression levels of Caspase 9 and it halved Bcl-2 gene expression levels in the spleen on day 1 p.i. (Figure 4.9). Poly(I:C) exposure of MacroGard® fed fish up-regulated p38 and Nemo gene expression ($p \leq 0.001$) but did not alter gene expression levels of apoptosis-related genes when compared to the PBS injected group of the same feeding regime. Oral administration of β-glucan supplemented feed changed the expression profile of Nemo and pro-apoptotic genes in Poly(I:C) injected fish, for instance Apaf-1 expression was increased 4-fold in MacroGard®/Poly(I:C) treated fish at day 1 p.i. and doubled at the later sampling point when compared to the control fed/Poly(I:C) treated fish.

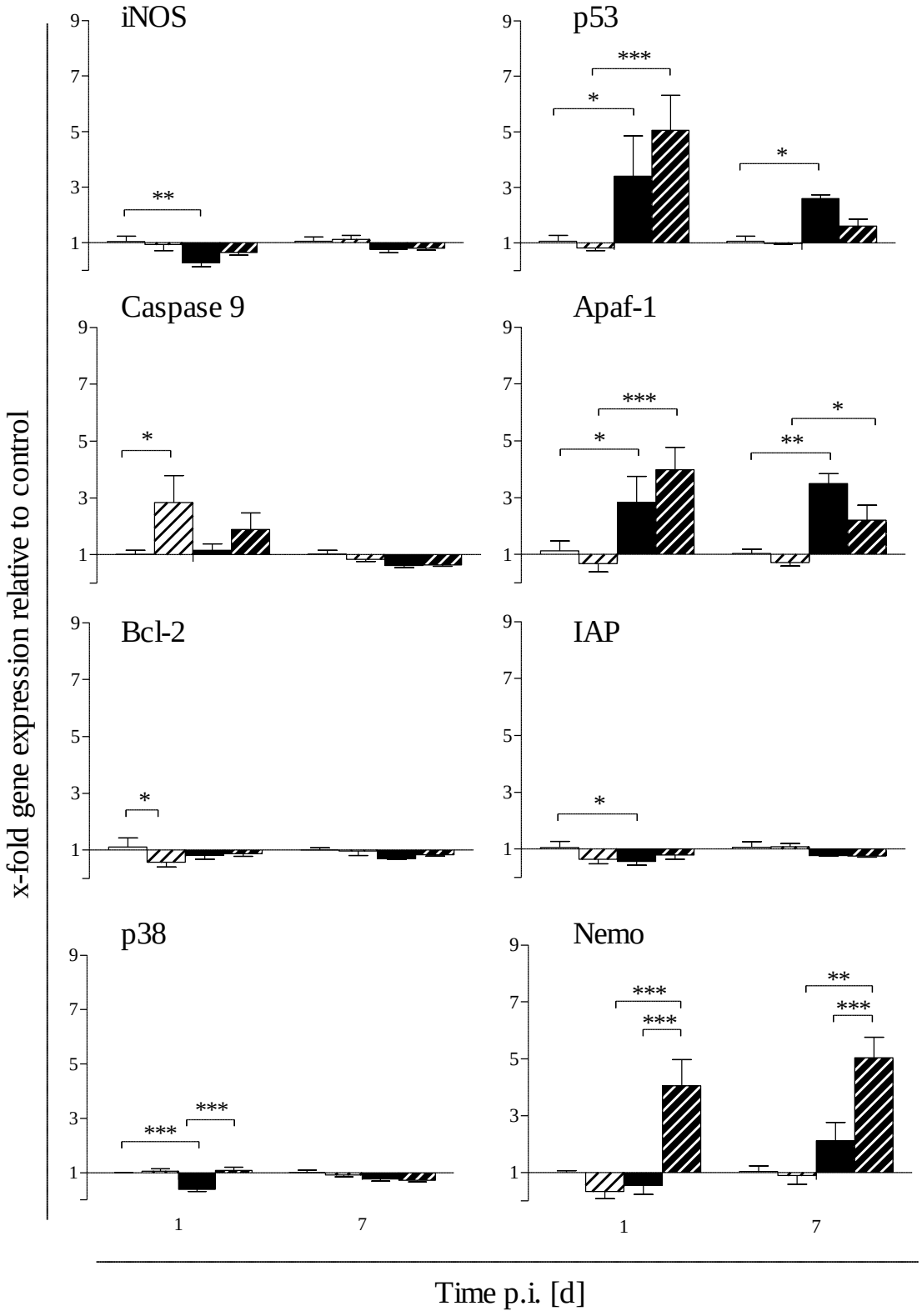

Figure 4.9: Gene expression in spleen during Poly(I:C) study

Carp fed with control or MacroGard® containing feed were injected with Poly(I:C). X-fold gene expression is expressed relative to control. White: control feed + PBS, white striped: control feed + Poly(I:C) injection, black: MacroGard® feed + PBS, black striped: MacroGard® feed + Poly(I:C). Graph shows mean ± SEM with n = 4. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$

Table 4.5: Summary of the influence of treatments on gene expression in the Poly(I:C) study*

	MacroGard®		Poly(I:C)		Poly(I:C) on MacroGard®		MacroGard® on Poly(I:C)	
Pronephros	1	**7**	**1**	**7**	**1**	**7**	**1**	**7**
iNOS						↑↑		↑↑
Caspase 9							↑	
Apaf-1		↑↑↑					↑	↑
P53		↑↑↑			↑		↑↑	↑↑
Bcl-2								
IAP		↑↑						
P38					↑			
Nemo		↑↑			↑↑		↑↑	↑↑
Spleen								
iNOS	↓↓							
Caspase 9			↑					
Apaf-1	↑	↑↑					↑↑↑	↑
P53	↑	↑					↑↑↑	
Bcl-2			↓					
IAP	↓							
P38	↓↓↓				↑↑↑			
Nemo					↑↑↑	↑↑↑	↑↑↑	↑↑↑
Gut								
iNOS	↓↓			↓↓				
Caspase 9					↑↑		↑↑↑	
Apaf-1		↑↑↑					↑↑	↑↑↑
P53		↑↑↑				↓	↑	↑
Bcl-2								
IAP		↑↑	↓		↑		↑↑	
P38		↑↑			↑		↑	
Nemo		↑↑↑						↑↑↑
Liver								
iNOS	↑						↑	
Caspase 9								
Apaf-1								
P53		↑↑↑						↑↑
Bcl-2	↑						↑↑	
IAP	↑↑↑		↑↑				↑	↑
P38	↑							
Nemo		↑↑↑						↑↑

*The "MacroGard®" column relates the two PBS injected feeding groups to each other, the "Poly(I:C)" column compares the PBS and Poly(I:C) treatments from the control fed group, "Poly(I:C) on MacroGard®" evaluates the difference from the two MacroGard® fed treatments groups and "MacroGard® on Poly(I:C)" compares the two Poly(I:C) treatment groups. ↑: enhanced gene expression, ↓ decreased gene expression. ↑: $p \leq 0.05$, ↑↑: $p \leq 0.01$, ↑↑↑: $p \leq 0.001$

LPS injection

Fish fed with control feed or feed supplemented with 0.1 % MacroGard® were subjected to injections of PBS or LPS. The results from the PBS injection are described later and therefore this section will solely focus on the LPS injected fish.

MacroGard® feeding and LPS injections influenced the expression of all analysed genes in the pronephros ($F = 3.4 – 21.2$, $p \leq 0.03$) except for anti-apoptotic genes, in which no significant differences between treatments were detected (Table 4.6). iNOS, Caspase 9, IAP and Nemo additionally displayed significantly different expression profiles during the time course of the experiment ($F = 6.2 – 17.2$, $p \leq 0.02$). The analysed pro-apoptotic genes as well as IAP and p38 were differently affected by the treatments over time, i.e. an interaction between the treatment and the time factor occurred ($F = 3.5 – 5.5$, $p \leq 0.03$).

Table 4.6: 2-way ANOVA analysis of x-fold gene expression in pronephros during LPS study

		Interaction		Treatment		Time	
		F	p	F	p	F	p
I	iNOS	1.36	0.28	5.41	0.006	6.29	0.02
+	p53	3.59	0.03	10.24	0.0002	3.75	0.07
	Caspase 9	5.20	0.007	3.45	0.03	11.72	0.002
	Apaf-1	3.55	0.03	21.16	< 0.0001	0.0009	0.98
-	Bcl-2	0.90	0.46	1.49	0.24	0.15	0.70
	IAP	5.54	0.005	2.27	0.11	17.19	0.0004
S	p38	3.49	0.03	3.57	0.03	2.46	0.13
	Nemo	2.46	0.09	9.77	0.0002	6.75	0.02

Grey backgrounds display significance. Gene function coding: I: involved in the immune response, +: pro-apoptotic, -: anti-apoptotic, S: involved in cell signalling.

Table 4.10 at the end of the LPS sub-section summarises the significant effects on gene expression observed in Figure 4.12 – 15. It is clear that LPS injection in control fed fish had no effect on expression levels of apoptosis-related genes. However in the fish that received dietary MacroGard® prior to LPS injection anti- as well as pro-apoptotic genes were up-regulated 1 day p.i. when compared to the MacroGard® fed control (Figure 4.10). Apaf-1 expression for example experienced a striking 16-fold enhancement ($p \leq 0.01$) and expression levels of Caspase 9 and IAP were approximately 2-fold enhanced ($p \leq 0.05$ and $p \leq 0.01$ respectively). Interestingly this influence of MacroGard® on gene expression after LPS injection was only noted in pronephros and not in any other organ. The cell signalling genes p38 and Nemo were also up-regulated in the group that received both MacroGard® and LPS ($p \leq 0.01$) and it is also noteworthy that iNOS gene expression in all studied organs was not significantly influenced by any of the LPS treatments. The comparison of the two LPS injected groups highlighted that MacroGard® mainly influenced pro-apoptotic genes e.g. the Caspase 9 expression level was approximately 4 times higher in the MacroGard® fed LPS injected group than in the injected control fed group on day 1 p.i. ($p \leq 0.001$). Also Apaf-1 was up-regulated circa 19-fold on day 1 ($p \leq 0.001$) and 10-fold on day 7 ($p \leq 0.01$) when MacroGard® and control fed LPS injected fish were compared.

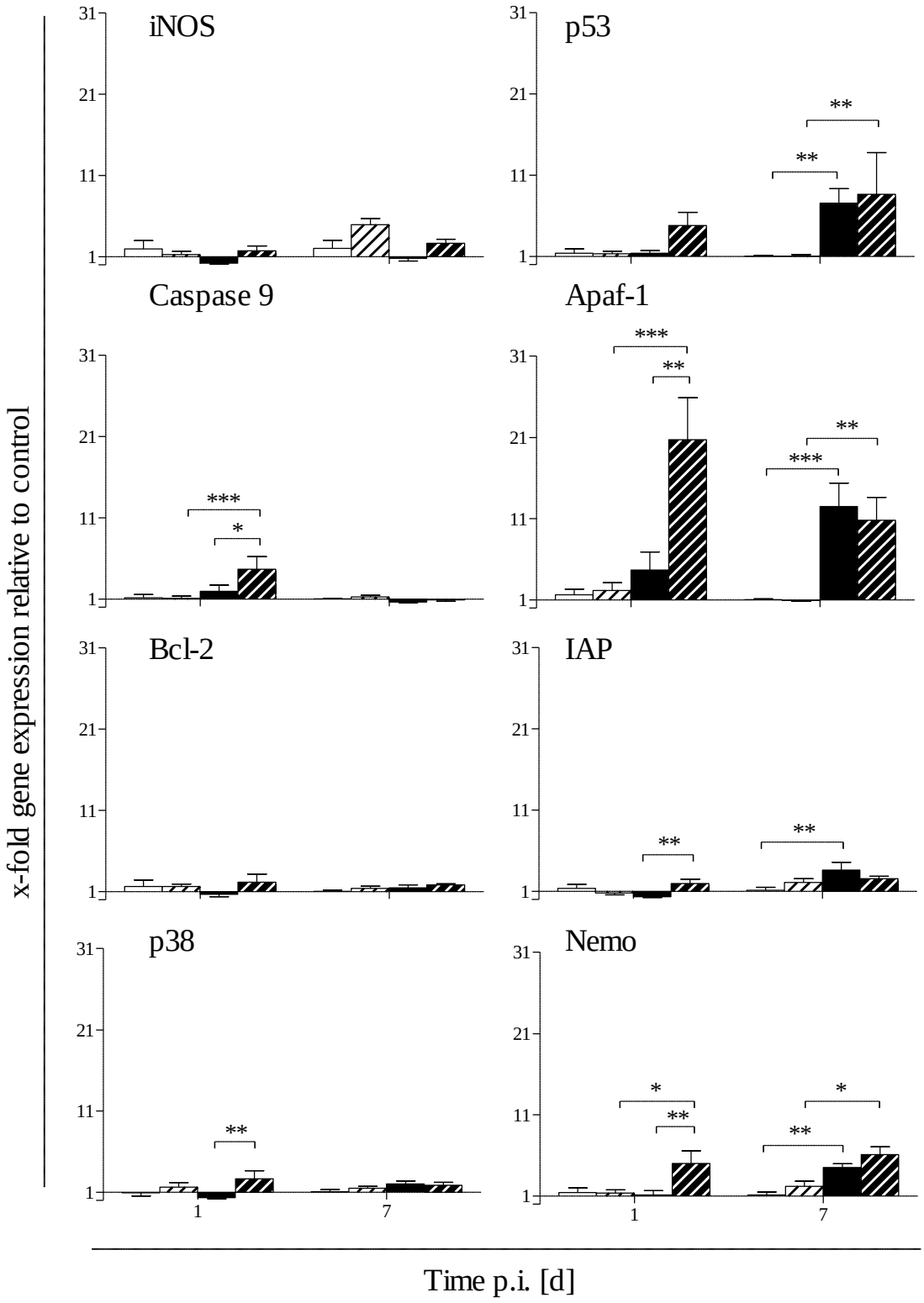

Figure 4.10: Gene expression during LPS study in pronephros

Fish previously subjected to control or MacroGard® supplemented feed were injected with LPS. X-fold gene expression is expressed relative to control. White: control feed + PBS, white striped: control feed + Poly(I:C) injection, black: MacroGard® feed + PBS, black striped: MacroGard® feed + Poly(I:C). Graph shows mean ± SEM with n = 4. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$

The gene expression profile in the spleen (Table 4.7 & Figure 4.11) was similarly affected as in the pronephros however in the spleen IAP gene expression was also influenced by the treatments ($F = 3.7 – 8.0$, $p \leq 0.3$). For iNOS, IAP and Nemo significant differences due to the time course of the experiment were found ($F = 6.9 – 18.3$, $p \leq 0.01$).

Table 4.7: 2-way ANOVA analysis of x-fold gene expression in spleen samples during LPS study

		Interaction		Treatment		Time	
		F	p	F	p	F	p
I	iNOS	1.29	0.30	4.32	0.02	10.3	0.004
+	p53	1.08	0.38	7.20	0.001	0.02	0.89
	Caspase 9	3.21	0.042	0.30	0.83	3.44	0.08
	Apaf-1	2.60	0.08	8.05	0.0007	0.31	0.58
-	Bcl-2	2.47	0.09	2.38	0.10	3.71	0.07
	IAP	0.94	0.44	4.11	0.02	6.93	0.02
S	p38	2.63	0.08	3.74	0.03	1.91	0.18
	Nemo	2.15	0.12	4.08	0.02	18.34	0.0003

Grey backgrounds display significance. Gene function coding: I: involved in the immune response, +: pro-apoptotic, -: anti-apoptotic, S: involved in cell signalling.

Post-hoc analysis revealed that in the control fed group the injection of LPS only affected the expression level of Bcl-2 gene (Figure 4.11), which was half of the expression in the control ($p \leq 0.05$). The LPS injection in the MacroGard® treated fish had little discernible effect however this treatment led to a reduction in Apaf-1 gene expression and an elevation of p38 mRNA levels when compared to the PBS injected fish. Additionally on day 1 p.i. Apaf-1 and p53 mRNA levels were approximately 3 times higher in the MacroGard® fed/LPS fish than in the control fed/LPS ones ($p \leq 0.01$). The Nemo gene expression was influenced by this treatment on day 7 where it experienced an up-regulation of circa 500 %.

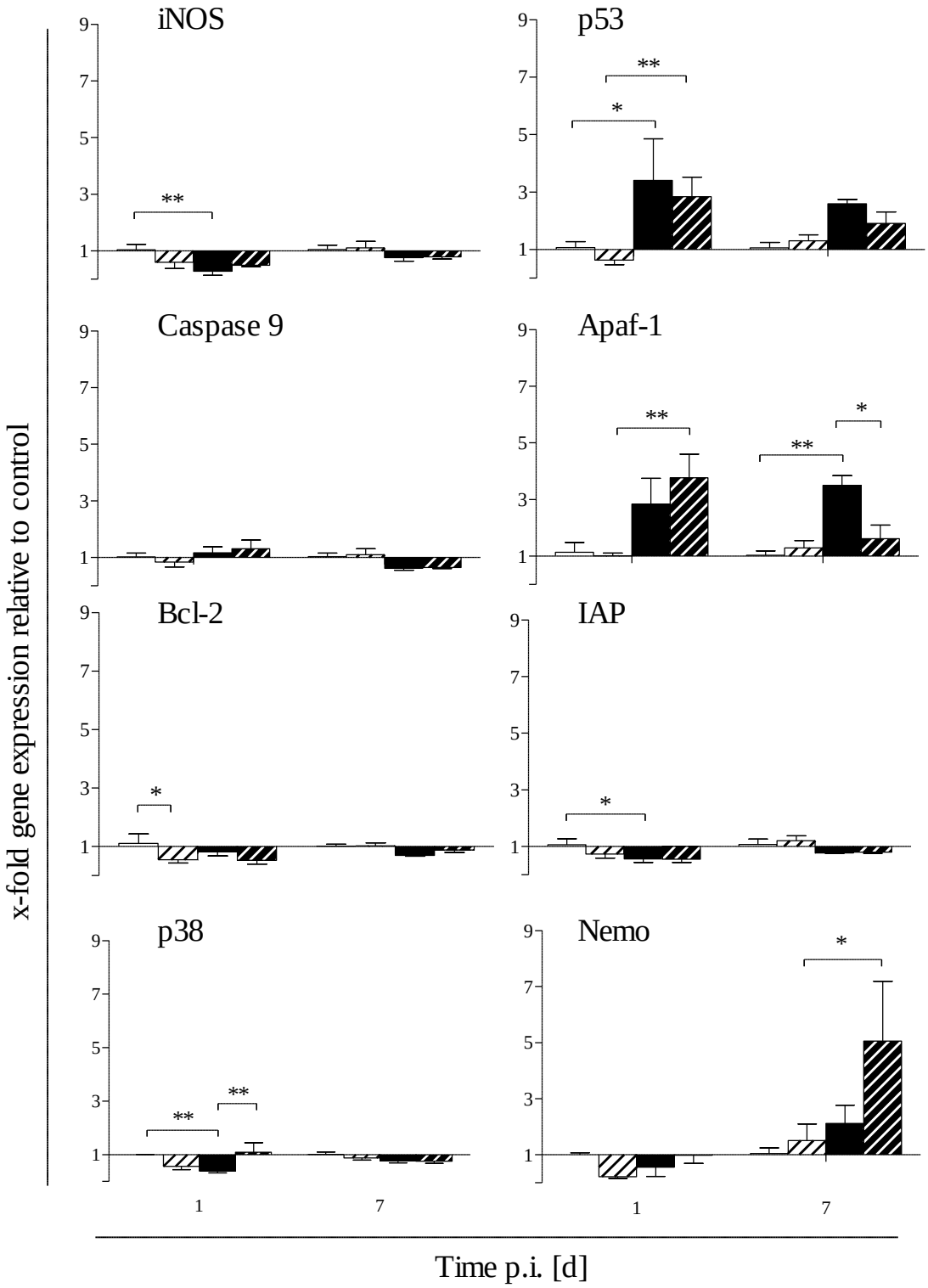

Figure 4.11: Gene expression during LPS study in spleen

Fish previously subjected to control or MacroGard® supplemented feed were injected with LPS. X-fold gene expression is expressed relative to control. White: control feed + PBS, white striped: control feed + Poly(I:C) injection, black: MacroGard® feed + PBS, black striped: MacroGard® feed + Poly(I:C). Graph shows mean ± SEM with n = 4. *: $p \leq 0.05$, **: $p \leq 0.01$

The least response to any of the treatments was observed in gut (Figure 4.12). In this organ no significant differences in the gene expression profile were noted when the LPS injected groups were compared to their respective controls. The significant differences between the treatments in gene expression of p53, Apaf-1 and Nemo ($F = 4.6 – 10.1$, $p \leq 0.01$) detected by the 2-way ANOVA (Table 4.8) were mainly due to the effects of MacroGard® feeding alone (Table 4.10) and to the differential mRNA levels in the two LPS injected treatment groups (Table 4.10). For instance in the LPS injected fish Apaf-1 from the MacroGard® group was 15 times more expressed on day 7 p.i. than in the respective control fed group ($p \leq 0.001$). In addition mRNA levels of p53 were 5-fold higher in MacroGard fed/LPS injected fish compared to the control fed/LPS injected fish.

Table 4.8: 2-way ANOVA analysis of x-fold gene expression in gut during LPS study

		Interaction		Treatment		Time	
		F	p	F	p	F	p
I	iNOS	0.64	0.60	2.92	0.06	0.01	0.92
+	p53	11.87	< 0.0001	14.55	< 0.0001	26.28	< 0.0001
	Caspase 9	0.85	0.48	0.68	0.58	0.22	0.65
	Apaf-1	5.25	0.006	32.63	< 0.0001	32.84	< 0.0001
-	Bcl-2	2.82	0.06	0.13	0.94	17.08	0.0004
	IAP	3.36	0.04	2.78	0.06	27.54	< 0.0001
S	p38	2.89	0.06	1.49	0.24	7.09	0.01
	Nemo	6.46	0.002	4.04	0.02	47.3	< 0.0001

Grey backgrounds display significance. Gene function coding: I: involved in the immune response, +: pro-apoptotic, -: anti-apoptotic, S: involved in cell signalling.

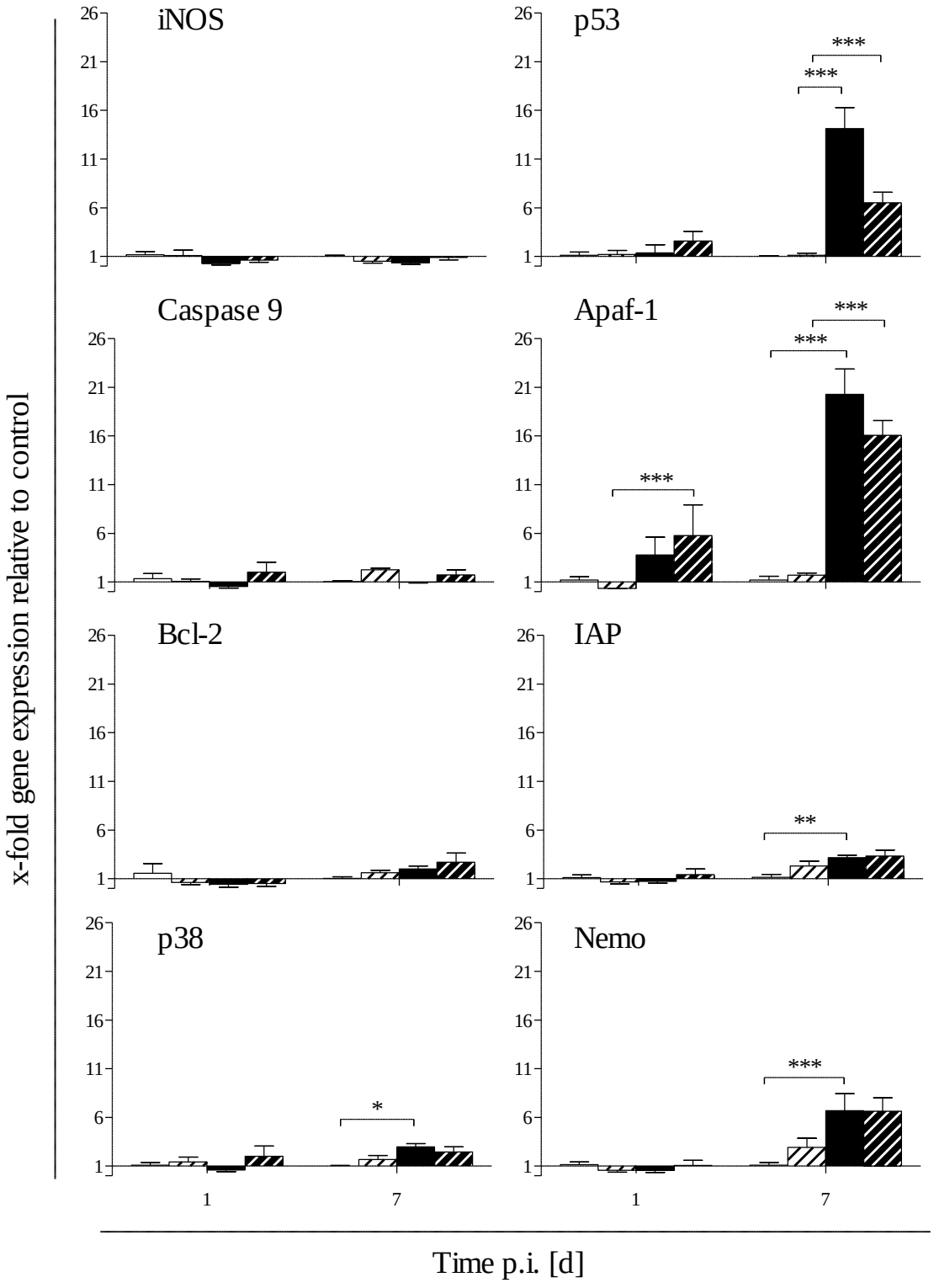

Figure 4.12: Gene expression during LPS study in gut

Fish previously subjected to control or MacroGard® supplemented feed were injected with LPS. X-fold gene expression is expressed relative to control. White: control feed + PBS, white striped: control feed + Poly(I:C) injection, black: MacroGard® feed + PBS, black striped: MacroGard® feed + Poly(I:C). Graph shows mean ± SEM with n = 4. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$

Only few effects on the gene expression profile were noted in the liver of LPS injected fish (Table 4.9 & Figure 4.13). The treatments differentially influenced the mRNA levels of p53, IAP, p38 and Nemo ($F = 4.6 – 10.1$, $p \leq 0.01$). It was observed that LPS injection caused up-regulation of p38 gene and the anti-apoptotic gene IAP in the liver of control fed fish ($p \leq 0.05$) while p53 gene expression was significantly different between the two LPS injected groups ($p \leq 0.05$).

Table 4.9: 2-way ANOVA of x-fold gene expression in liver samples during LPS study

		Interaction		**Treatment**		**Time**	
		F	p	F	p	F	p
I	iNOS	0.67	0.58	2.23	0.11	10.7	0.004
+	p53	6.21	0.003	10.1	0.0002	7.43	0.01
	Caspase 9	1.30	0.30	0.23	0.88	2.99	0.10
	Apaf-1	1.45	0.25	1.92	0.15	3.74	0.07
-	Bcl-2	1.21	0.33	2.52	0.08	8.54	0.008
	IAP	1.75	0.18	6.97	0.002	9.41	0.005
S	p38	1.32	0.29	4.64	0.01	6.09	0.02
	Nemo	1.43	0.26	5.13	0.007	10.82	0.003

Grey backgrounds display significance. Gene function coding: I: involved in the immune response, +: pro-apoptotic, -: anti-apoptotic, S: involved in cell signalling.

In general it was observed that the main immune organs, pronephros and spleen, were much more influenced by the feeding and injection treatments than organs with less immune activity, i.e. liver and gut.

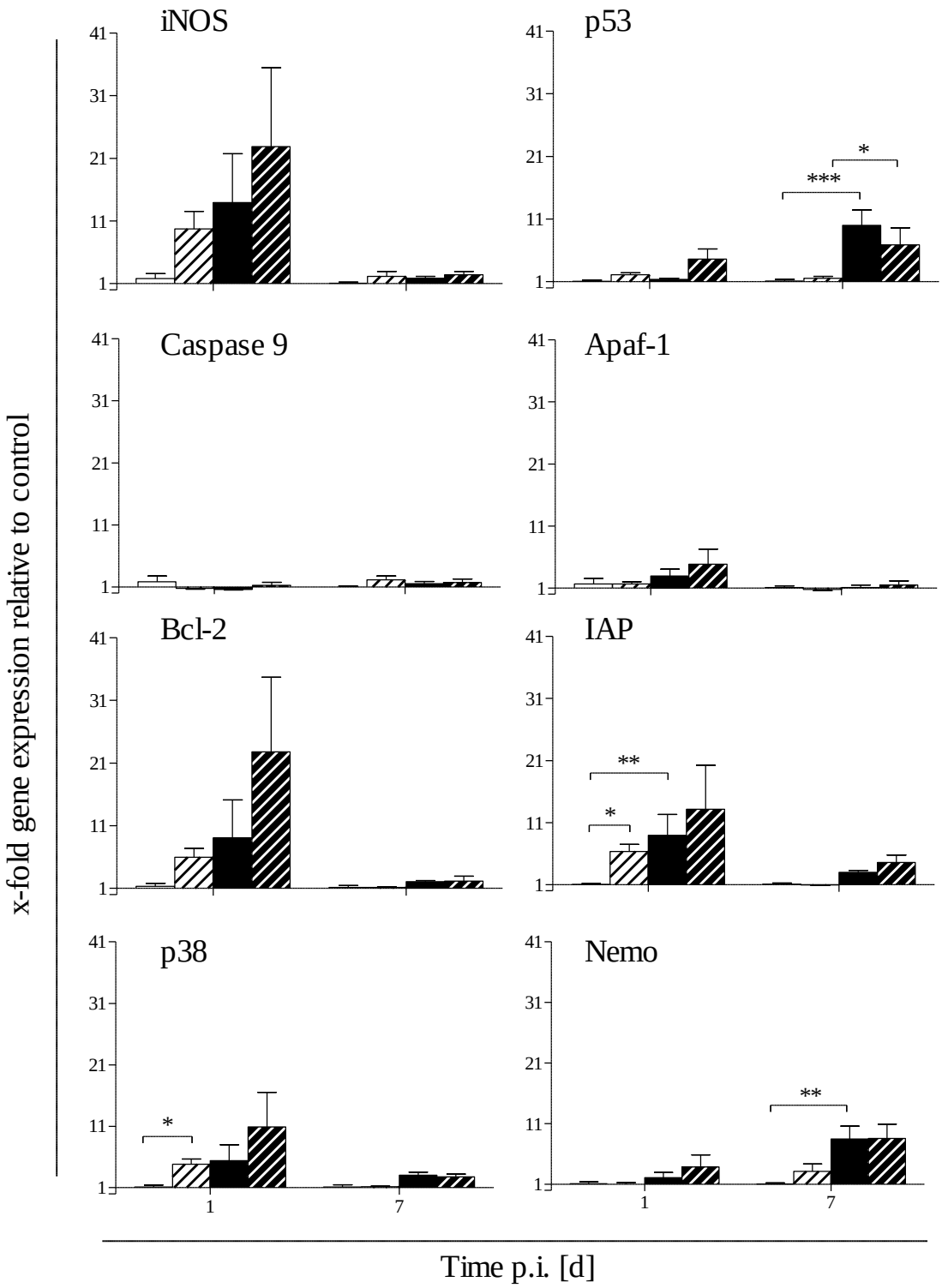

Figure 4.13: Gene expression during LPS study in liver

Fish previously subjected to control or MacroGard® supplemented feed were injected with LPS. X-fold gene expression is expressed relative to control. White: control feed + PBS, white striped: control feed + Poly(I:C) injection, black: MacroGard® feed + PBS, black striped: MacroGard® feed + Poly(I:C). Graph shows mean ± SEM with n = 4. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$

Table 4.10: Summary of influence of LPS treatments on gene expression*

	MacroGard®		LPS		LPS on MacroGard®		MacroGard® on LPS	
Pronephros	**1**	**7**	**1**	**7**	**1**	**7**	**1**	**7**
iNOS								
Caspase 9					↑		↑↑↑	
Apaf-1		↑↑↑			↑↑		↑↑↑	↑↑
P53		↑↑						↑↑
Bcl-2								
IAP		↑↑			↑↑			
P38					↑↑			
Nemo		↑↑			↑↑		↑	↑
Gut								
iNOS								
Caspase 9								
Apaf-1		↑↑↑					↑↑↑	↑↑↑
P53		↑↑↑						↑↑↑
Bcl-2								
IAP		↑↑						
P38		↑						
Nemo		↑↑↑						
Liver								
iNOS								
Caspase 9								
Apaf-1								
P53		↑↑↑						↑
Bcl-2								
IAP	↑↑		↑					
P38			↑					
Nemo		↑↑						
Spleen								
iNOS	↓↓							
Caspase 9								
Apaf-1		↑↑				↑	↑↑	
P53	↑						↑↑	
Bcl-2			↓					
IAP	↓							
P38	↓↓				↑↑			
Nemo								

*The "MacroGard®" column relates the two PBS injected feeding groups to another, the "LPS" column compares the PBS and LPS treatments from the control fed group, "LPS on MacroGard®" evaluates the difference from the two MacroGard® fed treatments groups and "MacroGard® on LPS" compares the two LPS treatment groups. ↑: enhanced gene expression, ↓ decreased gene expression. ↑: $p \leq 0.05$, ↑↑: $p \leq 0.01$, ↑↑↑: $p \leq 0.001$.

Influence on gene expression in PBS injected fish

In the MacroGard® fed control group mRNA levels of pro- and anti-apoptotic genes were elevated in pronephros and gut at 7 days after the injection treatment (Figure 4.6 & Figure 4.8). For example at this time point mRNA levels of Apaf-1 and p53 are approximately 7 - 10-fold increased in pronephros and 13 - 20-fold increased in gut ($p \leq 0.001$). Also IAP expression in MacroGard® fed fish is 3 times higher in both gut and pronephros when compared to the control fed group ($p \leq 0.01$). In contrast to pronephros and gut, the liver (Figure 4.7) was mainly affected by MacroGard® on day 1 p.i. At this time point an up-regulation of anti-apoptotic genes IAP (8.96 ± 3.37 –fold, $p \leq 0.001$) and Bcl-2 (9.06 ± 6.07, $p \leq 0.05$) was observed while only p53 as pro-apoptotic genes was up-regulated on day 7 p.i. ($p \leq 0.001$). In the spleen however expression of the pro-apoptotic genes Apaf-1 and p53 was increased 2.5 - 3-fold on both sampling days, whilst anti-apoptotic genes were unaffected (Figure 4.9) except for a reduction in expression of IAP levels on day 1 ($p \leq 0.05$).

The expression of iNOS is regulated differently between organs on day 1. Whilst iNOS gene expression is not affected in pronephros it was increased in the liver (circa 13-fold, $p \leq 0.05$) and reduced by approximately 75 % in spleen and gut ($p \leq 0.01$). Additionally it is interesting to note that p38 and/or Nemo are elevated in almost all organs in fish fed on MacroGard®, however p38 gene expression is down-regulated in spleen ($p \leq 0.001$).

4.4 Discussion

The aim of this chapter was to evaluate the effects of MacroGard® feeding on apoptosis in immune related organs in carp and to assess how disease associated PAMPs influence the relationship between MacroGard® and apoptosis. MacroGard® feeding did not induce apoptosis in the pronephros but it modulated apoptosis-related gene expression and influenced PAMP induced mRNA levels in an organ and time dependent manner. This highlights, for the first time, the complexity of interaction between the immunostimulant, PAMPs and the immune organs of fish.

4.4.1 Influence of MacroGard® feeding on apoptosis

The feeding of MacroGard® resulted in an organ specific response. The interpretation of these effects is constrained by the fact that, to date, it is unknown if yeast β-glucan, both the pure form and in feed formulations such as MacroGard®, can be taken up by the gastrointestinal tract in fish and there is no evidence in fish for a β-glucanase, the enzyme responsible for digestion of β-glucan (Dalmo *et al.,* 1994). Research in mice however has shown that orally administrated soluble β-glucan is absorbed by the GALT (gut associated lymphoid tissue) cells and can be detected in plasma after feeding (Rice *et al.,* 2005; Sandvik *et al.,* 2007). Likewise Dalmo *et al.* (1994) and Sveinbjornsson *et al.,* (1995) demonstrated that in Atlantic salmon (*Salmo salar*) both laminaran (i.e. β-(1,3/1,6)-glucan from algae) and soluble aminated β-(1,3)-D-polyglucose are absorbed by the posterior intestine. β-Glucan is thereby probably taken up by specialized enterocytes, which are able to pinocytose intact macromolecules. The authors found that β-glucan is transported via the blood to the various organs and accumulates in liver, spleen and

anterior as well as posterior kidney. It was found that liver accumulates most β-(1,3)-$_D$-polyglucose, probably because it is the first organ through which blood passes after leaving the gut. Curiously in the present study the least effect of the MacroGard® feeding was observed in liver. However it should be noted that Dalmo *et al.* (1994) and Sveinbjornsson *et al.,* (1995) studied β-glucan uptake by measuring the signal emitted by radiolabelled β-glucan. Hence it is not known if the polysaccharide is taken up in its intact form or if it is broken up and only certain products are pinocytosed as a result. Hence it is possible that the particulated MacroGard® β-glucan is not able to cross the intestine wall into the blood stream and does therefore not reach the liver. However this is not likely since spleen and pronephros were affected by this treatment and therefore another explanation for the limited effect on gene expression observed in the liver is that the immune cells in this organ are not stimulated by β-glucan. The liver is an immune privileged organ and hence induces tolerance rather than an immune response upon antigen detection (Knolle and Gerken 2000). These authors suggest that oral tolerance, i.e. tolerance to dietary antigens, depends on the concentration of the PAMP presented with low concentrations leading to a tolerance and high concentrations leading to an immune response. This could have affected the potential of reactivity of the liver to β-glucan. Whatever the case more research is needed to explain this organ-dependent effect and to elucidate if β-glucan from MacroGard® can be absorbed by the gut.

When carp were fed with the recommended dose of MacroGard® no significant increase in apoptosis in pronephric cells and no significant elevation in the expression of apoptosis-related genes in pronephros, spleen, liver and gut was noted after 7 days of feeding. In addition this short-term feeding had no effect on iNOS gene expression in

pronephros, spleen and gut, which is also in line with the absence of ROS production during 1 – 2 weeks of β-glucan feeding in rainbow trout (*Oncorhynchus mykiss*) and channel catfish (*Ictalurus punctatus*) (Chen and Ainsworth 1992; Jørgensen *et al.,* 1993). After 7 days enhanced iNOS levels were only observed in the liver. This observation might be due to the specific antigen-filtrating functions of the liver since this is the first site where foreign particles from the gastrointestinal tract encounter members of the innate immune response (Sveinbjornsson *et al.,* 1995; Knolle and Gerken 2000).

In contrast feeding the glucan supplemented diet for 25 days resulted in higher mRNA levels of iNOS in combination with enhanced gene expression of anti-apoptotic genes in gut and pronephros. In other long-term studies (2 – 6 weeks) exposure to yeast β-glucan led to the production of oxygen radicals in macrophages of rainbow trout (*O. mykiss*) and Atlantic salmon (*S. salar*) (Jørgensen *et al.,* 1993; Brattgjerd *et al.,* 1994). Such oxygen radicals (ROS and NO) are known to cause cell damage, which can lead to apoptosis (Dimmeler and Zeiher 1997; Risso-de Faverney *et al.,* 2001; Xiang and Shao 2003). However NO is known to have anti-apoptotic properties if produced at low levels by inhibiting activation of caspases and acting on anti-apoptotic signals that possibly lead to stabilisation of the mitochondrial membrane (Dimmeler and Zeiher 1997). Therefore the observed up-regulation of anti-apoptotic genes associated with iNOS up-regulation in gut and pronephros might have protective effects against apoptosis, as has been demonstrated for Bcl-2 in human and murine cells (Genaro *et al.,* 1995; Meβmer *et al.,* 1996).

On the other hand if NO is produced at high quantities it can act as an pro-apoptotic agent (Dimmeler and Zeiher 1997). Thus the observed iNOS up-regulation in spleen might be associated with the up-regulation of pro-apoptotic Caspase 9 gene expression in splenic cells. The detected up-regulation of the p53 gene in carp splenic cells on day 7 may have

resulted in an accumulation of p53 protein, which has been described as an early indicator of NO-induced apoptosis in mouse macrophages (Meβmer *et al.,* 1996). The same authors suggested that Bcl-2, which was also up-regulated in the present study, acts down-stream of p53 and leads to inhibition or delay of apoptosis. However in carp splenic cells enhanced levels of Caspase 9 gene expression additionally to up-regulated p53 were observed. Caspase 9 acts down-stream of Bcl-2, therefore it is suggested that in this case pro-apoptotic signals compensate the anti-apoptotic ones and thus apoptosis might be induced. The contrasting apoptotic effects of MacroGard® on the pronephros and spleen might be related to the higher sensitivity of splenic carp leucocytes to NO toxicity in comparison to pronephric leucocytes (Saeij *et al.,* 2002). This phenomenon is possibly related to different levels of antioxidant glutathione in the cells (Saeij *et al.,* 2003b). As the enhancement of pro-apoptotic genes was only noted on day 25 it is also possible that long-term feeding of β-glucan might be associated with increased levels of this form of cell death. This would explain the reduction in immune response factors, such as phagocytic activity and respiratory burst noted by some authors after feeding β-glucan supplemented diet for 8 weeks (Misra *et al.,* 2006a; Lin *et al.,* 2011). Therefore Bricknell and Dalmo (2005) suggested pulse-feeding over a period of 4 – 6 weeks, where the immune system oscillates between an activated and a resting state and thus does not experience immune fatigue.

The up-regulation of NF-κB essential modulator (Nemo) suggests the involvement of the NF-κB pathway in the β-glucan signalling pathway in common carp (See Figure 1.4). In the mammalian model NF-κB was shown to be involved in the signalling of various β-glucans. For example dietary administration of oat glucan ((β-1,3/1,4)-D-glucan)

led to an activation of NF-κB in leucocytes and enterocytes of gut in mice (Volman *et al.*, 2010). Also the production of TNF-α upon Zymosan A (β-(1,3)-glucan) exposure in a murine macrophage cell line was dependent on the NF-κB pathway (Young *et al.*, 2001). Additionally it has been proposed that the mammalian β-glucan receptor dectin 1 signals via the NF-κB pathway (Blonska and Lin 2011).

In carp fed with MacroGard® there was a differential expression of the Nemo gene in the organs examined. For example the spleen and gut displayed a greater gene expression response compared to the other organs. This might indicate an organ dependent recognition of β-glucan. At least in spleen the MAPK p38 pathway seems to be involved in the cellular signalling during MacroGard® feeding. Both of these pathways (Figure 4.14) have been associated with the induction of phagocytosis, iNOS expression, NO and ROS production and have been linked to apoptosis via p53 in mice (Bulavin *et al.*, 1999; Morin *et al.*, 2001; Park 2003; Yang *et al.*, 2008).

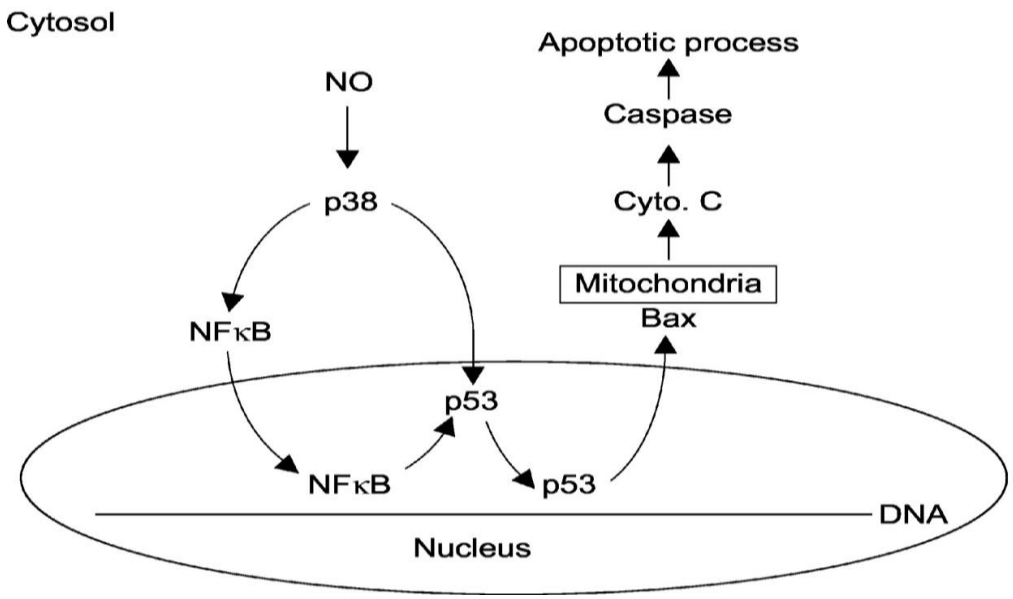

Figure 4.14: Relationship of NO, p38, NF-κB and apoptosis

Oxidative stress caused by nitric oxide (NO) and reactive oxygen species (ROS) leads to activation of the p38 MAPK pathway, which triggers the phosphorylation of p53 and can induce apoptosis. P38 is also involved in regulating NF-κB. Adapted from Kim *et al.*, (2002).

4.4.2 MacroGard® effects on PAMP induced gene expression

After 25 days of feeding, fish were injected with either PBS, LPS or Poly(I:C) to test the effects that additional PAMP exposure had on the MacroGard® – Apoptosis relationship. In these studies the expression of both pro- and anti-apoptotic genes was influenced in the MacroGard® fed PBS injected control group. This is in contrast to the results obtained during the "pure" feeding trial (prior to injection, see section 4.4.1) and is possibly due to a stress response caused by the handling of the fish. The influence on apoptosis-related genes is perhaps explained by the fact that the stress-induced glucocorticoid cortisol is known as an apoptosis-inducer in fish and therefore higher cortisol levels, probably induced by the handling, could affect the levels of apoptosis within the organs examined (Weyts *et al.,* 1997b). The results obtained from the PBS injection groups indicate a possible differential response to the handling in the fish fed with MacroGard® supplemented feed and the fish fed the control diet. Jeney *et al.,* (1997) reported a reduction of cortisol levels in the plasma of glucan fed rainbow trout. Further investigations of this effect will be necessary to evaluate the implications this has on the stress response in the animals and on immune cell apoptosis.

Poly(I:C) has been used by several workers to mimic the double stranded RNA (dsRNA) associated with many viral infections (e.g. Fortier *et al.,* 2004). The cell recognizes dsRNA via pattern recognition receptors (PRR) and subsequently induces an immune response. Indeed some efforts have been made to use Poly(I:C) as an immunostimulant to protect against viral diseases in salmon and other fish species as it has been shown to induce various cytokines, such as interferons, and inflammatory cytokines (Fortier *et al.,* 2004; Bricknell and Dalmo 2005; Falco *et al.,* 2007; Chettri *et al.,* 2011). In

addition it was linked to the induction of ROS and antiviral Mx protein both in mammals (Boltaña *et al.,* 2009; Yang and Su 2010) and in fish, e.g. catfish (*I. punctatus*) (Milev-Milovanovic *et al.,* 2009) and rainbow trout (*O. mykiss*) (Falco *et al.,* 2007). In mammals Poly(I:C) is recognized by TLR3 and recently this Toll-like receptor has also been identified in fish. For example in *C. carpio* it was shown that the TLR3 receptor is expressed in various immune and non-immune organs, i.e. gill, heart, intestine, kidney, liver, muscle and spleen (Yang and Su 2010). This specific Toll-like receptor signals via NF-κB, p38 MAPK and other pathways to induce the immune response (Lee and Kim 2007). In the present study gene expression associated with these two pathways was not affected in the control fed Poly(I:C) injected fish. However it cannot be excluded that the pathways were activated via phosphorylation of involved proteins without an increase in gene expression.

The gene expression of iNOS was also not affected in this treatment group (i.e. in spleen, liver and pronephros) and was reduced in the gut. This is in contrast to previous studies which have shown that Poly(I:C) exposure leads to an increased iNOS gene expression. For example *in vitro* exposure of a zebrafish hepatocyte cell line and rainbow trout pronephric leucocytes to 50 µg/ml and 30 µg/ml respectively resulted in induced iNOS gene expression (Falco *et al.,* 2007; Lepiller *et al.,* 2009). *In vivo* studies on zebrafish larvae bath exposed to 100 µg/ml and adults, which were injected with approximately 10 µg/fish, also reported induced gene expression of iNOS (Dios *et al.,* 2010). It is possible that the Poly(I:C) concentration used (i.e. 5 mg/kg) was not sufficient to induce iNOS expression, or that a possible iNOS expression peak was missed with the chosen sampling points. However due to the different fish species used it is not possible to relate the concentrations used and their effects to another. Since iNOS is normally induced

by cytokines it is also possible that the immune response to Poly(I:C) was not strong enough to induce this gene.

In many studies an association between double stranded RNA and apoptosis has been reported. For example Poly(I:C) induced apoptosis in human Jurkat E T-cells (Kalai *et al.,* 2002) and in a rainbow trout macrophage cell line (RTS11) (DeWitte-Orr *et al.,* 2005). In both mammals and fish Poly(I:C) is associated with the extrinsic as well as the intrinsic pathway of apoptosis (Der *et al.,* 1997; Kalai *et al.,* 2002; Mu *et al.,* 2010; Colli *et al.,* 2011; Jeong *et al.,* 2011). For example Fas, a member of the TNF receptor family, which is associated with the induction of the extrinsic apoptosis pathway, is up-regulated by stimulation with 5 μg/ml Poly(I:C) in rock bream (*Oplegnathus fasciatus*) peripheral blood leucocytes (Jeong *et al.,* 2011). Whilst Caspase 9, a member of the intrinsic apoptosis pathway, was induced in kidney and spleen of yellow croaker (*Pseudosciaena crocea*) after injection of 2.5 mg/kg Poly(I:C) (Mu *et al.,* 2010). Fibroblast and epithelial cell lines seem to be less sensitive to Poly(I:C) induced apoptosis and additional interferon exposure is required to induce cytotoxicity in this cell type (Stewart *et al.,* 1972; DeWitte-Orr *et al.,* 2005). Therefore it would appear that Poly(I:C) induction of apoptosis via the extrinsic and intrinsic pathway is dependent on species and cell type (Milev-Milovanovic *et al.,* 2009). The anti-apoptotic and the pro-apoptotic effects of Poly(I:C) noted in the present study support these previous observations. For example, anti-apoptotic IAP gene expression was up-regulated in liver. This observed induction of IAP expression was also reported in human cancer cells and catfish ovary fibroblasts after Poly(I:C) exposure (Milev-Milovanovic *et al.,* 2009; Friboulet *et al.,* 2010). In the spleen on the other hand Poly(I:C) down-regulated anti-apoptotic Bcl-2 gene expression and induced pro-apoptotic

Caspase 9 gene expression, both indicative of increased sensitivity to apoptosis. This is in agreement with the enhancement of pro-apoptotic caspases in spleen of the yellow croaker (*P. crocea*) after Poly(I:C) exposure (Mu *et al.,* 2010; Li *et al.,* 2011). It is possible that this differential response arises from the various organ functions and thus the different cell populations present in the organs. The liver and gut mainly consist of cells that are not involved in the immune response, i.e. hepatocytes and epithelial cells, therefore the sensitivity to mediators of the immune response, such as ROS and NO production, might be different in these cells. In addition a differential sensitivity of cell types to Poly(I:C) was demonstrated by Milev-Milovanovic *et al.,* (2009). They compared the influence of Poly(I:C) on gene expression in various channel catfish cell lines including ovary fibroblasts, a B cell line and a macrophage cell line and found a variation in the induced gene expression between lines. For instance, depending on the cell line used IAP gene expression was enhanced in the ovary fibroblast cell line and reduced in the macrophage cell line. Also other immune-relevant genes such as IFN and TLR3 were less expressed in fibroblasts compared to B cells and in macrophages the expression was even lower. These differences might be due to a differential regulation of the dsRNA receptor TLR3 in the cells, however data that would support this hypothesis is not yet available. As a clear difference in the effect of Poly(I:C) on the studied organs was detected in the present study a detailed investigation of the functional and systemic causes is needed to elucidate the effect of viral dsRNA on immune and non-immune cells.

In most mammalian studies Poly(I:C) only induced apoptosis or cytotoxicity if the cells were co-stimulated with interferon or another substance that sensitized the cells for apoptosis (Stewart *et al.,* 1972; Der *et al.,* 1997; Kalai *et al.,* 2002). Such a co-stimulating effect might be the cause for the observed differences in gene expression between the two

Poly(I:C) injected feeding treatments. This differential effect resulted in induced gene expression of both pro-apoptotic Caspase 9 and anti-apoptotic IAP in the MacroGard® fed group, which probably indicates a sensitization of the cells to apoptosis. The fact that morphological changes associated with apoptosis were not observed with the acridine orange staining may indicate that IAP inhibited the pro-apoptotic process or that apoptotic cells were not detected with this staining as it only detects late stages of apoptosis. Anyhow it can be concluded from this study that MacroGard® feeding in combination with virus related dsRNA causes the induction of pro- and anti-apoptotic genes associated with the intrinsic apoptosis pathway. However if this leads to pathology in the organs or has negative effects on the immune response has to be further investigated.

Lipopolysaccharide (LPS) has been associated with septic shock in mammals, however lower vertebrates appear to be more resistant to endotoxin (Swain *et al.,* 2008). One reason for this enhanced resistance to LPS in fish might lie in the recognition of this PAMP. In mammals LPS signals through TLR4, a member of the toll-like receptor family but there has been a debate if TLR4 is expressed in fish. To date the general opinion is that TLR4 was lost in most fish species (Palti 2011) since TLR4 has only been sequenced in some cyprinids e.g. zebrafish (*D. rerio*) and grass carp (*C. idella*) (Su *et al.,* 2009; Sullivan *et al.,* 2009), where a loss-of-function is being proposed (Sepulcre *et al.,* 2009). However, it was shown in several studies that LPS can induce an immune response in fish, as reviewed by Swain *et al.,* (2008), and it has been suggested that LPS signalling is TLR4 independent in teleosts (Sepulcre *et al.,* 2009). In the present study LPS did not affect the NF-κB pathway, represented by Nemo, which is in line with the study by Sepulcre *et al.,* (2009) who demonstrated that LPS receptor signalling is independent of NF-κB signalling

in fish. In the MacroGard® fed group however LPS and Poly(I:C) induced the NF-κB and the p38 signaling pathways. These PAMPs and β-glucan therefore might have cumulative effects on these pathways, which, in fish, appear to be time- and organ-dependent.

Even though the signalling in fish seems to differ from mammals, LPS still induces an immune response in fish. For example LPS exposure leads to production of cytokines, activation of the respiratory burst, and enhancement of phagocytosis, NO production, iNOS gene expression and antibody levels *in vivo* and *in vitro* (Laing *et al.*, 1996; 1999; Saeij *et al.*, 2000; Swain *et al.*, 2008). However the *in vitro* effect was shown to be time and concentration dependent (Tafalla and Novoa 2000) and *in vivo* feeding 0.1 % LPS from *Aeromonas salmonicida* to *C. carpio* juveniles did not affect the cytokine profile or iNOS expression levels in the gut (Huttenhuis *et al.*, 2006a). This is in line with my study where LPS injection did not significantly induce iNOS gene expression in the organs examined. This apparent contradiction to mammalian studies, which report induced iNOS expression after LPS exposure *in vivo* (Zhang *et al.*, 2000; Hickey *et al.*, 2002) is probably due to the lack of TLR4 receptors in fish (Novoa *et al.*, 2009; Sepulcre *et al.*, 2009).

The up-regulation of Bcl-2 observed in this study in response to LPS exposure corroborates the previously suggested delineation of the LPS induced apoptosis pathway between mammals and teleosts (Xiang *et al.*, 2008). These authors suggest that whilst in mammals apoptosis is mainly induced via a TNF-α dependent mechanism and mediated by death receptors (extrinsic pathway), in fish it seems to involve the mitochondrial pathway including the down-regulation of Bcl-2 and the enhancement of Bax and Caspase 9 gene expression (intrinsic pathway). This difference could be a result of the different signalling

pathways of LPS in mammals and fish. In my study only a reduction of Bcl-2 expression in spleen was observed but no enhancement of Caspase 9. This lack of apoptosis induction and up-regulation of pro-apoptotic genes, is in contrast to previous studies showing that LPS can induce apoptosis *in vivo* in the thymus of mice (Zhang *et al.,* 1993) and in brook trout ovary (MacKenzie *et al.,* 2006), and *in vitro* in fish lymphocytes (Xiang *et al.,* 2008). However as described above LPS effects on the immune system are concentration dependent which includes a stimulation of the immune response at lower concentration (1 EU) and a suppression at higher (10 EU) concentrations (Nayak *et al.,* 2008). This might indicate that the dose of LPS used, i.e. 4 mg/kg LPS, is an important factor in the induction of apoptosis in immune cells and organs in fish, and reflects the different sensitivity of the immune components in fish and mammals (Swain *et al.,* 2008). This effect has been shown *in vitro* where rainbow trout macrophages displayed lower sensitivity to LPS compared to mammalian macrophages (Iliev *et al.,* 2005) and, in general, concentrations of LPS used in *in vitro* studies in teleosts i.e. 0.1 – 500 µg/ml (Laing *et al.,* 1999; Brubacher *et al.,* 2000; Hirono *et al.,* 2000; Mackenzie *et al.,* 2003) are much higher than in mammalian studies i.e. 20 – 100 ng/ml (Bannerman *et al.,* 2001; Vinokurov *et al.,* 2006; Reimer *et al.,* 2008). Few studies on fish involve *in vivo* injections of LPS however the concentration of 4 mg/kg utilised in my investigations corresponds to the concentration used by other authors, for example injection with 3 mg/kg LPS in yellow perch (*Perca flavescens*), Atlantic salmon (*S. salar*) and tilapia (*Oreochromis mossambicus*) to study endocrine effects (Balm *et al.,* 1995; MacKenzie *et al.,* 2006; Haukenes *et al.,* 2011).

The effects of the combined treatment with dietary MacroGard® and LPS were mainly observed in the pronephros, where an up-regulation of pro-apoptotic genes and IAP

was detected. It has been reported that monocytes from β-glucan fed mice showed higher levels of apoptosis after LPS exposure than monocytes from control-fed mice. The same cells also had higher levels of TNF-α compared to the cells from the control-fed mice (Soltys and Quinn 1999). It is possible that in my studies the observed LPS-induced pro-apoptotic effect of β-glucan were associated with the production of cytokines like IL-1β and TNF-α (Falco *et al.*, 2012). The differential effect on organs may be related to the different cellular composition of the organs examined, as has been suggested in mammalian systems (Battle *et al.*, 1998; Soltys and Quinn 1999).

In summary it was shown in this chapter that PAMPs associated with viral and bacterial diseases can influence the apoptotic pathway of immune-related organs. It was also demonstrated that the β-glucan containing immunostimulant MacroGard® influences this process. In the next chapters it is therefore evaluated how viral and bacterial pathogens affect apoptosis and how this is modulated by MacroGard® exposure.

5 Apoptosis in relation to *Aeromonas salmonicida* and immunostimulation *in vivo*

In the previous chapter (chapter 4) it was observed that effects of dietary MacroGard® on the apoptotic process seem to depend on the duration of the feeding and on the organ studied. In the same chapter it was established that exposure to a molecular pattern associated with bacterial infection (i.e. LPS) had little effect on the apoptotic process. In this chapter it is investigated how a live bacterium affects the apoptotic process and how these effects might be affected by oral immunostimulation.

5.1 Introduction

As described in chapter 1 high densities in fish farms lead to disease outbreaks. One such disease, furunculosis, which is caused by the non-motile gram-negative bacterium *Aeromonas salmonicida* (McCarthy 1975), is an important disease in salmonids and in freshwater fish, e.g. goldfish, common carp, koi, eels, but it also poses a problem on marine fish. *A. salmonicida* is a member of the facultative anaerobic *Aeromonas* species, which are associated with many diseases in endo- and ectothermic animals including humans. For example *A. hydrophila* is associated with fatal hemorrhages in freshwater fish and amphibians and can cause gastroenterititis in humans (Shotts Jr *et al.,* 1972; Hubbard 1981; Sha *et al.,* 2002). However in contrast to other members of this family, such as *A. hydrophila*, which are motile and mesophil bacteria, *A. salmonicida* is non-motile and

psychrophil. *A. salmonicida* measures 0.4 - 0.6 x 0.8 - l .5 µm (Jeney and Jeney 1995) and studies have shown that it can stay pathogenic in freshwater for up to 9 months (Michel and Duboisdarnaudpeys 1980). Clinical signs of furunculosis include skin ulcers, which are involved in the propagation of the pathogen to other animals. The outbreak of this disease is often associated with an immunosuppression of the animals due to stress such as handling and high temperatures (Noga, 2000). Furunculosis can be classified according to the severity of the infection into acute, subacute, chronic, or latent. Other clinical signs associated with this disease include anorexia, lethargic movement, a darker pigmentation, exophthalmia and a distended abdomen. Internally the infected fish may suffer from gastroenteritis, hemorrhagic septicemia, edematous kidney, and an enlarged spleen (Hoole *et al.,* 2001). This disease is often fatal to the fish.

In general bacterial diseases are mainly treated with antibiotics, which are also often given as prophylaxis against infection. However various resistances to antibacterial compounds have been demonstrated for *A. salmonicida* (Inglis *et al.,* 1991). In addition great effort has been put into the development of vaccines against furunculosis, however many of the developed vaccines have either adverse effects due to the oil adjuvants or give only short-term protection (Villumsen *et al.,* 2012). It is therefore becoming increasingly important to develop a consumer- and environment-friendly disease prevention strategy.

β-Glucan is an ideal target for this task since it occurs naturally in the environment and therefore raises less concern in regards to the environment and human health compared to antibiotics (Gannam and Schrock, 2001). Reports have already shown that β-glucan enhances protection against *Aeromonas* spp. infections in various fish species. For example Yano *et al.,* (1991) and Selvaraj *et al.,* (2005) demonstrated that β-glucan administered by i.p. injection increased the survival rate of common carp (*C. carpio*) infected with

Aeromonas hydrophila. In regard to MacroGard® feeding it was demonstrated that it reduces the mortality of tench (*Tinca tinca*) after an *A. hydrophila* infection (Siwicki *et al.*, 2010). VitaStim-Taito, another dietary β-glucan, protected juvenile Chinook salmon (*Oncorhynchus tshawytscha*) against infection with *A. salmonicida* (Nikl *et al.,* 1993).

Many bacterial pathogens are linked to the induction of apoptosis in the host, which can be either initiated by the host as a defence mechanisms against the pathogen or it can be modulated by the pathogen to avoid the host's immune response (Hoole and Williams 2004). Host induced apoptosis can be a side-effect of the activation of the innate immune system, which kills pathogens by phagocytosis, production and release of bactericidal products such as reactive oxygen species (ROS), nitric oxide (NO) and lysozyme (DeLeo 2004; Murphy *et al.,* 2008). Certain bacteria, e.g. *Shigella flexneri*, *Salmonella typhimurium*, induce apoptosis in the immune cells of the host by activation of pro-apoptotic and inactivation of anti-apoptotic genes and proteins or by up-regulation of apoptosis inducing receptor/ligand systems (Grassme *et al.,* 2001). For the host this induction of apoptosis in the immune cells is disadvantageous since it impairs the immune response and therefore the clearance of the infection. Other bacterial pathogens such as *Chlamydia* spp. and *Salmonella enterica* can block apoptosis by inhibiting activation of pro-apoptotic genes and inducing anti-apoptotic genes in the host cell. *Salmonella enterica* can even distinguish between cell types and inhibit apoptosis in epithelial cells and induce it in macrophages (Faherty and Maurelli 2008). In fish some bacteria have been shown to be associated with apoptosis of host cells. For example *Vibrio anguillarum* seems able to inhibit the respiratory burst and caspase gene expression in leucocytes of sea bass (*Dicentrarchus labrax*) (Sepulcre *et al.,* 2007). *Piscirickettsia salmonis* on the other hand

induces apoptosis in macrophages and monocyte-like cells from from rainbow trout *in vitro* (Rojas *et al.*, 2010).

Even though *A. salmonicida* has been intensely studied (e.g. (Reith *et al.*, 2008; Fast *et al.*, 2009; Daher *et al.*, 2011) little is known about its apoptotic properties. The *Aeromonas* family in general has been shown to induce apoptosis *in vitro* utilising various cell types e.g. human intestinal epithelial cells (Galindo *et al.*, 2004), murine macrophages (Majumdar *et al.*, 2009), and lymphocytes of goldfish (*C. auratus*) (Shao *et al.*, 2004). However, *in vivo* studies on apoptosis involving the viable bacterium are rare, although several workers (Silva *et al.*, 2008b) have highlighted the importance of such investigations in relationship to the pathology induced by the infection. Therefore this chapter aims to elucidate possible apoptotic effects of *A. salmonicida in vivo* on the morphologic and genetic level and to reveal the effects of dietary immunomodulation on this process.

5.2 Materials & Methods

The study described in this chapter was carried out at the Institute of Ichthyobiology & Aquaculture of the Polish Academy of Sciences, in Golyz, Poland in collaboration with Nicolas Pionnier, Patrick Frost, Ganna Sych, Dr. Alberto Falco and Dr. Ilgiz Irnazarow.

5.2.1 Fish

C. carpio with an average weight of 78.4 g of the Ukrainian line (Białowąs *et al.*, 2008) were kept in tanks with UV-treated recirculating water and bio-filters at 20 ºC in the facilities of the Polish Academy of Sciences, Golyz. All experimental fish were fed control feed (0 % MacroGard®) for a 3 week acclimatization phase. Fish were then divided into two groups and subjected to a 14 day feeding regime, during which fish were either fed a diet with a 0 % MacroGard® control diet or the same diet but supplemented with 0.1 % MacroGard®. The composition of these diets is described in chapter 2.4 and came from the same batch as the diets described in chapter 4 - 7 (provided by Tetra GmbH, Germany).

5.2.2 Bacterial infection

An *Aeromonas salmonicida* strain (provided by Alicija Kozinska from the National Veterinary Institute Research Institute in Pulawy, Poland) with low virulence (Polish strain A449) (Reith *et al.*, 2008) was chosen for the injection to ensure survival of the fish over the experimental period. Bacteria were stored at – 80 °C and were seeded on agar plated before they were grown in lysogeny broth for 18 hours at 25 ºC. Afterwards bacteria were concentrated by centrifugation at 1600 g for 10 min, and the bacterial pellet reconstituted in PBS. Bacterial concentration was determined via OD measurement at 540 nm (UV-1601

PC, UV-Visible Spectrophotometer, Shimadzu) and alignment with a McFarland scale. Fish were either exposed to a non-lethal dose of *A. salmonicida* carried out by intraperitoneal injection (1 x 10^8 bacteria/fish in 250 µl PBS) or PBS alone. All the work regarding *Aeromonas salmonicida* growth and injection were carried out by the team of the Polish Academy of Sciences.

In summary 4 treatments were carried out:

- Control feed + PBS
- Control feed + *A. salmonicida*
- MacroGard® feed + PBS
- MacroGard® feed + *A. salmonicida*

These treatments were carried out in separate individual tanks with 35 fish each. The PBS treatments of the control feed and the MacroGard® feed were carried out in four tanks each, while the *A. salmonicida* treatments of both feeding regimes were divided up over six tanks each. Sampling was conducted by taking fish from a different tank each day to minimize stress if the fish.

5.2.3 Sample preparation & analysis

Feeding was stopped after the *A. salmonicida* injection and 5 fish were sampled for each treatment at 0 h (just before the injection), 6 h, 12 h, 24 h, 72 h and 120 h injection. Fish were sacrificed with a lethal dose of 0.2 % Prospicin (2 % etomidate, produced by Inland Fisheries Institute, Poland) (Kazuń and Siwicki 2001), pronephros and mid-gut samples were removed and processed for microscopical as well as gene expression analysis

as described in chapter 2. Pronephric cells were isolated in RPMI+ without β-mercaptoethanol and were stained with acridine orange as described in chapter 3.3.4 to be analysed with a UV microscope (Nikon Eclipse E400) with a B-2a filter.

In this chapter the expression of the following genes was studied:

Pro-apoptotic p53, Caspase 9 and Apaf-1, anti-apoptotic Bcl-2, IAP and Mcl-1, and the inducible NO synthase (iNOS) as well as p38, which is part of the p38 MAPKK signalling pathway. Pronephros and mid-gut were analysed since they are key players in immunity and dietary immunomodulation.

Comparison of sample preparation procedures (pooled vs. individual)

The sample preparation and analysis for the gene expression was carried out at the School of Life Sciences at Keele University. Two possible procedures exist in analysing gene expression in large sample sizes. The quicker and less time and cost consuming procedure relies on the hypothesis that the gene expression of pooled samples is equal to the mean gene expression in individual samples. In this chapter it was explored if the analysis of "pooled" samples (i.e. samples of the same organ, treatment and time point were pooled together before RNA extraction) gives similar results to "individual" samples (i.e. samples were not pooled at any point of the analysis and the mean of the gene expression was calculated). This test was carried out to evaluate which procedure should be adopted in later studies.

The "pooled" samples were prepared by pooling organ sections of approximately 2 mm^2 for each treatment and time point (i.e. pool of 5 fish each). An effort was made to

use organ sections from the same region (e.g. mid-gut) to reduce variation. However for pronephros, which does not have a clear shape after extraction from the animal, this region-equality could not be maintained. These pooled samples were then processed for real-time PCR analysis as described in chapter 2.5. Only a basic set of genes was analysed to test the hypothesis that results from pooled samples will match results from individual samples. The genes were selected to give a representation of iNOS induction as well as of pro- (i.e. Apaf-1) and anti-apoptotic effects (i.e. IAP, Bcl-2).

Individual samples were prepared as described in chapter 2.5 and were analysed with real-time RT-PCR. Statistical analysis using 2-way ANOVA and Bonferroni Post-hoc test was carried out as described earlier (chapter 2.6) with significance defined as $p \leq 0.05$.

5.3 Results

5.3.1 Apoptosis levels in pronephric cells

The level of apoptosis in the pronephric cells was analysed for each sample individually (Figure 5.1) and differed between the treatment groups ($F = 13.69$, $p < 0.0001$) as *A. salmonicida* caused a rise in the number of cells with fragmented nuclei ($p \leq 0.05$). This effect occurred independent of the feeding regime and was not affected by MacroGard® feeding in any of the treatment groups. The apoptotic effect of *A. salmonicida* on pronephric leucocytes was time dependent and peaked at day 1 post injection ($F = 2.25$, $p = 0.04$). In addition an interaction between the time and treatment factor was detected ($F = 2.19$, $p = 0.07$), which resulted in significantly different treatment effects depending on the analysed time point.

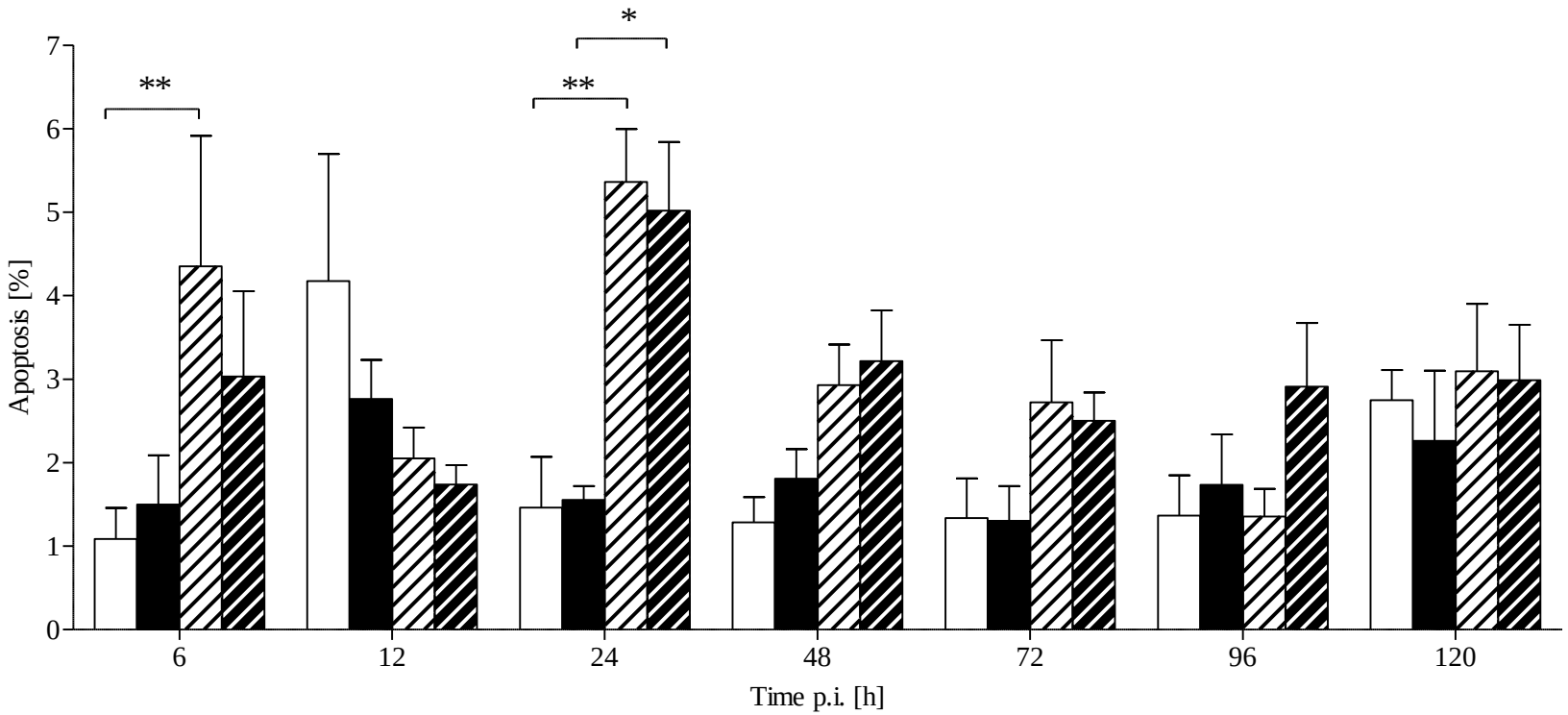

Figure 5.1: Percentage of apoptotic cells in the pronephros after A. salmonicida and MacroGard® treatment.

Carp were fed for 14 days with a control diet (i.e. 0 % MacroGard) or a diet containing 0.1 % MacroGard®. Afterwards fish were injected with PBS or *Aeromonas salmonicida* and the levels of apoptotic cells (i.e. cells with fragmented nuclei) was analysed microscopically with acridine orange staining. The graph shows mean ± SEM with n = 5. Bars: white = Control feed/PBS, black = MacroGard® feed/PBS, white with stripes = Control feed/*A. salmonicida*, black with stripes = MacroGard® feed/*A. salmonicida*. $p \leq 0.05$, **: $p \leq 0.01$

5.3.2 Gene expression in pooled samples

When samples were analysed from both the pooled gut and pooled pronephros (Table 5.1) the only significant difference between treatments was observed in iNOS in the pronephros ($F = 5.18$, $p = 0.02$). The only significant effects on gene expression occurred at 6 hours p.i. in the control fed/*A. salmonicida* injected group (Figure 5.2) with anti-apoptotic genes (i.e. IAP, Bcl-2) and iNOS gene ($p \leq 0.05$) being up-regulated. No significant change in gene expression was detected for the pro-apoptotic Apaf 1 in both organs and for IAP, Bcl-2 and iNOS in gut. However all of these genes showed trends for enhanced gene regulations at 6 hours and/or at 72 hours p.i., which is reflected by a significant time effect for all genes except iNOS in gut ($F = 3.86$, $p \leq 0.03$).

Table 5.1: 2-way ANOVA of x-fold gene expression in pooled samples*

	Gut				**Pronephros**			
	Treatment		Time		Treatment		Time	
Genes	F	p	F	p	F	p	F	p
iNOS	1.57	0.25	2.88	0.07	5.18	0.02	5.01	0.01
Apaf-1	0.38	0.77	3.86	0.03	1.59	0.24	5.77	0.008
IAP	1.48	0.27	5.93	0.007	2.04	0.16	5.60	0.007
Bcl-2	1.39	0.29	3.86	0.03	1.64	0.23	6.62	0.005

*grey boxes represent significant effects

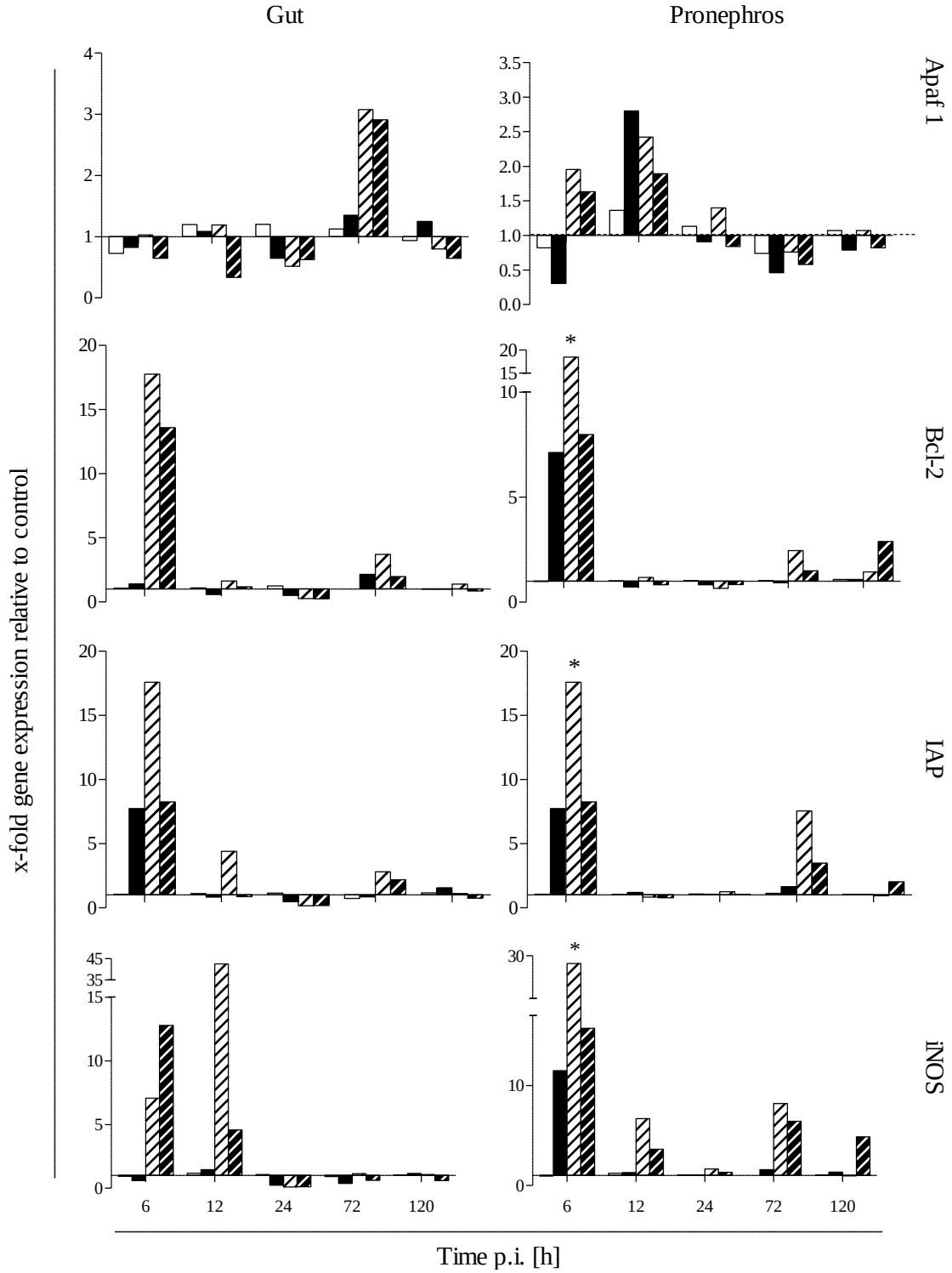

Figure 5.2: Expression analysis of pooled samples.

Fish were fed with a 0 % control diet or a 0.1 % MacroGard® containing diet. Organ samples of 5 fish were pooled and gene expression in these pooled samples was analysed. Graph shows the x-fold expression relative to the respective control of the time point. White bars: control feed/PBS, black bars: MacroGard® feed/PBS, white bars with stripes: control feed/*A. salmonicida*, black bars with white stripes: MacroGard® feed/*A. salmonicida*. *: significant difference to control fed/PBS group with $p \leq 0.05$.

5.3.3 Gene expression in individual samples

Effect of MacroGard® feeding prior to injection

Samples were taken prior to the injection (i.e. 0 hours) to establish the effects of MacroGard® feeding (Figure 5.3). This revealed that at 14 days of feeding there was no significant effect on the expression of the analysed genes, except for a down-regulation of pro- and anti-apoptotic genes in gut, i.e. p53 ($p \leq 0.01$) and IAP ($p \leq 0.05$), and pro-apoptotic Caspase 9 gene in pronephros ($p \leq 0.01$).

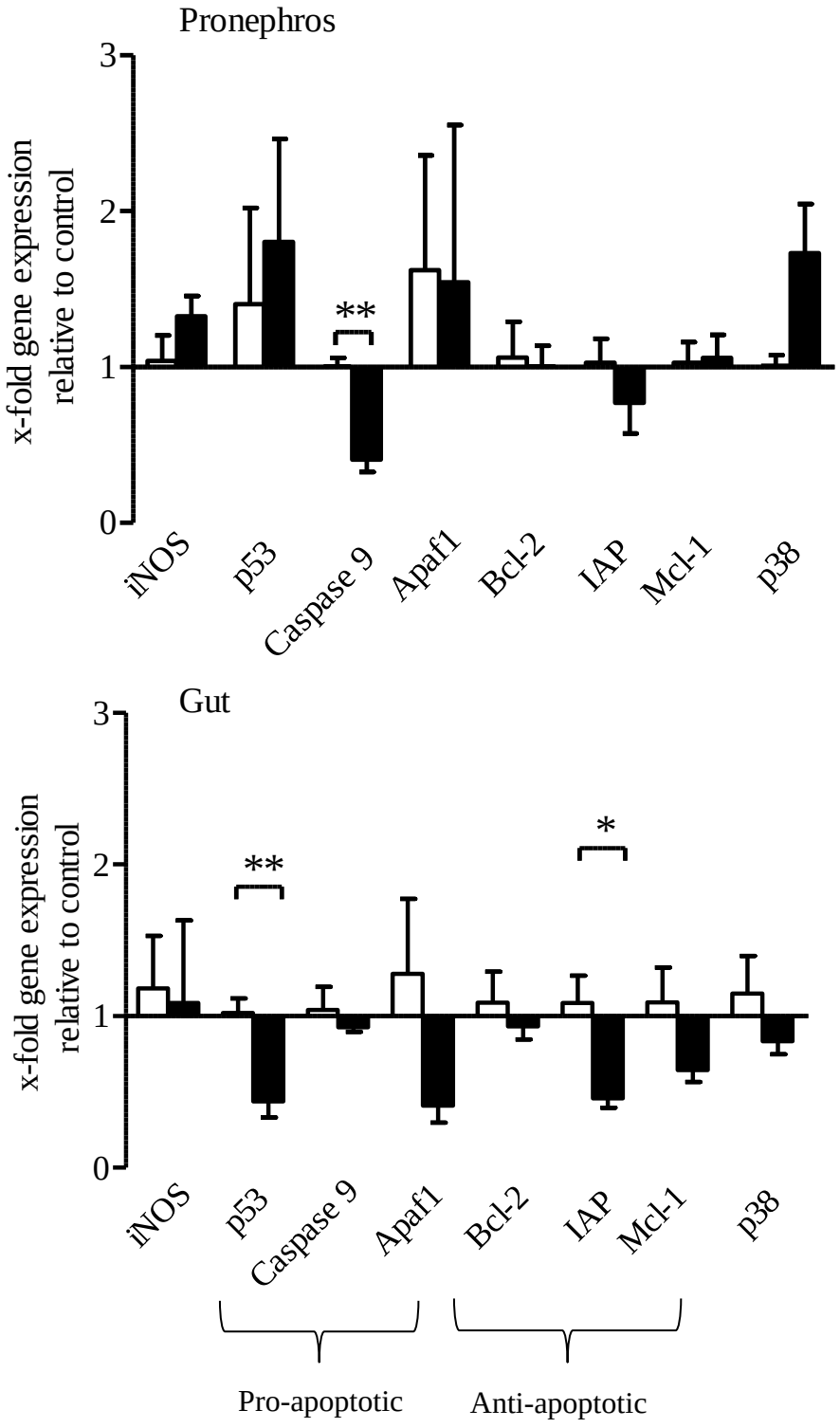

Figure 5.3: Gene expression at 14 days of MacroGard® feeding.

Carp were fed with either a control feed or a 0.1 % MacroGard® containing feed for 14 days and x-fold gene expression relative to control was analysed. White bars: control feed, black bars: MacroGard® feed. The graphs show mean ± SEM, n = 5. *: significant difference to control fed/PBS group with $p \leq 0.05$, **: significant differences to control with $p \leq 0.01$.

Effects of MacroGard® and Aeromonas salmonicida

The expression of all analysed genes in the pronephros (Table 5.2) differed significantly between treatments ($F = 2 – 13$, $p \leq 0.05$) and in addition the gene expression also differed between sampling points ($F = 3 – 8$, $p \leq 0.01$) which resulted in higher gene expression levels at 6 hours p.i. (Figure 5.4). However this latter effect was absent in the anti-apoptotic genes IAP and Bcl-2. In the gut (Table 5.2) only the gene expression of iNOS and IAP were affected by the treatments ($F = 3$, $p \leq 0.05$), whilst experimental duration altered the expression of all genes analysed with the exception of Apaf-1 ($F = 2 – 11$, $p \leq 0.05$). For some genes (p53, Mcl-1b, p38) this time dependency effect led to significant changes in gene expression at 24 h p.i., whilst others (iNOS, Caspase 9) exhibited changes at 6 hours p.i. In both organs a dependency of the treatment effect on the sampling time point was observed ($F = 1.91 – 3.24$, $p \leq 0.05$), however the affected genes differed. In pronephros the interaction between these two factors occurred in iNOS, IAP, Caspase 9 and Apaf-1 whilst in gut only Mcl-1 was affected.

Table 5.2: 2-way ANOVA analysis of x-fold gene expression during *A. salmonicida* exposure

			2-way ANOVA analysis					
			Interaction		Treatment		Time	
		Pronephros	F	p	F	p	F	p
Effect on cell*	B	iNOS	2.98	0.002	13.32	< 0.0001	8.37	< 0.0001
	-	Bcl-2	0.60	0.84	3.04	0.03	1.32	0.27
		IAP	1.91	0.05	5.34	0.002	3.68	0.009
		Mcl-1	0.72	0.73	2.86	0.04	0.48	0.75
	+	Apaf-1	2.27	0.015	2.27	0.02	12.26	< 0.0001
		p53	1.34	0.22	4.59	0.005	1.58	0.19
		Caspase 9	2.42	0.01	1.26	0.29	40.24	< 0.0001
	S	p38	1.68	0.09	2.78	0.05	5.30	0.0008
	Gut							
Effect on cell*	B	iNOS	1.12	0.35	3.92	0.01	3.97	0.006
	-	Bcl-2	1.23	0.28	2.17	0.10	4.53	0.002
		IAP	0.96	0.50	3.08	0.03	2.58	0.04
		Mcl-1	1.92	0.04	0.85	0.47	11.01	< 0.0001
	+	Apaf-1	1.13	0.35	1.25	0.30	1.76	0.15
		p53	1.34	0.21	1.10	0.36	4.32	0.003
		Caspase 9	3.24	0.0009	6.12	0.0009	9.14	< 0.0001
	S	p38	2.57	0.007	0.88	0.45	10.22	< 0.0001

*B: bactericidal effect, -: Anti-apoptotic effect, +: pro-apoptotic effect, S: cell signalling; grey background: significant changes with $p \leq 0.05$.

MacroGard® in combination with a PBS injection affected two pro-apoptotic genes in the pronephros at 6 h p.i.: Apaf-1 was reduced by approximately 50 % ($p \leq 0.05$) and gene expression of Caspase 9 was 12-times higher than the value for the control fed/PBS injected animals ($p \leq 0.001$). All other analysed genes in gut and pronephros of fish injected with PBS were not significantly affected by the MacroGard® feeding (Figure 5.4 &Figure 5.5).

In contrast, *A. salmonicida* injection in fish from both feeding groups caused a substantial (approximately 15-fold) enhancement of iNOS expression in pronephros ($p \leq 0.001$) in combination with an up-regulation of IAP gene expression ($p \leq 0.01$). iNOS gene expression was also enhanced in gut of the control fed/*A. salmonicida* fish ($p \leq 0.05$) but at a lower level (~ 3-fold) than in the pronephros. This iNOS up-regulation occurred

together with higher mRNA levels of Mcl-1b ($p \leq 0.05$) and lowered expression of p38 ($p \leq 0.001$), which was down-regulated to approximately 10 % of the expression of PBS injected control fed group on day 1.

The group of fish that had been fed with MacroGard® prior to the bacterial injection experienced a greater influence on gene expression in the pronephros than in gut. In the pronephros of fish receiving an *A. salmonicida* injection in combination with MacroGard® feeding not only an enhanced gene expression of iNOS and anti-apoptotic IAP was noted but also the pro-apoptotic gene p53 ($p \leq 0.05$) was up-regulated when compared to the PBS injected MacroGard® fed fish at 6 h p.i. In the combined MacroGard®/*A. salmonicida* treatment the cell signalling factor p38 was the only gene that was influenced in both organs, where it experienced a slight up-regulation. However this occurred at different time points; p38 was up-regulated in pronephros at 6 h p.i. ($p \leq 0.01$) whilst in gut this effect was seen at 3 d p.i. ($p \leq 0.05$). In the comparison of the two bacterial injection groups (i.e. control feed versus MacroGard® feed) differences in gene expression were noted between the two feeds in the gut and pronephros. In the pronephros MacroGard® caused an enhancement of expression of the gene encoding p38 while in gut an up-regulation of the p53 gene was observed. Table 5.3 summarizes the effects observed due to the various treatments.

Table 5.3: Influence of treatments on the gene expression profile

Gene*	MacroGard®		*A. salmonicida*		*A. salmonicida* on MacroGard®		MacroGard® on *A. salmonicida*	
Pronephros	6h	24h	6h	24h	6h	3d	6h	24h
iNOS			↑↑↑		↑↑↑			
IAP			↑↑		↑			
Apaf1	↓							
p53					↑			
Caspase 9	↑↑↑							
P38					↑↑		↑	
No effects on Mcl-1b and Bcl-2								
Gut								
iNOS				↑				
Mcl1b				↑				
p53								↑
Caspase 9			↑↑↑				↑↑↑	
P38		↓		↓↓↓		↑		
No effects on Bcl-2, IAP, Apaf-1								

*colours indicate the cellular effects of the genes: black: cell signalling, red: anti-apoptotic, green: pro-apoptotic, blue: bactericidal, ↑: up-regulation, ↓: down-regulation, the light grey boxes highlight significant up-regulations, dark-grey highlights significant down-regulations. ↑: $p \leq 0.05$, ↑↑: $p \leq 0.01$, ↑↑↑: $p \leq 0.001$

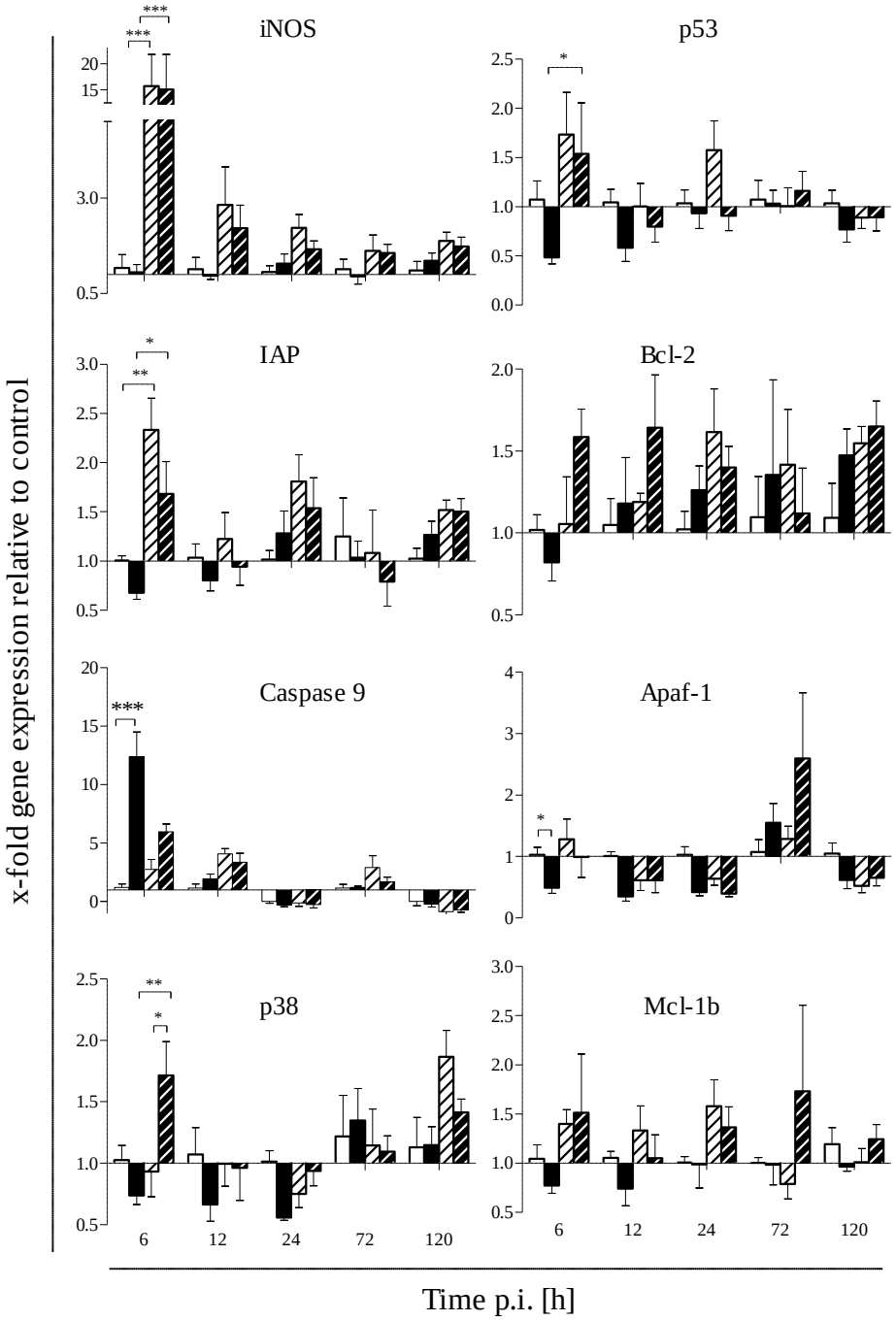

Figure 5.4: Gene expression in pronephros during *A. salmonicida* exposure.

Carp were subjected to a 14 day feeding regime with either a control feed (0 % MacroGard®) or a 0.1 % MacroGard® containing feed. Fish were sampled over a 5 day period. Graphs show mean ± SEM of the x-fold gene expression relative to the control of the day from n = 5. White bars: Control feed/PBS, black bars: MacroGard® feed/PBS, white bars with stripes: Control feed/*A. salmonicida*, black bars with white stripes: MacroGard® feed/*A. salmonicida*. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

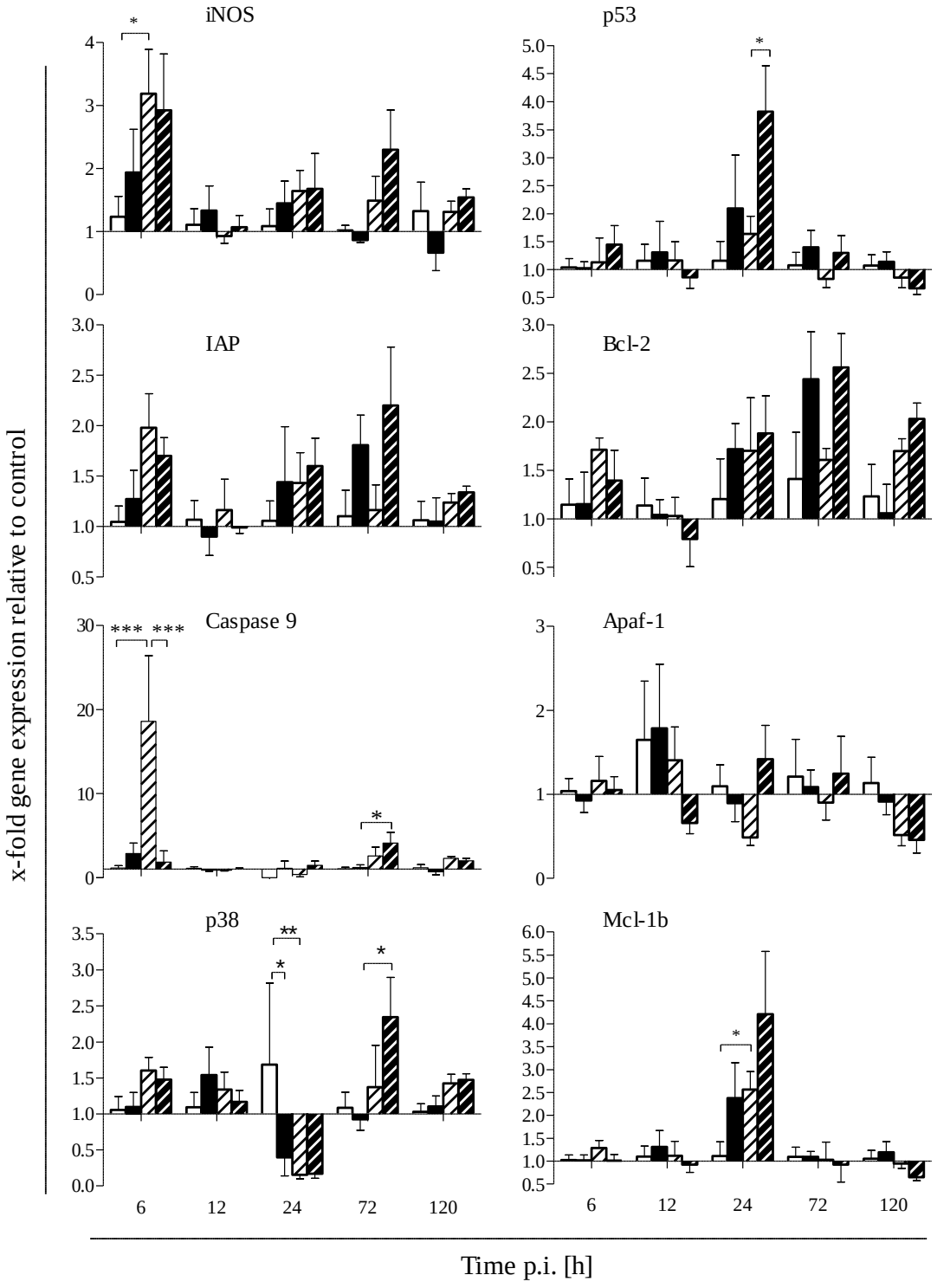

Figure 5.5: Gene expression in gut during *A. salmonicida* exposure.

Carp were subjected to a 14 day feeding regime with either a control feed (0 % MacroGard®) or a 0.1 % MacroGard® containing feed. Fish were sampled over a 5 day period. Graphs show mean ± SEM of the x-fold gene expression relative to the control of the day from n = 5. White bars: Control feed/PBS, black bars: MacroGard® feed/PBS, white bars with stripes: Control feed/*A. salmonicida*, black bars with white stripes: MacroGard® feed/*A. salmonicida*. *: $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$.

5.4 Discussion

5.4.1 Use of pooled data

In the first part of this chapter the hypothesis was tested that the analysis of pooled organ samples will equal the mean of the individual samples. However when comparing gene expression from these two examinations (see Table 5.4) a clear differences were observed between the pooled and individual data.

Table 5.4: Comparison of pooled and individual samples

	Aeromonas		***Aeromonas* & MacroGard®**	
	Pooled	Individual	Pooled	Individual
Apaf-1	1.03	1.16	0.65	1.05
Bcl-2	17.76	1.71	13.57	1.40
IAP	7.74	1.98	8.26	1.70
iNOS	0.61	3.19	12.79	2.93

Table shows the gene expression (x-fold in relation to control) in pooled and individual samples from gut at 6 hours p.i. were compared.

These differences indicate that the results from pooled and individual data is not comparable, which is in line with findings from Talbot *et al.,* (2009). The observed variability between data sets could be due to the differences in sample preparation. In the individual samples it can be easily ensured that equal amounts of each sample are used for the cDNA preparation (i.e. standardized RNA concentration). In samples that are pooled on the organ level however the equality of the sample size was judged by eye and therefore it is possible that some individual fish had more influence on the resulting gene expression due to a higher proportion of their mRNA in the pool. This effect could possibly be

avoided by pooling of normalised mRNA or cDNA of the individual fish rather than pooling on the organ level. Even though this does not save time or money on the sample preparation side it can still reduce the number of samples run for the estimation of which samples to analyse individually. However Talbot *et al.,* (2009) have shown that pooled data is often influenced by individual fish that deviate from the rest of the group. Similar effects were observed in the presented data set as some individual fish displayed gene expression levels that did not correspond to the mean expression levels of their sampling group. The analysis of pooled data should therefore not be used for conclusions about the treatment effects and should be backed up by analysis of individual samples.

5.4.2 Effect of MacroGard® feeding

The effects observed during the 14 days of feeding MacroGard® prior to the injection are similar to those observed during the 25 day feeding period in chapter 4. In this study a down-regulation of pro- and anti-apoptotic genes, i.e. p53 and IAP, in gut and a reduction of pro-apoptotic Caspase 9 in pronephros were noted. These observations indicate suppression rather than induction of apoptosis in gut and pronephros due to MacroGard® feeding. This is in line with the finding that expression of pro-apoptotic genes was not enhanced in gut and pronephros during 25 days of MacroGard® feeding (Figure 4.3 & 4.4). Differences between the gene expression profiles constructed from these two data sets (Table 5.5) may have arisen as a consequence of the different carp strains utilised (UK strain vs. Polish strain) and sampling schedules used. In the feeding study conducted previously (chapter 4) samples were taken after 7 and 25 days whilst in the present study samples were analysed after 14 days of feeding.

Table 5.5: Comparison of gene expression profiles due to feeding MacroGard®

	Gut Time [d]			Pronephros Time [d]		
	7	14	25	7	14	25
iNOS			↑			↑
p53		↓				
Caspase 9					↓	
Apaf-1						
Bcl-2			↑			↑
IAP		↓	↑			
P38						
Nemo			↑			

Gene expression (x-fold relative to the control) was compared between the feeding study described in chapter 4 (i.e. samples analysed at day 7 and 25 of feeding) and chapter 5 (i.e. samples analysed at day 14, grey background). Arrows indicate significant up- (↑) and down- (↓) regulation of gene expression observed in the two studies.

In the previous chapter (chapter 4), MacroGard® feeding had an extensive influence on the gene expression pattern in response to a PBS injection. In the present study however the only changes observed were an enhanced gene expression of Caspase 9 and reduced levels of gene expression of Apaf-1 in pronephros and p38 in gut. The synchronistic up- and down-regulation of pro-apoptotic genes in pronephros is difficult to interpret. However since apoptosis was absent on the cellular level (Figure 5.1) plus a general trend to reduced mRNA levels in all apoptosis-related genes, it is possible that the resulting effect was an apoptosis-inhibiting one.

The difference in gene expression patterns between the two studies (chapter 4 and chapter 5) might be due to different levels of stress experienced by the fish. Since the injections were carried out by different researchers and at different institutes it is possible

that this difference in handling induced different responses in the fish. Additionally in the study described in the present chapter, the fish were inoculated after 14 days of feeding whilst in the previous study fish were injected after a 25 day feeding period. Therefore the duration of MacroGard® administration might have an influence on the stress response of the fish and the resulting gene expression. In the study conducted by Jeney *et al.,* (1997) rainbow trout (*Oncorhynchus mykiss*) elicited a lower stress response after 4 weeks of β-glucan feeding in comparison to the control fed group. It is therefore possible that stress-reducing effects only occur after a longer feeding period. This however will have to be explored in future investigations.

5.4.3 Effect of *Aeromonas salmonicida* and MacroGard® feeding

In this study it was shown for the first time that injection of a non-lethal dose of *A. salmonicida* affects apoptosis in carp and that dietary β-glucan affects this cell death process. The *A. salmonicida* injection led to morphological changes in the pronephros associated with apoptosis during the first 24 hours. This is in agreement with other studies that also report apoptosis induction by members of the *Aeromonas* family *in vitro*. It was shown that *Aeromonas hydrophila* induced apoptosis in lumphocytes of goldfish (*Carassius auratus*) and in haemocytes of the giant fresh water prawn (*Macrobrachium rosenbergii*) (Shao *et al.,* 2004; Hsu *et al.,* 2005). In mammals this is also a well-known occurrence as *Aeromonas* spp. exposure led to apoptosis in murine macrophages and human intestinal epithelial cells (Galindo *et al.,* 2004; Krzyminska *et al.,* 2009). However *in vivo* studies have not been conducted to ascertain the apoptotic effect of live aeromonads in teleosts. Thus this is the first study to show that a member of the *Aeromonas* family, i.e. *A. salmonicida,* can induce apoptosis *in vivo* in pronephric cells from a fish, *C. carpio*. The

pronephros is a very heterogeneous organ consisting of various cell types and Verburg-van Kemenade *et al.,* (1994) identified lymphocytes, neutrophilic and basophilic granulocytes and macrophages in suspensions of isolated pronephric cells. In order to understand the mechanisms underlying the bacteria-induced apoptosis in the host it will be necessary to identify the cell type(s) affected. This knowledge of the affected cell type may shed light on the immunological effects since for example neutrophil granulocytes have a greater ability to produce ROS compared to macrophages, and hence have a lower bactericidal capacity. Macrophages on the other hand are longer lived and due to their antigen presenting properties they are involved in the mediation of the specific immune response (DeLeo 2004). Thus the loss of specific cell types will influence the immune response differently. In addition it has been proposed that apoptotic cell death of macrophages is a cellular mechanisms for the clearance of infected cells (DeLeo 2004) and hence to fully understand the host-pathogen interaction it will be of importance to clarify if the observed apoptosis is an anti-host or anti-pathogen response.

The pathway of apoptosis induction by this pathogen was further investigated through analysis of the gene expression. Bacterial pathogens are able to induce apoptosis in host immune cells via various pathways depending on the infecting bacterium and the host cell type (Ashida *et al.,* 2011). The only information on apoptosis-related genes in association with *A. salmonicida* arises from general gene regulation studies. These studies reported an up-regulation of Mcl-1 in the pronephros of Atlantic cod (*Gadus morhua*) 6 hours post injection with formalin-killed *A. salmonicida* (Feng and Rise 2010). In the present study such an influence of *A. salmonicida* exposure on Mcl-1 was observed in gut after 24 hours p.i. but was not seen in pronephros. Mcl-1 is mainly involved in the intrinsic pathway by inhibiting the release of cytochrome c by interaction with pro-apoptotic

targets, e.g. Bim, Bak (Michels *et al.,* 2005). However Mcl-1 can by spliced into two isoforms with two distinct functions: the short form acts as apoptosis inducer whilst the longer isoform is anti-apoptotic (Bae *et al.,* 2000; Feng and Rise 2010). Therefore without knowledge of the protein conformation it is difficult to interpret the function of this gene. Nonetheless Mcl-1 up-regulation indicates the involvement of the intrinsic apoptosis pathway. At an earlier time point (6 h p.i.) the gut displayed increased levels of Caspase 9 mRNA. This gene is also involved in the intrinsic apoptosis pathway and therefore it can be concluded that in gut, *A. salmonicida* is linked to intrinsic apoptosis. The additional up-regulation of iNOS is a sign for oxidative stress, which can induce this mitochondria dependent pathway.

In comparison to the gut, iNOS up-regulation in the pronephros was drastically higher, but IAP was the only apoptosis-related gene that was enhanced by the bacterial injection. This is also in contrast to the increased apoptosis levels that were observed on the cellular level in this organ, which might be due to various reasons. For example, the short term control of apoptosis occurs post-transcriptionally via cleavage and phosphorylation of proteins (Hengartner 2000). Hence it is possible that the subsequent changes in gene expression might have been missed with the chosen sampling points. On the other hand the lack of gene expression could also indicate that the induction of apoptosis in this organ is via the extrinsic pathway as the genes analysed are primarily involved in the intrinsic pathway. Since only little data is available for the apoptosis inducing effects of *A. salmonicida* consideration of the results from *A. hydrophila* might give some indications on the possible pathways involved. In an infection with a highly virulent strain of *A. hydrophila*, Apaf-1, a component of the intrinsic pathway, was up-regulated in pronephros of common carp (Cols Vidal 2006). On the other hand several

authors (Galindo *et al.,* 2004; Majumdar *et al.,* 2009) reported that *A. hydrophila* virulence factors can induce apoptosis via the extrinsic and the intrinsic pathway. This is plausible as it was shown that *Aeromonas* spp. infection can lead to mitochondria dependent apoptosis through ROS and NO production in human epithelial cells (Krzyminska *et al.,* 2011). However, *A. salmonicida* infection induced production of pro-inflammatory cytokines that can induce extrinsic and intrinsic apoptosis (dos Santos *et al.,* 2008; Fast *et al.,* 2009; Grunnet *et al.,* 2009; Falco *et al.,* 2012).

Indeed, it is interesting to note that in other gene expression studies associated with this present experiment, published by Falco *et al.*, (2012), it was observed that TNF-α gene expression was enhanced in pronephros at 6 h p.i. but not in gut. This pro-inflammatory cytokine has been associated with the extrinsic pathway of apoptosis (dos Santos *et al.*, 2008). In contrast at 6 h p.i IL-1β, which is associated with the intrinsic apoptosis pathway (Grunnet *et al.,* 2009), was up-regulated in gut tissue (Falco *et al.,* 2012). Therefore it seems that the pathway by which *Aeromonas salmonicida* leads to apoptosis differs between organs and future studies exploring the regulation of genes involved in the extrinsic apoptosis pathway are required to further elucidate the mechanisms involved.

The association between *A. salmonicida* and the intrinsic and extrinsic pathways of apoptosis appears to be affected by the feeding of MacroGard®. As a result the detected up-regulation of Caspase 9 is absent in the MacroGard® fed/*A. salmonicida* group, which supports the previously suggested anti-inflammatory effects of MacroGard® (Falco *et al.*, 2012). Additionally the elevation of Mcl-1 and iNOS mRNA levels induced by bacterial exposure in the control fed group was absent in the gut of MacroGard® fed fish. This lack of bacterial induced gene expression is due to elevated mRNA levels in the non-infected MacroGard® fed fish which was not accompanied by additional enhancement due to the

bacteria. Thus it is possible that the immunostimulation leads to elevated baseline levels of iNOS (and NO production) which were not further stimulated by bacterial infection. These results as well as the results from the LPS injections (i.e. mimicked bacterial infection) in combination with the MacroGard® feeding (chapter 4) indicate that the feeding regime can influence the gene expression pattern of apoptosis-related genes.

In conclusion it was demonstrated in this chapter that the bacterial pathogen *Aeromonas salmonicida* is capable of inducing apoptosis in the pronephros in common carp. It was also speculated that the apoptotic pathways involved, either intrinsic or extrinsic, differ between organs and cell types. However future research is needed to elucidate the exact mechanisms of the apoptosis induction.

6 Viral infection in relation to apoptosis and immunostimulation *in vitro*

In chapter 4 it was established that the viral mimic Poly(I:C) alone had little effect on the apoptotic process but that dietary immunostimulation influenced the gene expression pattern. In order to evaluate the effect of β-glucan on viral infections the present chapter focusses on the apoptosis modulation caused by two carp viruses alone and in combination with β-glucan *in vitro*.

6.1 Introduction

As described in the introduction to this thesis (chapter 1) viral diseases are a major threat to aquaculture and have high economic costs. The World Organisation of Animal Health (OIE) lists nine notifiable fish diseases of which seven are caused by viral pathogens including the spring viremia of carp virus (SVCV) and the koi herpes virus (KHV) (OIE 2012).

SVCV, the causative agent of the spring viremia of carp disease (SVC), causes high mortalities in farmed and wild carp in Europe and North America, and also affects other cyprinids in which it tends to be less virulent (Hoole *et al.,* 2001; Garver *et al.,* 2007). This disease is characterized by lethargy, protruding eyes (exophthalmia), inflammation of the intestine and the peritoneum (enteritis, peritonitis), abdominal distension (ascites), thickening of the swim bladder, petechial haemorrhages in internal organs, skin and muscle and fluid accumulation in kidney, liver and spleen (Hoole *et al.,* 2001; Goodwin 2009). As

indicated in the name “spring viremia of carp” (SVC) outbreaks mainly occur in spring with the rise of the water temperature. The low water temperatures during winter, and with this the less active immune system of the fish, allow the virus to spread horizontally (i.e. from fish to fish) during this season. When the water temperature rises towards 10 °C during spring fish develop clinical signs and mortalities occur, which peak at 15 – 17 °C. At these temperatures the acquired arm of the immune response in carp is impaired (i.e. no antibody production) but at higher temperatures (above 20 °C) carp can develop humoral antibodies against SVCV, which protects them against following SVCV infections. This is probably the reason why mainly young fish (1^{st} and 2^{nd} years of age) are affected by this disease (mortality up to 70 %) whilst older fish rarely show signs of SVC (mortality below 30 %) (Hoole *et al.,* 2001; Ahne *et al.,* 2002; Goodwin 2009). Over recent years there has been some effort to develop vaccines against this disease and although some are commercially available the pathogen still remains a threat to carp culture (Gomez-Casado *et al.,* 2011).

The spring viremia of carp virus has been identified as a member of the rhabdoviridae family in the order of the mononegavirales and the genus vesiculovirus. Other members of this genus include the “eel virus American”, the “eel virus European X”, pike fry rhabdovirus and the ulcerative disease rhabdovirus, and in mammals the vesicular stomatitis Indiana and the vesicular New Jersey virus (Fu 2005). SVCV, in accordance with most members of the rhabdoviridae family, has a bullet shaped morphology and a genome that is composed of one molecule of non-segmented, linear, single stranded negative-sense RNA. This RNA strand encoded 5 genes (Figure 6.1), i.e. N (core nucleocapsid protein), P (a major phosphoprotein), M (matrix protein), G (glycoprotein),

and L (virus own RNA-dependent RNA polymerase) (Ahne *et al.*, 2002; Licata and Harty 2003).

Figure 6.1: SVCV genome

SVCV genome organization and the predicted molecular weight (MW) in kilodaltons (kd) of the individual genes. Adapted from (Purcell *et al.*, 2012).

SVCV enters the host cell by binding of its surface glycoproteins to cellular receptors and thereby inducing viral endocytosis (Granzow *et al.*, 1997; Ahne *et al.*, 2002) and replicates in the cytoplasm of the cell. *In vitro* the virus replicates best at temperatures between 20 – 25 °C (Ahne *et al.*, 2002), which corresponds to the optimal temperature of the viral RNA polymerase (Roy and Clewley 1978). Although two independent studies have shown that SVCV infection of the EPC cell line *in vitro* induces apoptosis at the morphological level (Björklund *et al.*, 1997; Kazachka *et al.*, 2007), the mechanism by which SVCV induces apoptosis at the molecular level still requires elucidation.

The other virus that was investigated in this study is the koi herpes virus (KHV), also termed cyprinid herpes virus 3 (CyHV-3). This virus, which was previously identified as carp interstitial nephritis and gill necrosis virus (CNGV) (Shapira *et al.*, 2005), leads to mortalities of 80 – 100 % of infected wild and cultured common and koi carp (Garver *et al.*, 2010; Eide *et al.*, 2011). This disease has been observed in farmed and wild fish during spring and autumn when water temperatures range between 18 – 28 °C, and has been

shown to be highly specific to carp since experimental challenges were not able to infect other fish species such as goldfish (*Carassius auratus*), grass carp (*Hypophthalmichthys molitrix*), tilapia (*Oreochromis niloticus*), and silver perch (*Bidyanus bidyanus*) (Perelberg *et al.,* 2003). The clinical signs of KHV are lethargy, increased respiratory frequency and gasping movements on the water surface, gill necrosis, sunken eyes, pale patches on the skin and increased mucus production (Pokorova *et al.,* 2005; Hanson *et al.,* 2011). Internal signs include lesions within the skin, gill, kidney, liver and gut. Changes in gills are marked by loss of lamellae and inflammation in the filaments, and the tubuli of the kidney lose their integrity, which causes problems with the osmoregulation (Negeborn 2009). Because of the high mortalities caused by this disease efforts have been made to develop a vaccine against it, which is available in Israel (Michel *et al.,* 2010).

The KHV virus is classed as a member of the *Herpesvirales* order (Waltzek *et al.*, 2005), which is widely spread among vertebrates. Characteristics of this order are a high degree of host specificity, the ability to interact with the host's immune system and the establishment of long-term latency. Generally herpesviruses cause mild diseases in natural conditions but can be highly pathogenic in an immune compromised host or in an environment that promotes the transfer of high doses of viruses to a naive host. Three separate families have been identified in the *Herpesvirales*: *Herpesviridae*, which mainly infect mammals, birds and reptiles, *Alloherpesviridae*, pathogens of fish and amphibians, and *Malacoherpesviridae*, which was identified in oyster. These three families are distantly related to one another although there is almost no similarity in their genome sequence between them (Hanson *et al.*, 2011).

Fourteen herpesviruses have been identified in fish including three cyprinid herpes viruses (CyHV-1 (carp pox herpesvirus), CyHV-2 (goldfish haematopoietic necrosis

virus), CyHV-3/KHV), which are all members of the *Alloherpesviridae* (Hanson *et al.*, 2011). These cyprinid herpes viruses are also closely related to the channel catfish virus (CCV). KHV is a double stranded DNA virus and is the largest known herpesvirus with a genome size of 295 kb, which encodes 156 predicted genes (Aoki *et al.,* 2007). Some of these encoded genes are related to the immune system, such as interleukin-10 (IL-10) and tumor necrosis factor receptor (TNFR-1 and TNFR-2) (van Beurden *et al.*, 2011).

As mentioned in chapter 1.4 viruses are able to influence apoptosis within a range of host cells leading to three possible outcomes:

1) The host itself can induce apoptosis. This can occur via an immune driven process, whereby viral peptides are presented on the infected host cell, which are recognized by cytotoxic T-cells, which then cause cell lysis by apoptosis or necrosis in the infected cell. Another pathway of host induced apoptosis is cell-autonomous where the cell goes into apoptosis after recognizing an unscheduled activation of the cell cycle via viral proteins (O'Brien 1998).
2) The virus itself promotes apoptosis to spread to other cells without an inflammatory response.
3) Viruses can inhibit host apoptosis to maximize progeny.

Which strategy is employed depends on the evolutionary history of the virus and its relationship with the host cell. In general RNA viruses, such as the rhabdovirus SVCV, have a small genome, which does not appear to encode genes that can influence the apoptotic process of the host. In contrast, DNA viruses, such as KHV, have large genomes and are often known to interfere with the host's immune response (Licata and Harty 2003;

van Beurden *et al.,* 2011). SVCV and KHV can therefore be expected to have different survival strategies and are thus good models to study viral interaction with the host apoptotic pathways. SVCV has been shown previously to cause morphological changes associated with apoptosis, but very little attempt has been made to analyse the genes involved (Björklund *et al.,* 1997) and no information is available regarding apoptosis in relation to KHV.

Various prevention strategies have been used to protect fish against viral diseases. Vaccines are a very specific approach and can only protect against a small range of pathogens, for example a KHV specific vaccine cannot protect carp against SVCV. Therefore more general approaches have to be undertaken to ensure a better survival of the farmed fish and β-glucan has been previously reported to reduce viral infections. MacroGard® injections for instance protected rainbow trout (*Oncorhynchus mykiss*) against hematopoietic necrosis virus (IHNV) (LaPatra *et al.,* 1998) and grass carp (*Ctenopharygodon idella*) injected with fungal β-glucan displayed higher survival rates after challenge with grass carp hermorrhage virus (GCHV) (Kim *et al.,* 2009b).

In chapter 4 it was shown that MacroGard® influences the apoptosis-related gene expression after exposure to a viral mimic (i.e. Poly(I:C)). The aim of this chapter therefore was the investigation of the morphological and molecular changes associated with the apoptotic process induced by two viral infections and its modulation by MacroGard® *in vitro*.

6.2 Materials & Methods

This study was carried out at the Veterinary University Hanover in Germany under the supervision of Mikolaj Adamek and Dr. Dieter Steinhagen.

6.2.1 CLC cells

Cells of the carp leucocyte cell line (CLC) cells were cultured as described in chapter 2.3 at Keele University and prior to use of these cells at the Veterinary University Hanover, Germany it was established that this cell line was free of Mycoplasma contamination as described in section 2.3.1. Results (Figure 6.2) indicated the absence of Mycoplasma contamination.

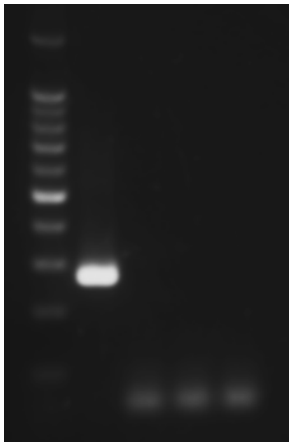

Figure 6.2: Test for Mycoplasma contamination in CLC cell line
Three individual cDNA samples from CLC cell cultures were tested for Mycoplasma using a PCR detection kit. Lines are M: marker, +: positive control, S1 – 3: samples 1 – 3.

Previous studies have indicated that some stocks of the CLC cell line were contaminated with other cell types which are not of carp origin (G. Wiegertjes personal communication). It was therefore ensured that the CLC cell line used in this study is derived from carp (see section 2.3.5). The Cyprinus Carpio Brain (CCB) cell line was used as a positive control. Figure 6.3 indicates that both cell lines show products of the same size and hence it was concluded that both cell lines are of *C. carpio* origin.

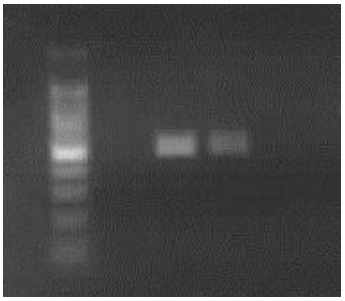

Figure 6.3: Test for *C. carpio* origin of CLC and CCB cells
cDNA samples from CLC cells and CCB cells were tested by PCR for their carp origin by using carp specific primers (Forward: CTTGTATTTGGTGCCTGA, Reverse: GTGTTTGGTATTGGGAGATG). Expected product length is expected to be 486 bp.
M corresponds to the marker (i.e. 100 bp DNA ladder), while L indicates the CLC and B indicated the CCB cell line (i.e. positive control).

After it was established that the CLC cell line had not been contaminated, subcultures of it were sent to the Veterinary University Hanover, Germany for the study in a cool-box with several ice-packs to keep the cells cool but not frozen. Here cells were cultured in T75 flasks (Nunc, Germany) with RPMI 1640 supplemented with 5 % foetal bovine serum, 2.5 % specific pathogen free heat inactivated carp serum, 2 mM L-

glutamine, 10 % water, 200 IU/ml penicillin and 200 µg/ml streptomycin (all ingredients from Sigma, Germany) and were incubated at 25 °C in a humid atmosphere with 2 % CO_2 (Thermo Scientific Heraeus CO_2 Incubator). Confluent cells from the flasks were trypsinised, diluted 1:3 and seeded to 24-well plates, whereby one flask was used to seed three 24-well plates (Nunc, Germany). The cells were checked daily to ensure that they grew into a monolayer (approximately 1 day post seeding) which was then used for experimentation. CLC cells did not readily adhere to the bottom of the well and had to be handled very carefully since they tended to detach if the monolayer was disturbed (see Figure 6.4).

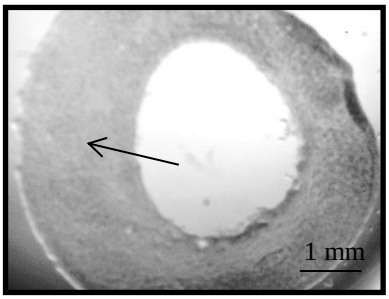

Figure 6.4: Detached CLC monolayer
The monolayer of CLC cells tended to detach from the bottom during handling. The arrow indicates the detached monolayer, which had formed a ring. © Alexandra Taylor

6.2.2 CCB cells

The Cyprinus Carpio Brain (CCB) Cell line is a fibroblastic cell line, which was established in (1999) by Neukirch *et al*. CCB cells were cultured in minimum essential medium (MEM) with Earle's salts supplemented with Non-Essential Amino Acids (NEAA), 10 % foetal bovine serum, 0.35 % glucose, 100 IU/ml penicillin and 100 µg/ml streptomycin (all ingredients from Sigma, Germany). Cells were kept at 25 °C in a humid atmosphere with 2 % CO_2. Confluent cells were seeded into 24-well plates and were used for the studies the next day.

6.2.3 MacroGard® treatment

Aliquots of MacroGard® powder, stored at -20 °C at the Veterinary University Hanover, were prepared in deionised water, sonicated and heated as described in chapter 2.4. Dilutions were then prepared in cell medium.

The protocol for the MacroGard® treatment was established prior to the infection study. For this purpose CLC and CCB cells were incubated with a range of MacroGard® concentrations for 24 hours. A 3 min exposure to UV light served as a positive control for the apoptosis induction. CLCs were treated in triplicate while CCBs were processed in duplicate. For both cell lines, analysis with acridine orange indicated baseline levels of apoptosis in all treatments, and a concentration of 50 µl/ml MacroGard® was utilised.

For the following studies cells were seeded into 24-well plates and the next day they were exposed for 24 hours to medium alone or medium containing 50 µg/ml MacroGard® in a humid atmosphere with 2 % CO_2 and at the temperature appropriate to the viral challenge (i.e. SVCV 20 °C; KHV 25 °C).

6.2.4 SVCV infection

SVCV (isolate 56 – 70) was kindly donated by Prof. N. Fijan in 1979 to the Veterinary University Hanover. SVCV was propagated in CCB cells at 20 °C with 2 % CO_2 in a humid atmosphere. The Tissue Culture Infective Dose ($TCID_{50}$/ml) was estimated to be $1x10^7$ by Mikolaj Adamek according to the method established by Reed and Muench (1938). For this method monolayers of cells were incubated with serial dilutions of the virus stock and the CPE is evaluated for each dilution after a certain incubation period. The $TCID_{50}$ equals the dilution at which 50 % of the replicates display cytopathic effects.

For the infection CCB cells were pre-treated with MacroGard® as described above, after 24 hours the spent medium was removed and cells were treated with fresh medium alone or with fresh medium containing SVCV with a virus titre of 1×10^3 $TCID_{50}$/ml. The treatments were carried out in six replicates (Figure 6.5) and the medium was replaced by fresh medium after 1 hour. Cells were cultured at 20 °C (see above) and were checked daily for CPE by light microscopy, and on day 1, 2, 3, and 4 days post infection (p.i.) samples were harvested with trypsin. For this purpose the medium was removed from the wells and the cells were washed with 500 µl 0.5x trypsin in PBS. Cells were then incubated with 200 µl 0.5x trypsin in PBS and were observed for detachment of the cells. When approximately 70 % of the monolayer had become detached (~ 10 min) the trypsin was deactivated by adding 800 µl of cell medium. Cells were detached and suspended by drawing them through a pipette 40 times. Three of the six replicates of each treatment (i.e. control, MacroGard®, SVCV and MacroGard®/SVCV) were processed for the gene expression analysis by centrifuging the cells (100 g for 5 min) at room temperature (Thermo Scientific Hereaus Megafuge 1.0), washing the cell pellet with Dulbeco's PBS (without Mg^{2+}, Ca^{2+}), re-centrifuging (900 g for 5 min) and reconstituting the cell pellet in

300 µl lysis buffer from the RNeasy kit (Qiagen, UK). These samples were stored at -80 °C until further processing for the gene expression analysis. Figure 6.5 shows a schematic outline of the experimental procedure.

The other three replicates were used to determine the level of apoptosis in the cells by acridine orange staining and flow cytometry. For this purpose cells were concentrated by centrifugation (100 g for 5 min at room temperature) and the pellet was resuspended in 150 µl of the supernatant. Approximately 120 µl of the suspension was used for the flow cytometry (see section 6.2.7) while 10 µl were used for the immediate analysis with acridine orange staining as described in chapter 3.3.4 and 20 µl were used to confirm infection as described in section 6.2.6. The UV microscope used for the analysis of the acridine orange staining was a Zeiss Axiophot with a FITC filter and photographs were taken with a digital camera (Nikon Coolpix P5000).

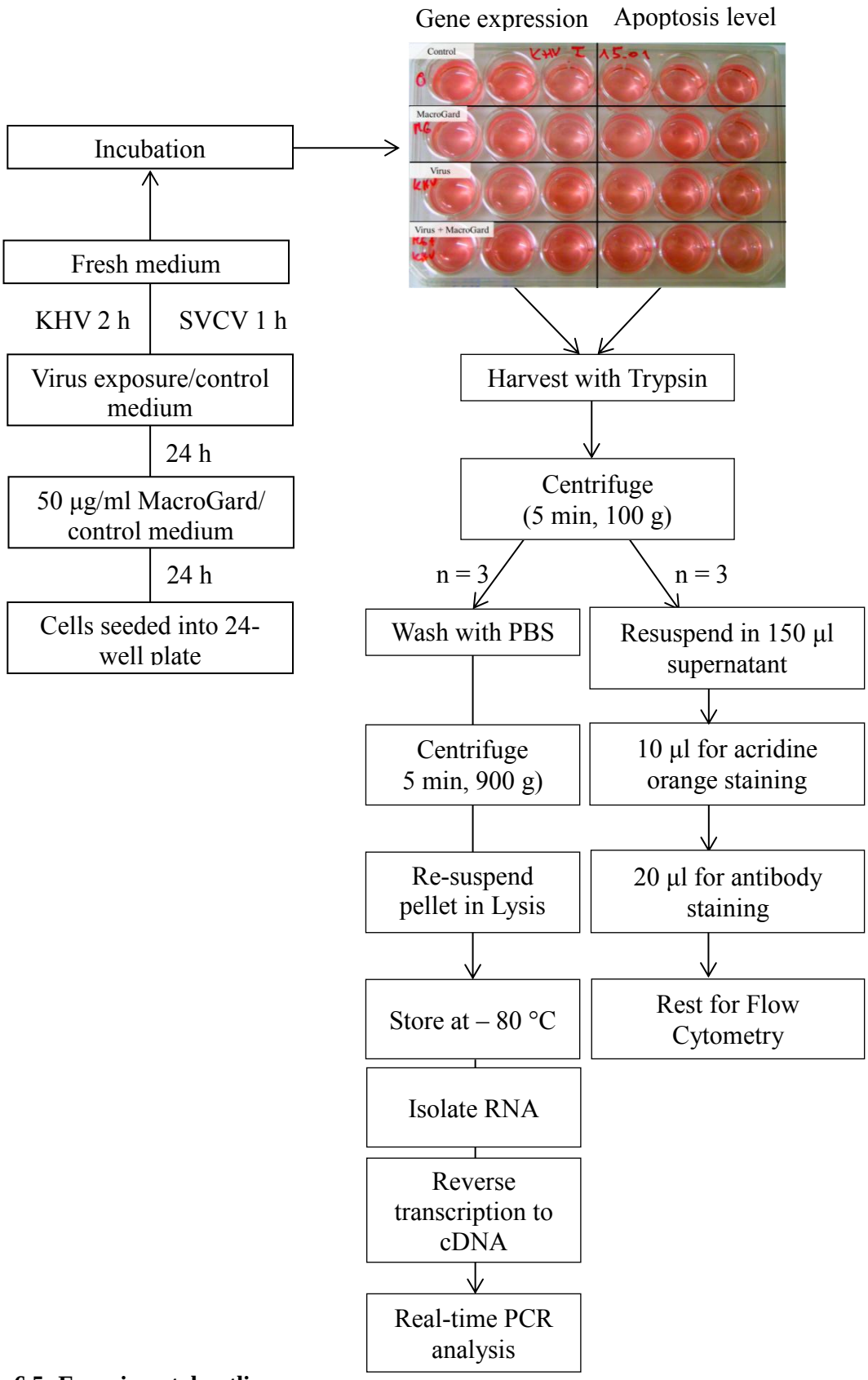

Figure 6.5: Experimental outline

This flow chart shows the outline of the study described in this chapter. The study starts with the seeding of the cells into the well plate end describes the process of sample preparation and how the replicates are divided up.

6.2.5 KHV infection

KHV of the strain KHV-I, kindly provided by the Friedrich-Loeffler-Institute, Germany (Hedrick *et al.*, 2000), was re-isolated from skin of common carp that had been infected with this virus by intraperitoneal injection with the virus. The virus was then propagated in CCB cells over two passages (see section 6.2.2). On day 5 days post inoculation a clear CPE was visible and the culture was mechanically lysed and cellular debris were removed by centrifugation (15 min, 3000 g) as describes in Adamek *et al.*, (2012). The supernatant was used to to infect the cell lines as described below.

This virus displayed a titre of 1×10^3 $TCID_{50}$/ml and the same infection procedure was applied as described for SVCV with slight modifications. Cells of the CCB and CLC cell lines were incubated with 1.5×10^2 $TCID_{50}$/ml of the virus for 2 hours and were then cultured at 25 °C. CLC cells were harvested at 4, 5, 6 days p.i. whilst CCBs were sampled at 1, 2, 3, 4, 5, 6, and 9 days p.i. as described above. CLC cells detached very easily and the PBS washing step during the trypsin treatment was therefore abolished and cells were immediately incubated with trypsin. After isolation of the cells the samples were treated as described in section 6.2.4. The three replicates used for the gene expression analysis were stored at -80 °C, whilst the remaining three replicates were used for morphological analysis with antibody staining, acridine orange and flow cytometry as described for SVCV.

6.2.6 Confirmation of virus infection by immunocytochemistry

Fluorescence immunocytochemistry (FICC) was used to detect viral proteins in the infected cell cultures to confirm the presence of SVCV and KHV. At all sampling points CCB and CLC cells (3 replicates each) were placed on superfrost plus glass slides (Menzel-Glaeser, Germany) and left to dry overnight at 25 °C (Thermo Scientific Heraeus

CO_2 Incubator). The cells were then fixed with acetone for 10 min, the slides dried by evaporation, incubated for 30 min with 50 µl of blocker solution (1 % suspension of bovine albumin fraction V in PBS) and washed twice with PBS. The slides were dried by carefully blotting with tissue paper and the cells from the KHV study were then incubated with 20 µl of mouse monoclonal antibody P14 Anti-Koi Herpesvirus (Aquatic Diagnostics Ltd., Scotland). The stock solution of this antibody (i.e. 200 µg/ml PBS) was diluted 1:10 in PBS before use. For the SVCV study 20 µl of the 1:10 diluted BIO 331 SVC11A31 monoclonal antibody (Li StarFish, Italy) in PBS was used. In both cases cells were incubated with the antibody for 1 hour. After washing with PBS 20 µl fluorescence isothiocyanate conjugated rabbit anti mouse antiserum (RAM-FITC, Dako Cytomation, Denmark), which had been diluted 1:200 with RPMI 1640, was added and cells were incubated for 1 hour in the dark. Slides were then washed twice with PBS and a drop of Dabco buffer (2.5 % 1,4 diazobicyclo (2,2,2)-octan (Dabco) in 90 % glycerol and 10 % water plus 100 µl propidium iodide 2 mg/ml) was applied to the cells, which were covered with a cover slip, and analysed with a UV microscope (Axiophot, Zeiss) with FITC filter. Samples that were positive for virus protein were distinguished on the basis of green fluorescence (Figure 6.6). Propidium iodide (PI) is a nucleic stain, which in contrast to acridine orange only enters cells with damaged membrane and it can therefore be used to establish cell viability. In this case however cells were fixed and PI was used to visualize cells in general.

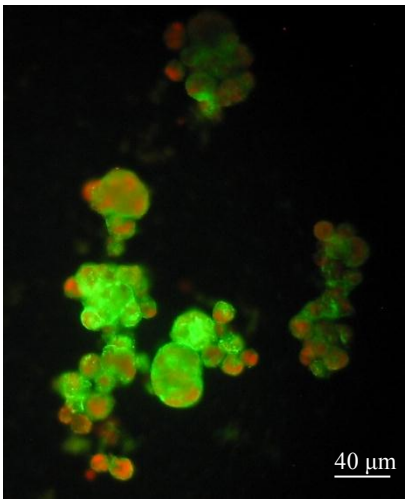

Figure 6.6: Antibody staining of CCB cells to visualise KHV infection

Virus infection was confirmed by staining CCB cells with P14 Anti-Koi Herpesvirus monoclonal antibody and conjugated fluorescence isothiocyanate. Green fluorescence indicates viral protein while propidium iodide (red staining) visualized cells.

6.2.7 Flow Cytometry

Principle

Flow cytometry is a widely used method to analyse the properties of individual cells by analysing light scatter and fluorescence emission.

A cell solution is placed into the flow cytometer and a stream of single cells is produced by hydrodynamic focussing, which flows past a light source. The light is scattered by the cells and fluorochromes are excited into a higher energy state (Figure 6.7). The forward scatter (FS) channel detects light that is scattered in the forward direction and its intensity relates to the cell size. Light detected by the side scatter (SS) channel, i.e. light that had been scattered in an angle of 90 ° from the light source, is related to the granular content of a cell. FS and SS are thus unique for every cell. The scattered light and the fluorescence emission are detected and converted into electrical pulses by photodetectors and are processed by linear or log amplifiers. Log amplifiers are thereby most often used for fluorescence studies since this enhances low signals and compresses strong signals. The signals are then converted by an analogue to digital converter and the analysis is carried out using specialized software (Rahman, 2006).

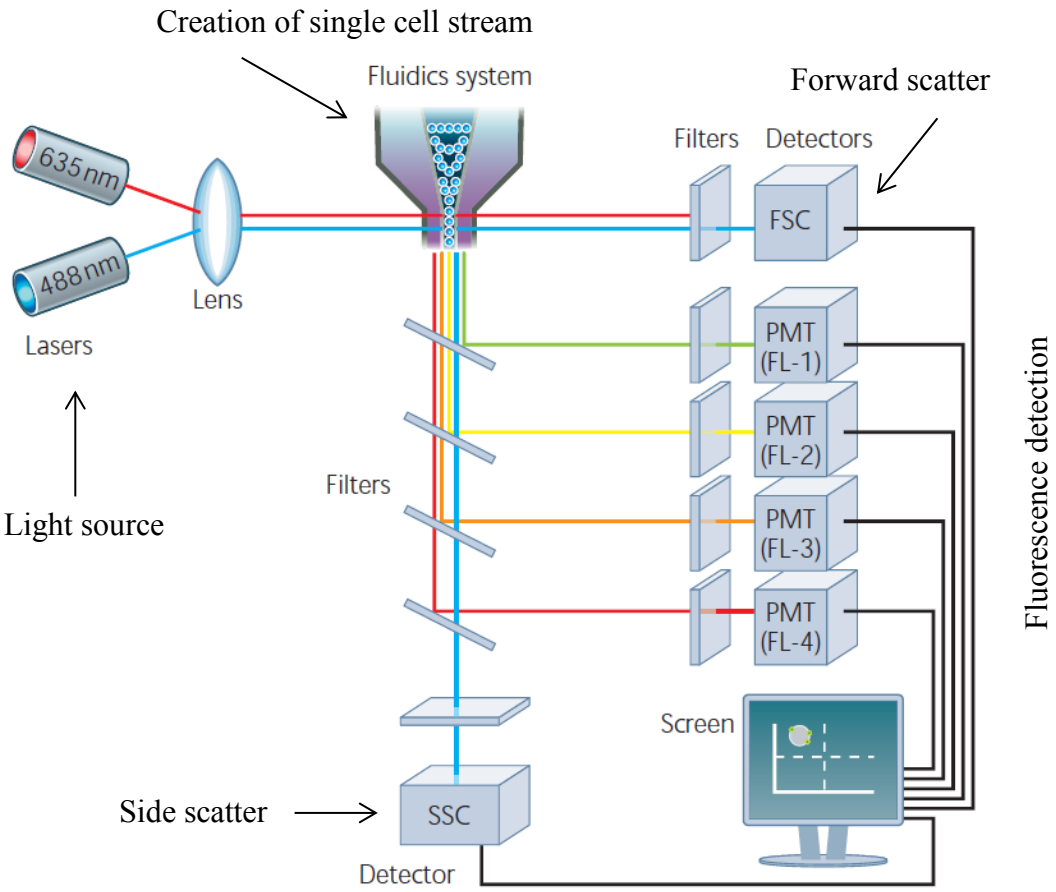

Figure 6.7: Principle of Flow Cytometry

The cell solution is compressed into a stream of single cells (blue balls) which passes a light source (i.e. lasers). The light is scattered by the cellular properties (size, granularity) and is detected by detectors (FSC, SSC) that measure the electric current. Fluorochromes are excited by the lasers and can be detected by detectors as well (PMT). This information is then analysed by software. Adapted from Rahman (2006).

In a process called "gating", cells of interest can be selectively visualized and unwanted particles (e.g. cell clusters and debris) can be excluded. The data obtained can be displayed in one-parameter and two-parameter histograms. In the former histogram cell count (i.e. number of cells) is plotted against the measurement parameter (Forward-, side-scatter or fluorescence), whilst in the two-parameter histogram analysis two parameters are plotted against each other and the particle counts are shown by density.

Apoptosis analysis by Flow Cytometry

Apoptotic cells were detected by CaspaTag staining (CaspaTag^{TM} Pan-Caspase In situ Assay Kit, Fluorescein, Millipore APT400). The principle of this method has already been described in chapter 3.3.2 and was adapted for flow cytometry analysis. For this purpose ~ 130 µl of concentrated cell solution (see section 6.2.4) were incubated with 10 µl freshly prepared 30X FLICA reagent (i.e. 2 µl FLICA reagent + 8 µl PBS) and the samples were incubated at 25 °C for 1 hour at 5 % CO_2 in the dark. Tubes were swirled every 20 minutes during this time. The 1.5 ml tubes were topped up with wash buffer (included in the kit) and centrifuged at 400 x g for 5 min at room temperature. The supernatant was removed and the cells were gently vortexed to prevent cell clumping. This washing step was repeated with 1 ml of wash buffer. The cell pellet was resuspended in 300 µl Sheath buffer (i.e. sterile PBS) with propidium iodide. Samples were stored on ice and analysed immediately.

Flow cytometry analysis was carried out with a Beckman Coulter Epics XL. An analysis protocol was set up to analyse forward and side scatter as well as the fluorescence signals of propidium iodide and CaspaTag. The flow rate was adjusted to allow a good resolution of the analysis and this flow rate was utilised throughout the whole experiment.

Before and after sample analysis an internal cleaning program was carried out utilising, Cleanse and Sheath buffers. The sample was pipetted into a round bottom polystyrene test tube, inserted into the flow cytometer and the analysis program run to analyse a maximum of 10.000 cells.

Flow cytometry analysis

Analysis of the cellular characteristics was carried out with FlowJo 7.6.5. software. To analyse the measured cell properties a gate was set to exclude cell clumps (high forward scatter (FS) and high side scatter (SS)) and debris (low FS and high SS) in a pseudocolour density plot of side scatter (cell granularity) against forward scatter (cell size) the (Figure 6.8 A). The selectively visualized CCB cells were then analysed for their cell size and granularity by applying a quadrant tool (Figure 6.8 B) which divided the cell population into four quadrants:

Quadrant	Cell size	Granularity
1	Low	High
2	High	High
3	High	Low
4	Low	Low

In a separate step the gated CCB cells were sub-divided into propidium iodide (PI) positive (pi) and negative cells (-pi) (Figure 6.8 C). Only the PI negative group was used for the analysis of the CaspaTag staining, because this identifies the apoptotic and living cells. For the purpose of quantifying CaspaTag positive cells a one-parameter histogram was created by displaying the cell size against the FITC channel. A low value for the FITC channel thereby indicates cells that displayed only low emission at the wavelength specific

for FITC fluorescence whilst higher values are measured in cells with higher fluorescence. The latter are thus positive for CaspaTag and can be considered as apoptotic. On this basis two regions were created within the histogram, one for CaspaTag negative (non-apoptotic) and one for CaspaTag positive cells (apoptotic) (Figure 6.8 D).

In summary the following factors were analysed by flow cytometry:

- Cell size
- Cell granularity
- Propidium Iodide staining
- CaspaTag staining

The FlowJo software automatically calculates the percentages of the various applied regions and this data was exported into Excel and statistically analysed with GraphPad Prism. Since the data obtained is in the form of percentages an arcsin-transformation was carried out before analysis by 2-way ANOVA and Bonferroni Post-hoc test.

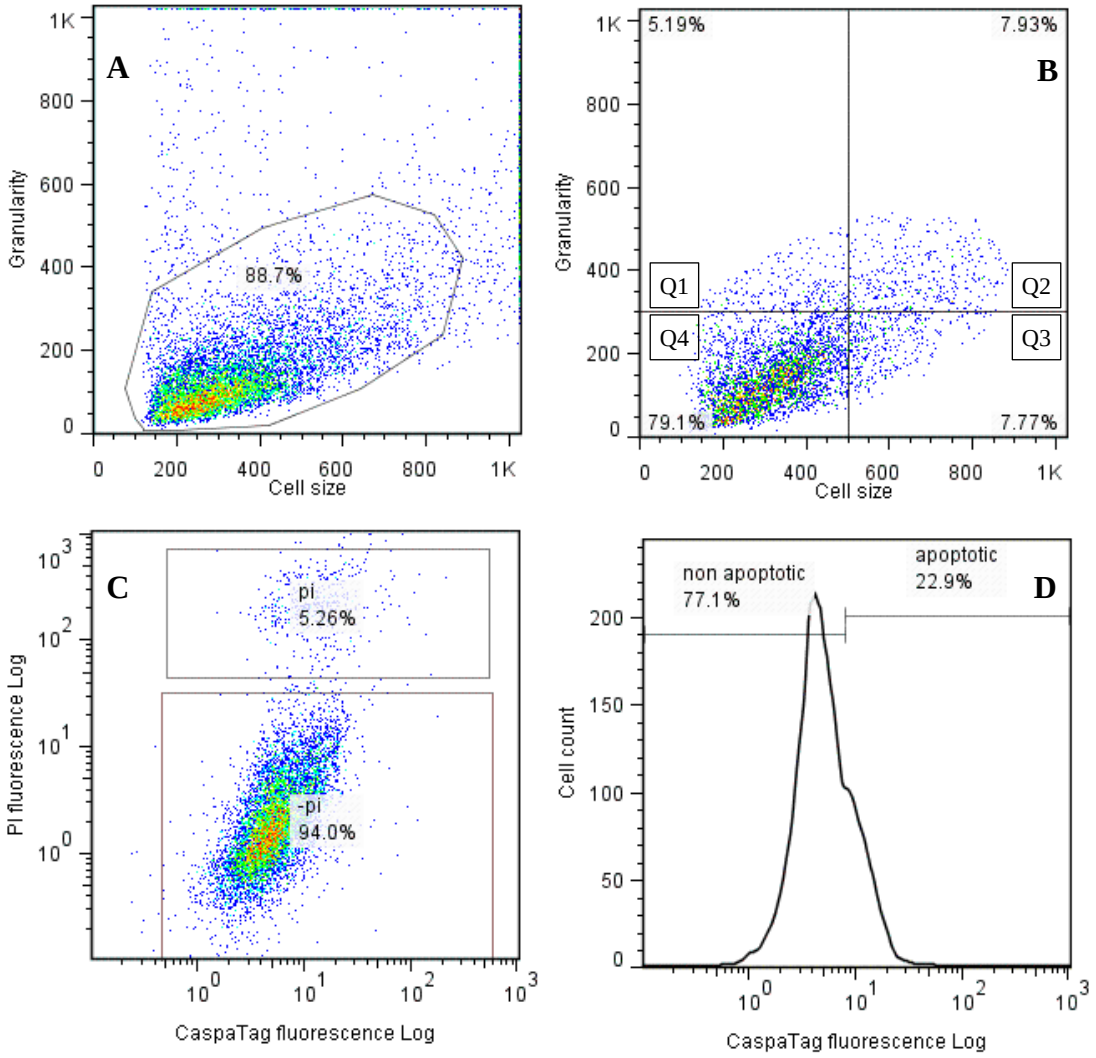

Figure 6.8: Gating

The cells detected by Flow Cytometry were gated to selectively visualize cells of interest. In the first step (A) only cells of the intended population were selected to exclude cell clusters and fragments. In step (B) a quadrant was applied to divide the cells into four types of morphology and the proportions of these were analysed (Q1: small cell size, high granularity; Q2: large cell size, high granularity; Q3: large cell size, low granularity; Q4: small cell size, low granularity). In order to analyse the percentage of PI positive and negative cells the gated cells from (A) were divided into two regions (C). Only cells from region –pi were used to analyse the CaspaTag staining (D).

6.2.8 Preparation of samples for gene expression

For the gene expression analysis RNA was isolated with the RNeasy Mini kit (Qiagen, UK) as described in chapter 2.5.1 and was normalized to a common concentration of 30 ng/ml. Since the DNA-virus KHV contributed DNA to the cell samples a DNase step was added to the procedure. For this purpose 1 µg RNA was added to a mixture of 1x RQ1 RNase free DNase buffer, 1 ng RQ1 RNase free DNase (both supplied by Promega) and 10 U RNase Inhibitor (Invitrogen). The sample was adjusted to 10 µl with DEPC treated water (Invitrogen) and incubated at 37 °C for 30 minutes. The reaction was stopped by adding 1 µl RQ1 DNase stop solution (Promega) and the DNase was inactivated by incubating at 65 °C for 10 min. All incubations were carried out in a thermocycler (GeneAmp PCR System 9700, Applied Biosystems). The preparation of cDNA from these samples was then carried out as described in chapter 2.5.2.

The virus load of SVCV was analysed by studying the expression of an innate glycoprotein G with the following primers (Alberto Falco, personal communication): Forward primer 5' – GCT ACA TCG CAT TCC TTT TGC – 3' and reverse primer 5' – GCT GAA TTA CAG GTT GCC ATG AT – 3'. The expression of KHV was studied using the thymidine kinase gene, which is innate to this virus using the following primers (Mikolaj Adamek, personal communication): Forward primer: 5' – TGG CTA TGC TGG AAC TGG TG – 3' and Reverse primer: 5' – GCT GGT CTA TGG CGT GCT TG – 3'.

The following primers were chosen to analyse cell signalling and apoptosis:

- Nemo and p38 was used to analyse cell signalling because viruses and β-glucan are known to affect the NF-κB pathway (Brydon *et al.*, 2005).
- iNOS was used to analyse the involvement of NO
- p53, Caspase 9, Apaf-1, Bcl-2, IAP were studied to determine the modulation of the intrinsic apoptosis pathway.

6.2.9 Data analysis and statistics

The virus load was calculated as x-fold gene expression relative to the control on day 1 and analysed with a 1-way ANOVA and a Tukey's Post-hoc test. The data for all other gene expression and apoptosis levels were analysed with 2-way ANOVA to compare gene expression and apoptosis levels within a KHV or SVCV treated cell line. Bonferroni Post-hoc analysis was carried out to test for differences between treatment groups. Significance was defined as $p \leq 0.05$. Graphs show mean ± SEM with $n = 3$.

6.3 Results of SVCV infection

6.3.1 Cytopathic effects

The infection with SVCV caused extensive damage to the cell layer (cytopathic effect CPE) 3 days post-infection (Figure 6.9). Visually, MacroGard® had no effect on the CPE level caused by the virus but caused differences in the density of the cell layer (black arrow in Figure 6.9).

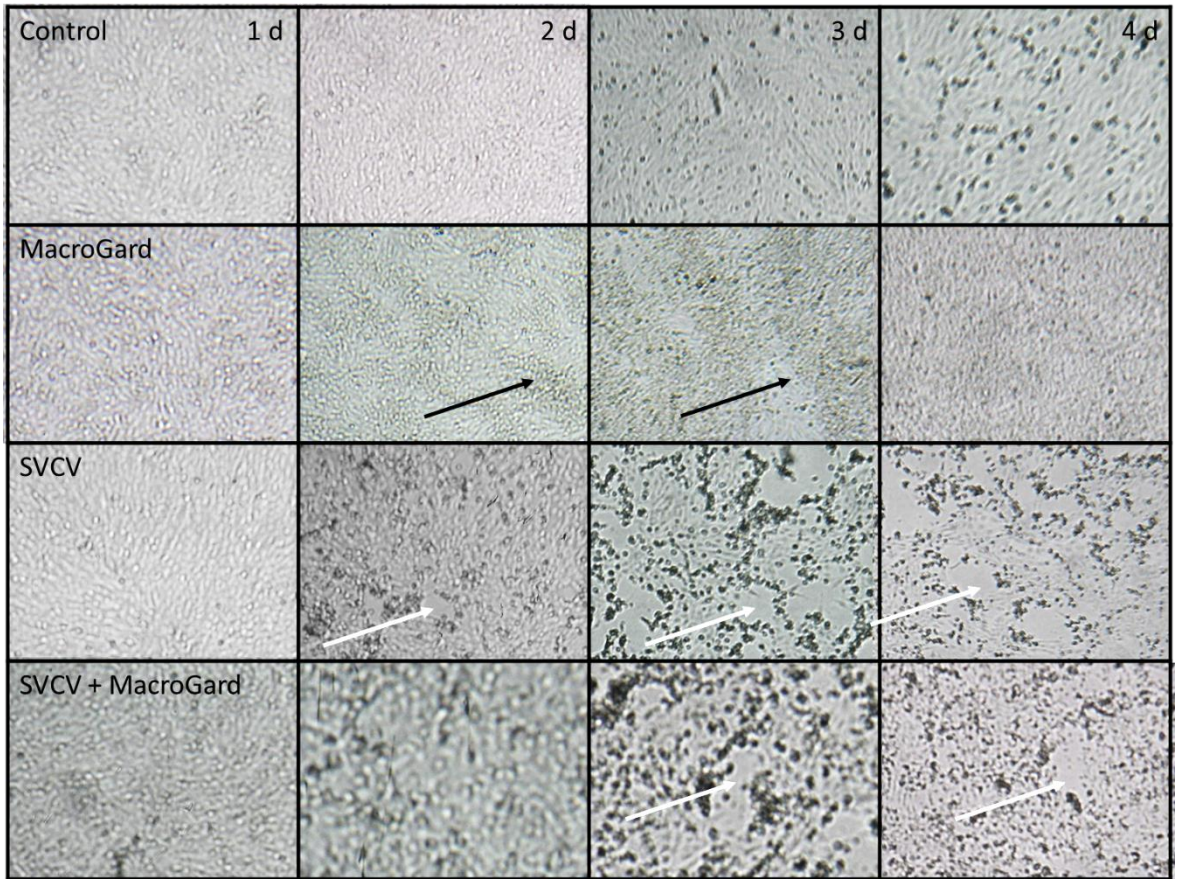

Figure 6.9: Monolayer of CCB cells infected with SVCV

Monolayers of CCB cells were infected with 1 x 10^3 $TCID_{50}$/ml and the CPE was observed over a period of 4 days. White arrows indicate CPE, black arrows indicate higher densities in the monolayer due to the treatment with 50 µg/ml MacroGard®. Photos are representative of three replicates taken at a magnification of 100x.

6.3.2 Apoptosis levels

The infection of CCBs with SVCV led to significantly elevated apoptosis levels on day 3 (8.7 ± 2.5 %) and 4 p.i. (13.2 ± 1.7 %) (Figure 6.10) as ascertained with the acridine orange staining. Exposure of non-infected cells to 50 µg/ml MacroGard® did not induce a significant effect on the level of apoptosis; however with MacroGard® there was a trend towards increased levels of apoptosis in SVCV infected cells i.e. 23.2 ± 7.1 % apoptosis on day 4; 10 times higher than the level in the cells that were only infected but not MacroGard® treated.

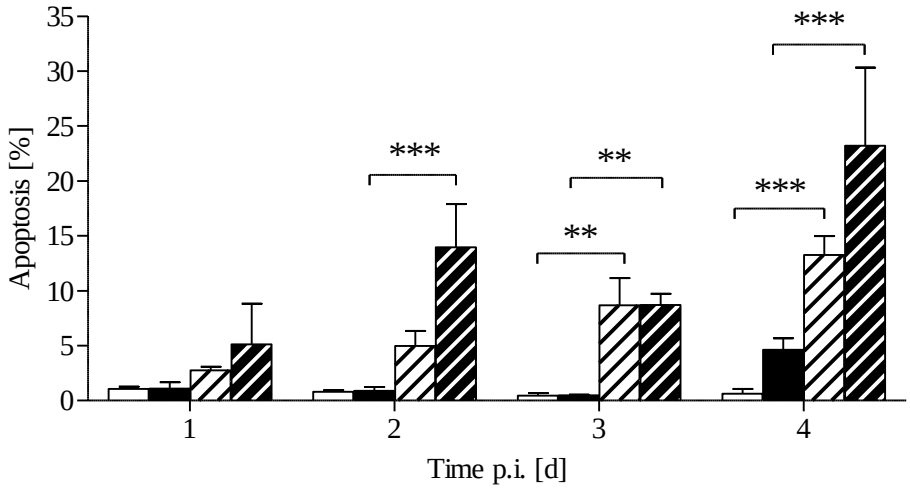

Figure 6.10: Apoptosis level in SVCV infection

CCB cells were exposed to 50 µg/ml MacroGard® and/or infected with 1 x 10^3 $TCID_{50}$/ml of SVCV. Apoptosis levels were recorded by ascertaining the percentage of apoptotic cells in the cell population using acridine orange. Bars: white = control, black = MacroGard®, white with stripes = SVCV infected, black with stripes = SVCV + MacroGard®. n = 3, graph shows mean ± SEM, statistical significances: **: 0.01, ***: 0.001.

6.3.3 Flow cytometry with SVCV infection

Cell size and granularity

The proportion in which the different recorded cell morphologies (Figure 6.11), expressed as cell size and granularity, existed changed significantly with the applied treatment ($F = 3.02 – 55.9$, $p \leq 0.04$), the time course of the experiment ($F = 13.3 – 39.03$, $p < 0.0001$) and the interaction of these two factors ($F = 4.3 – 8.3$, $p \leq 0.0009$).

Cells that displayed a small size and low granularity in the analysis were defined as cells with normal morphology and will be referred to as “normal cells”. This group of cells make up the major proportion of the analysed cells (around 80 %). SVCV infected cells showed slightly higher levels (~ 3 %) of normal cells throughout the experiment ($p \leq 0.001$). The number of cells with a higher granularity (independent of cell size) decreased in the infection treatments in comparison to the untreated control (i.e. non-infected, non-MacroGard® treated cells). The incubation of the cells with 50 µg/ml MacroGard® had no effect on the cell morphology as detected by flow cytometry.

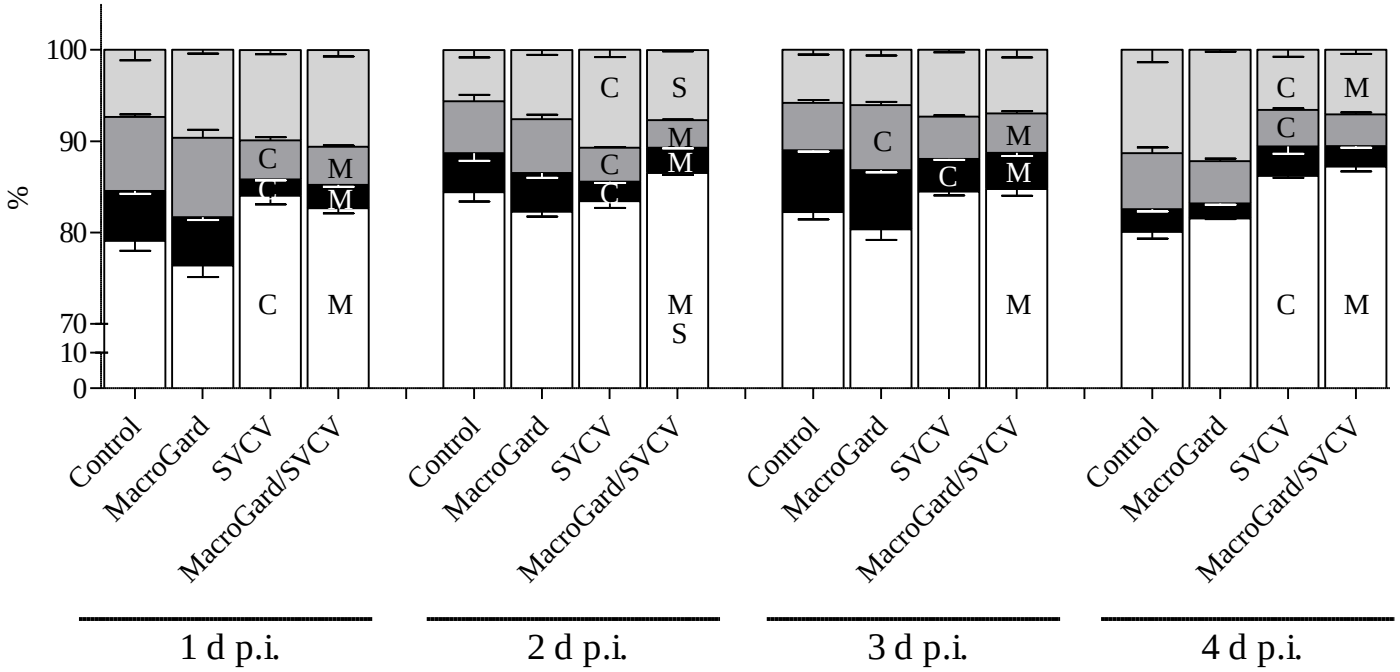

Figure 6.11: Changes is cellular morphology during SVCV infection

The percentage of cells with certain morphologies was measured by Flow Cytometry. White: small cells with low granularity, black: small cells with high granularity, light grey: large cells with high granularity, dark grey: large cells with low granularity. Statistical significance ($p \leq 0.05$) within morphology type is indicated by capital letters. C indicates significant difference to control, M = significant difference to MacroGard® group and S = significant difference to SVCV infected group. The graph displays mean ± SEM of n = 3.

Propidium Iodide staining

Live and dead cells (independent of their ability to be stained by CaspaTag) were distinguished by propidium iodide (PI), which stains cells with a permeable membrane, i.e. dead cells. This revealed that the levels of PI positive cells was dependent on the treatment ($F = 6.1$, $p = 0.002$), the time since start of the study ($F = 4.7$, $p = 0.008$) and the interaction between these two parameters ($F = 3.1$, $p = 0.009$). The mean control level of propidium iodide positive cells (Figure 6.12) was 6.0 ± 1.1 % with levels being higher during the first two days (7 – 8 %) and lowest level on the last day of experimentation (2.9 ± 0.7 %). The levels of PI positive cells in the SVCV treated samples (SVCV and MacroGard®/SVCV) were low (~ 4 %) throughout the study and thus significantly different to the non-infected control levels (Control and MacroGard®) during the first two experimental days ($p \leq 0.05$).

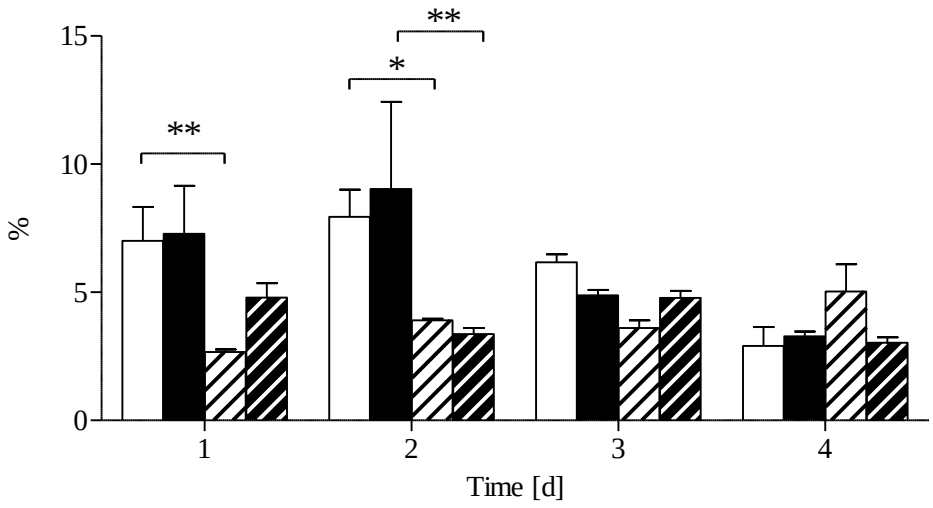

Figure 6.12: Levels of propidium iodide positive cells during SVCV infection

Cells were incubated with 50 µg/ml MacroGard® and infected with SVCV. A propidium iodide staining was employed and cells were analysed by flow cytometry. Bars: white = control, black = MacroGard® exposed, white with stripes = SVCV infected, black with stripes = MacroGard® exposed and SVCV infected. Graph shows mean ± SEM of n = 3. Statistical significance is defined as $p \leq 0.05$. * = $p \leq 0.05$, ** = $p \leq 0.01$.

CaspaTag staining

The amount of CaspaTag positive and PI negative cells was dependent on the time course of the experiment ($F = 17.2$, $p < 0.0001$) but also the interaction of the treatment and time factor influenced the percentage of positive cells ($F = 4.8$, $p = 0.0005$). In all treatments the average level of CaspaTag positive cells was around 25 % (Figure 6.13) and the only significant difference was detected on day 3, when 17 % more cells were CaspaTag positive in the SVCV treated group than in the control group ($p \leq 0.01$).

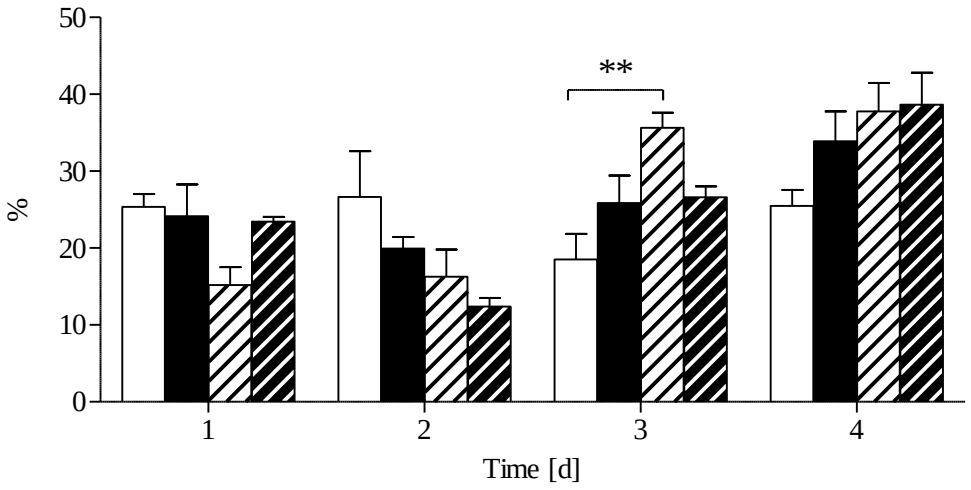

Figure 6.13: Levels of CaspaTag positive cells during SVCV infection

CCB cells were exposed to MacroGard® and infected with SVCV before being stained with CaspaTag. The analysis was carried out by flow cytometry. Bars: white = control, black = MacroGard® exposed, white with stripes = SVCV infected, black with stripes = MacroGard® exposed and SVCV infected. Graph shows mean ± SEM of n = 3. Statistical significance is defined as $p \leq 0.05$, ** = $p \leq 0.01$.

6.3.4 Gene expression with SVCV infection

The virus load of SVCV (Figure 6.14) was analysed by studying virus innate glycoprotein and the x-fold gene expression relative to the control on day 1 was calculated and a significant difference was detected ($F = 244.2$, $p \leq 0.0001$). As expected the non-infected samples were free of virus (expression ~ 1) and the virus expression in the infected samples was significantly (~ 19,000 fold) increased ($p \leq 0.001$). The virus load in the infected samples peaked on the second day post-infection and decreased in the following days to similar values as recorded on day 1. MacroGard® did not influence the virus load.

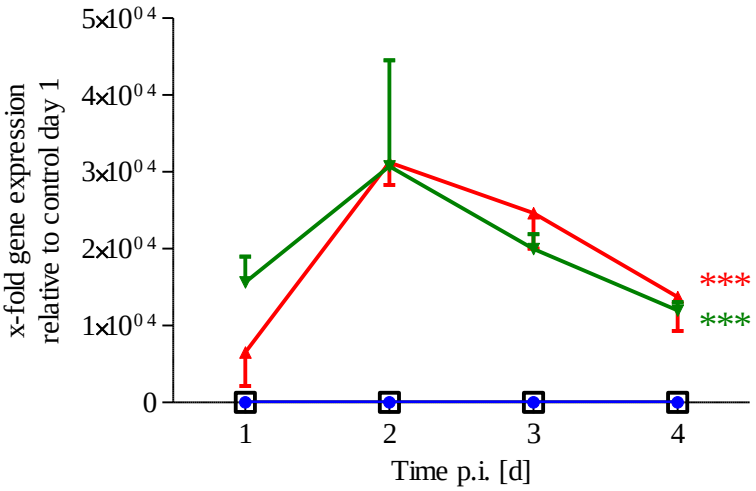

Figure 6.14: Viral gene expression in SVCV infected cells

CCB cells were exposed to 50 µg/ml MacroGard® and/or infected with 1×10^3 $TCID_{50}$/ml. Presence and replication of the virus was established by measuring expression of viral glycoprotein. n = 3 with mean ± SEM.

□: control, ●: MacroGard®, ▼: SVCV, ▲: SVCV + MacroGard®. Glycoprotein expression was significantly higher in both SVCV treatments with $p \leq 0.001$ (= ***). Graph shows mean ± SEM, n = 3

The analysis of variances (Table 6.1) revealed that the applied treatments (MacroGard® exposure and/or viral infection) influenced the expression of all genes investigated ($F = 3.2 – 68.5$, $p \leq 0.04$). The same applies for the influence of the time course of the experiment ($F = 6.4 – 14.0$, $p \leq 0.002$). The influence of the treatment was also different at the analysed time points, since generally lower levels of expression were observed in the earlier time points (day 1 – 2 p.i.) ($F = 2.4 – 11.6$, $p \leq 0.004$). Only the expression of the NF-κB related gene, Nemo, was not significantly influenced by the interaction.

Table 6.1: 2- way ANOVA analysis of x-fold gene expression during SVCV infection*

	Time		Treatment		Interaction	
	F	p	F	p	F	p
iNOS	10.0	0.0001	68.5	< 0.0001	7.4	< 0.0001
p53	14.2	< 0.0001	25.5	< 0.0001	11.6	< 0.0001
Caspase 9	6.6	0.002	14.11	< 0.0001	3.9	0.004
Apaf-1	6.6	0.002	7.7	0.0008	3.8	0.004
Bcl-2	6.4	0.002	3.7	0.02	2.4	0.04
IAP	13.0	< 0.0001	26.6	< 0.0001	7.3	< 0.0001
Mcl-1b	7.4	0.001	3.2	0.04	3.4	0.007
p38	9.3	0.0002	11.9	< 0.0001	4.0	0.003
Nemo	6.5	0.002	6.0	0.003	2.0	0.08

*the grey background highlights significant effects

With respects to the expression of apoptosis-related genes analysed (Figure 6.15) exposure to MacroGard® induced an effect on gene expression i.e. a reduction of p53 expression of approximately 70 % on day 4 ($p \leq 0.05$).

The viral infection with SVCV (Figure 6.15) led to increased levels of iNOS gene expression, which peaked on day 1 p.i. (7.3 ± 1.2 -fold) and declined on subsequent days reaching a minimum on day 4 (2.2 ± 0.6 -fold). In addition, there was significant influence of MacroGard® on the virus induced gene expression for iNOS except for a significant difference between SVCV infection and the combined MacroGard®/SVCV treatment on day 1 ($p \leq 0.001$). At this time point the gene expression in the combined treatment (MacroGard®/SVCV) was however not elevated from the control level (0.99 ± 0.24 -fold). Gene expression levels of the pro-apoptotic gene p53 were increased on day 3 ($p \leq 0.001$) and day 4 post-SVCV infection ($p \leq 0.05$). Incubation with MacroGard® caused a doubling of the SVCV induced p53 mRNA levels 2 ($p \leq 0.01$) and 4 ($p \leq 0.05$) days post infection. On the first sampling day (1 d p.i.) however this treatment led to a down-regulation of the p53 gene expression ($p \leq 0.05$). The other two pro-apoptotic genes analysed, i.e. Caspase 9 and Apaf-1, were not significantly influenced by the viral infection. Nevertheless in the samples that were exposed to MacroGard® prior to the infection the expression of these genes was significantly elevated. For Caspase 9 this up-regulation was apparent on day 2 ($p \leq 0.001$) and 4 ($p \leq 0.01$) of MacroGard® treatment, and for Apaf-1 on day 4 p.i. ($p \leq 0.001$). The only significant difference between the two infection treatments was recorded in the expression of Caspase 9 gene on day 1, where the expression in the SVCV treated cells was approximately 3 times higher than in the MacroGard®/SVCV treatment ($p \leq 0.01$).

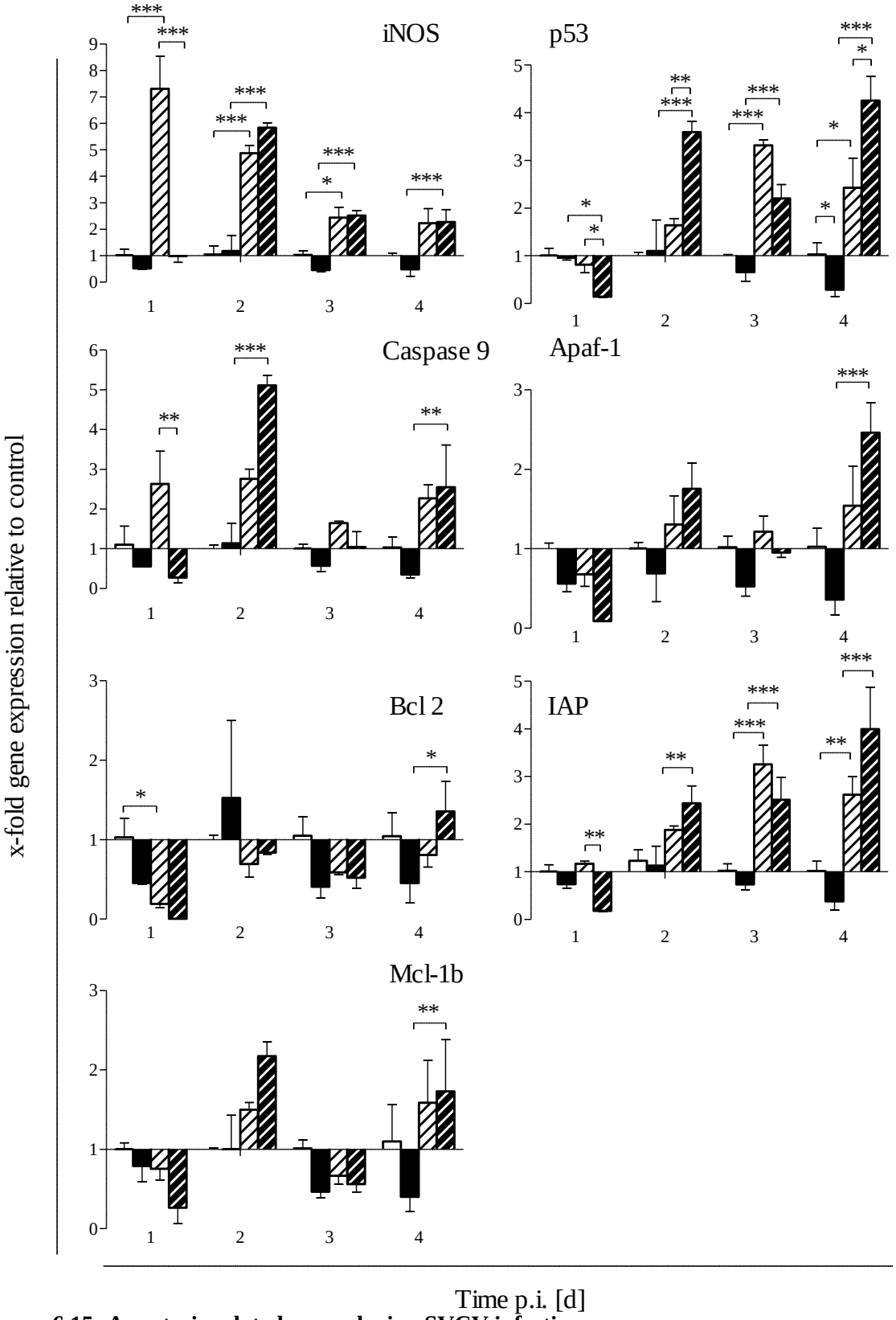

Figure 6.15: Apoptosis related genes during SVCV infection

CCB cells were incubated with 50 µg/ml MacroGard® or control medium for 24 h and then infected with SVCV virus. Graphs show x-fold gene expression relative to control. Bars: white = control, black = MacroGard®, white w/stripes = SVCV, black w/stripes = MacroGard®/SVCV, n = 3, graphs show mean ± SEM, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

The expression of the anti-apoptotic genes Bcl-2 and Mcl-1b showed little discernible response to the MacroGard® and infection treatments. A general down-regulation in all the treatments is observed for Bcl-2 though the decrease due to SVCV infection was significant only at day 1 ($p \leq 0.05$). The only up-regulation in Bcl-2 gene expression occurred on day 4 in the combined SVCV/MacroGard® treatment when compared to the MacroGard® control. This latter effect is also observed in Mcl-1b on day 4 p.i. ($p \leq 0.01$). The expression profile of the other anti-apoptotic gene, IAP, stands in contrast to these previous results since this gene is strongly affected by the infection. On day 3 and 4 post-SVCV infection caused a 2.5 – 3-fold increase in IAP mRNA levels ($p \leq 0.001$ and 0.01 respectively). The samples that were exposed to MacroGard® prior to the virus treatment were similarly affected as the samples without the pre-treatment since in both groups the IAP gene expression was elevated from day 2 onwards ($p \leq 0.01$) when compared to the respective controls. In the comparison of the two infection treatments, i.e. SVCV and SVCV/MacroGard®, the immunostimulant induced an elevation of IAP gene expression in the SVCV treated samples on day 4 (4.0 ± 0.9 -fold) compared to the samples that were only treated with the virus (2.6 ± 0.4 -fold). Interestingly this expression profile of IAP reflects the profile of p53.

Nemo and p38 are genes that are involved in important cell signalling pathways and both genes are modulated by SVCV (Figure 6.16). On day 3 p.i. p38 is up-regulated in both SVCVC treatments, i.e. SVCV alone and in combined treatment (SVCV/MacroGard®), when compared to their respective controls ($p \leq 0.05$). For the combined treatment this elevation of gene expression continued on day 4 when it was approximately 2.5 times higher than in the MacroGard® control. MacroGard® treatment led to a decrease in p38 expression, which was significant on day 4 p.i. when expression was decreased by approximately 70 % compared to the untreated control. Nemo was significantly affected only on the last sampling day, when both SVCV treated groups displayed higher gene levels (9 – 12 -fold) compared to their controls ($p \leq 0.01$).

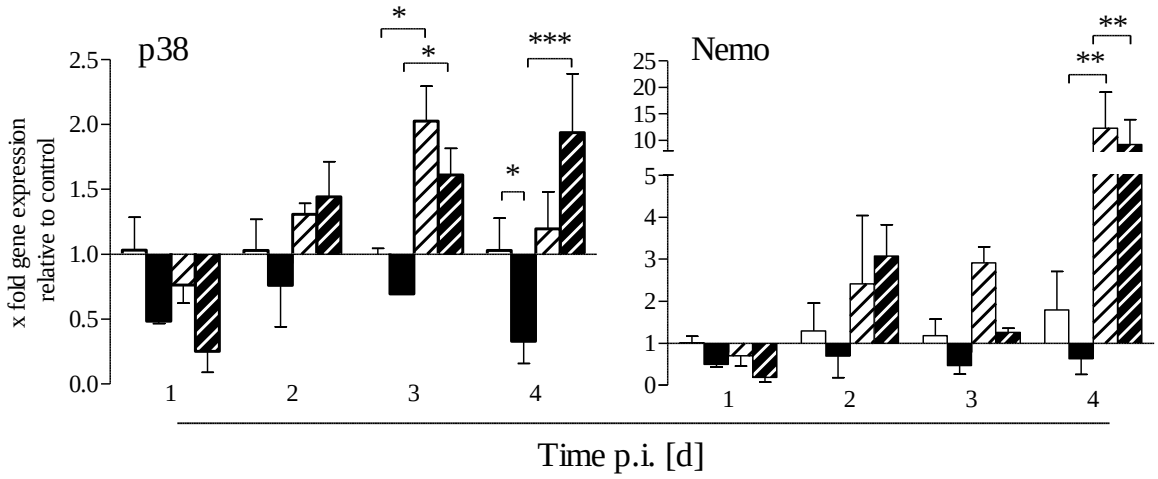

Figure 6.16: Cell signalling genes during SVCV infection

CCB cells were incubated with 50 µg/ml MacroGard® or control medium for 24 h and then infected with SVCV virus. Graphs show x-fold gene expression relative to control. Bars: white = control, black = MacroGard®, white with stripes = SVCV, black with stripes = MacroGard® & SVCV, n = 3, graphs show mean ± SEM, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

6.4 Results of KHV infections

6.4.1 Cytopathic effects

KHV had differential effects on the CCB and CLC cell lines. The fibroblastic cell line, CCB, displayed clear cytopathic effects (CPE) from day 5 of exposure to KHV whilst no such effects were ever noted in the leucocyte cell line (CLC) treated with KHV (Figure 6.17). The plaques of cells observed in the CLC cell line were caused by disturbance of the monolayer during the exposure treatments. Additionally it was observed that staining with anti-KHV antibody did not detect the virus associated with CLC cells while CCB cells were clearly infected with KHV.

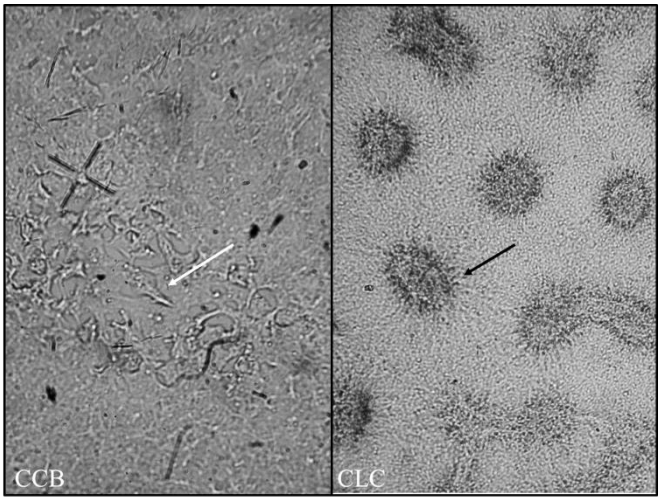

Figure 6.17: CCB & CLC 5 d.p.i. with KHV

CCB and CLC cells were infected with 1.5 x 10^2 $TCID_{50}$/ml KHV. After 5 days post infection CCB cells showed cytopathic effects (CPE, white arrow) while no CPE was observed in CLC. Due to disturbance of the monolayer during the treatment CLC displayed plaques in the monolayer (black arrow).

The KHV infection induced extensive CPE in CCB cells during the later days of the infection (day 5 – 9 p.i.) to an extent that the monolayer was completely destroyed on day 9 (Figure 6.18). As already noted in the SVCV study, MacroGard® did not have any visual effect on the level of CPE but did cause differences in the density of the cell layer.

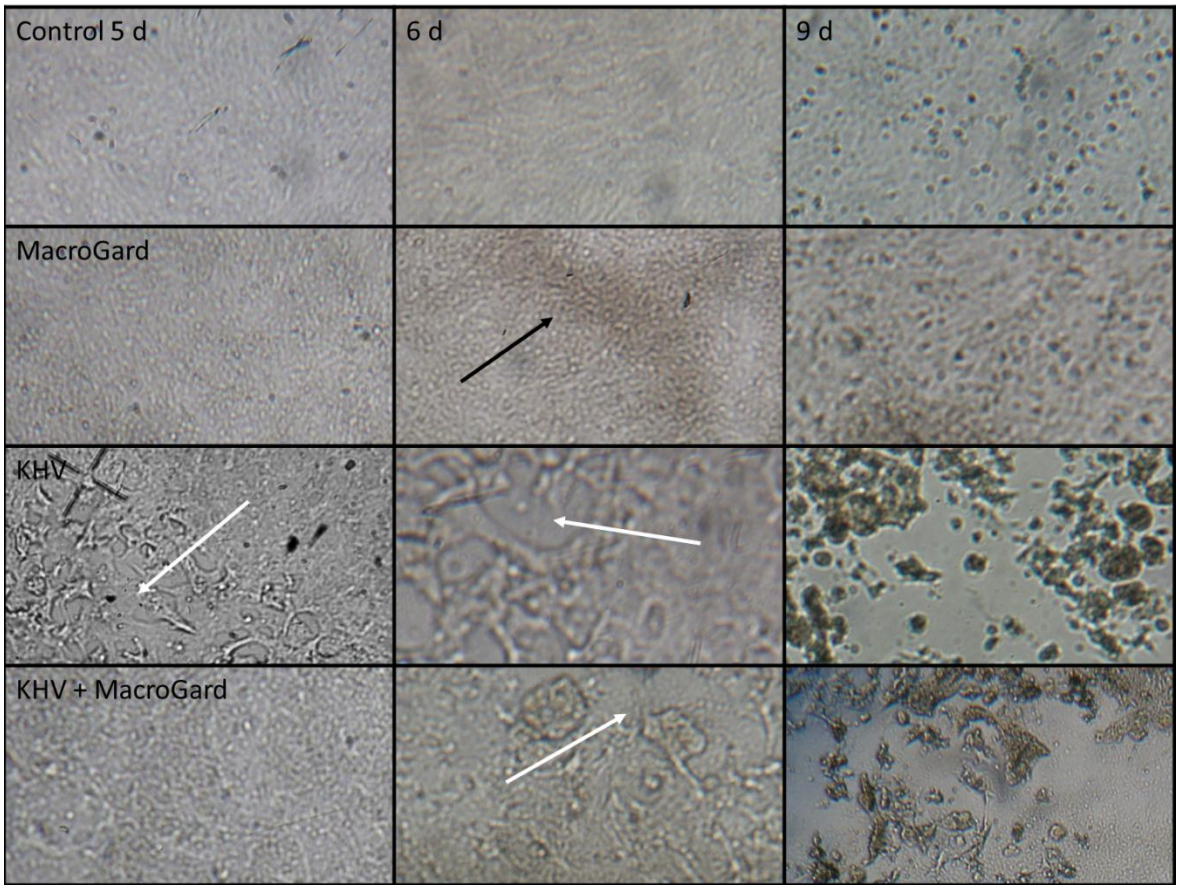

Figure 6.18: Monolayer of CCB cells after KHV infection

CCB cells were infected with KHV at a dose of 1.5×10^2 $TCID_{50}$/ml. KHV treated samples displayed CPE (white arrow) and at 9 d.p.i. complete destruction of the monolayer was observed. In the MacroGard® (50 µg/ml) treated samples higher densities of the monolayer were observed (black arrow). Pictures are a representative of 3 replicates. Cells were observed with 100x magnification.

6.4.2 Apoptosis levels and cellular morphology

The infection of CCB and CLC cells with KHV had no effect on the percentage of apoptotic cells (Figure 6.19) detected with acridine orange, and also there were no other discernible effects on the morphology of these cells. In addition, apoptosis levels in both cell types were not affected by MacroGard® (Figure 6.19).

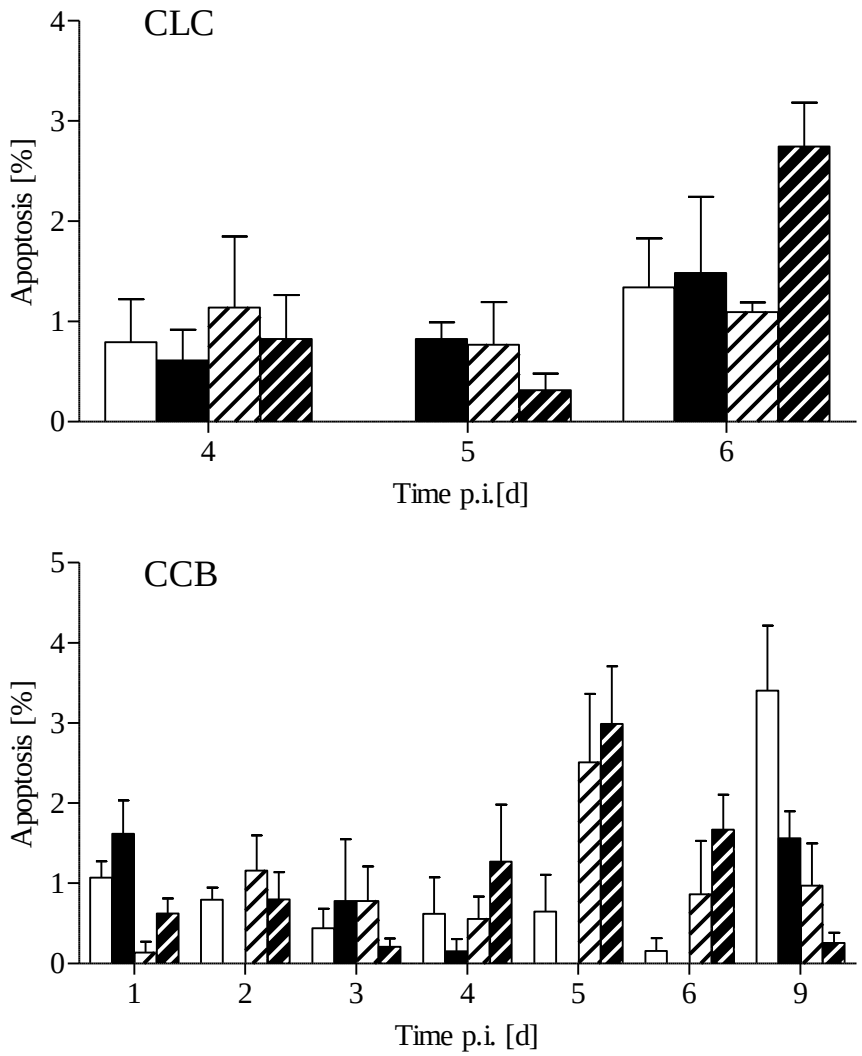

Figure 6.19: Apoptosis levels in KHV infected cells

CLC and CCB cells were subjected to 50 µg/ml MacroGard® and/or infected with 1.5×10^2 $TCID_{50}$/ml KHV and percentage of apoptotic cells within the cell population was observed with acridine orange staining. White bars: control treated cells, black bars: cells treated with MacroGard®, white with black stripes: KHV infected cells, black with white stripes: cells treated with KHV and MacroGard®. n = 3, data presented as mean ±SEM.

However, KHV infection of the CCB cells did induce morphological changes during the late sampling days (day 5 – 9), which resulted in the formation of giant cells and giant multinucleated cells (GMC) (Figure 6.20). These GMCs appeared to undergo lysis with time and released their content into the medium.

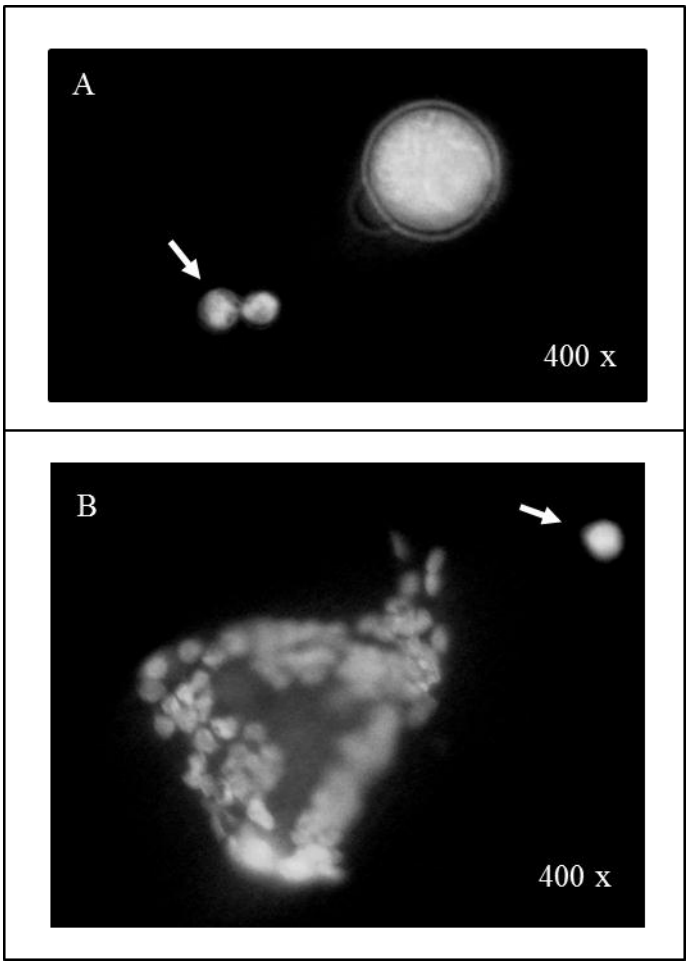

Figure 6.20: Morphological changes due to KHV infection

CCB cells were infected with 1.5 x 10^2 $TCID_{50}$/ml KHV and stained with acridine orange. Giant cells (A) and giant multinucleated cells (B) were observed and set in relation to normal sized cells (white arrows).

6.4.3 Flow cytometry with KHV infection in CCB cells

Cell size and granularity

The treatments ($F = 12.4 – 28.2$, $p < 0.0001$), the time since the infection treatment and the interaction ($F = 8.4 – 66.8$, $p < 0.0001$) of these two factors ($F = 3.3 – 9.4$, $p \leq 0.0003$) significantly influenced the size and the granularity of the cells analysed (Figure 6.21).

In the control and the MacroGard® treated group the level of the cells that were regarded as normal (i.e. small cell volume and low granularity) were between 77 and 86 % throughout the experiment. In the two KHV infected groups these levels of normal cells were significantly lower on the last two sampling days compared to the earlier sampling days (day 1 – 5 p.i.) ($p \leq 0.001$), i.e. day 6 levels approximately 74 %; day 9 approximately 70 %. Cells that displayed morphology with a large volume and a low granularity in the flow cytometry analysis were regarded as Giant Cells and occurred more frequently in the two groups that were KHV infected. On the last three sampling days (i.e. day 5, 6, 9) the percentage of Giant Cells were significantly higher than in the non-infected cells ($p \leq 0.001 – 0.05$) with the highest levels occurring on day 9 (KHV: 15.2 ± 0.5 %, KHV/MacroGard®: 14.2 ± 0.2 %). Similarly large cells with a high degree of granularity (i.e. defined as Multinucleated Giant Cells) were more common in cells exposed to KHV during the last two days of the study. At these days the number of cells with this morphology was doubled (day 6) or tripled (day 9) compared to the non-infected cells ($p \leq 0.001$). There was also a difference in the number of small cells with high granularity, which can be regarded as apoptotic cells. These cells occurred more frequently in the non-infected treatments than in the cells infected with KHV at the 2nd, 5th and 6th sampling day ($p \leq 0.001 – 0.05$). MacroGard® had little discernible influence on CCB cells at 24 h

incubation although on day 2 p.i. higher levels of large cells with low granularity ($p \leq 0.01$) and lower levels of small cells with high granularity ($p \leq 0.05$) when compared to the control was noted. The MacroGard® treatment also led to a higher percentage of small cells with high granularity in KHV infected cells in comparison to solely KHV treatment ($p \leq 0.01$).

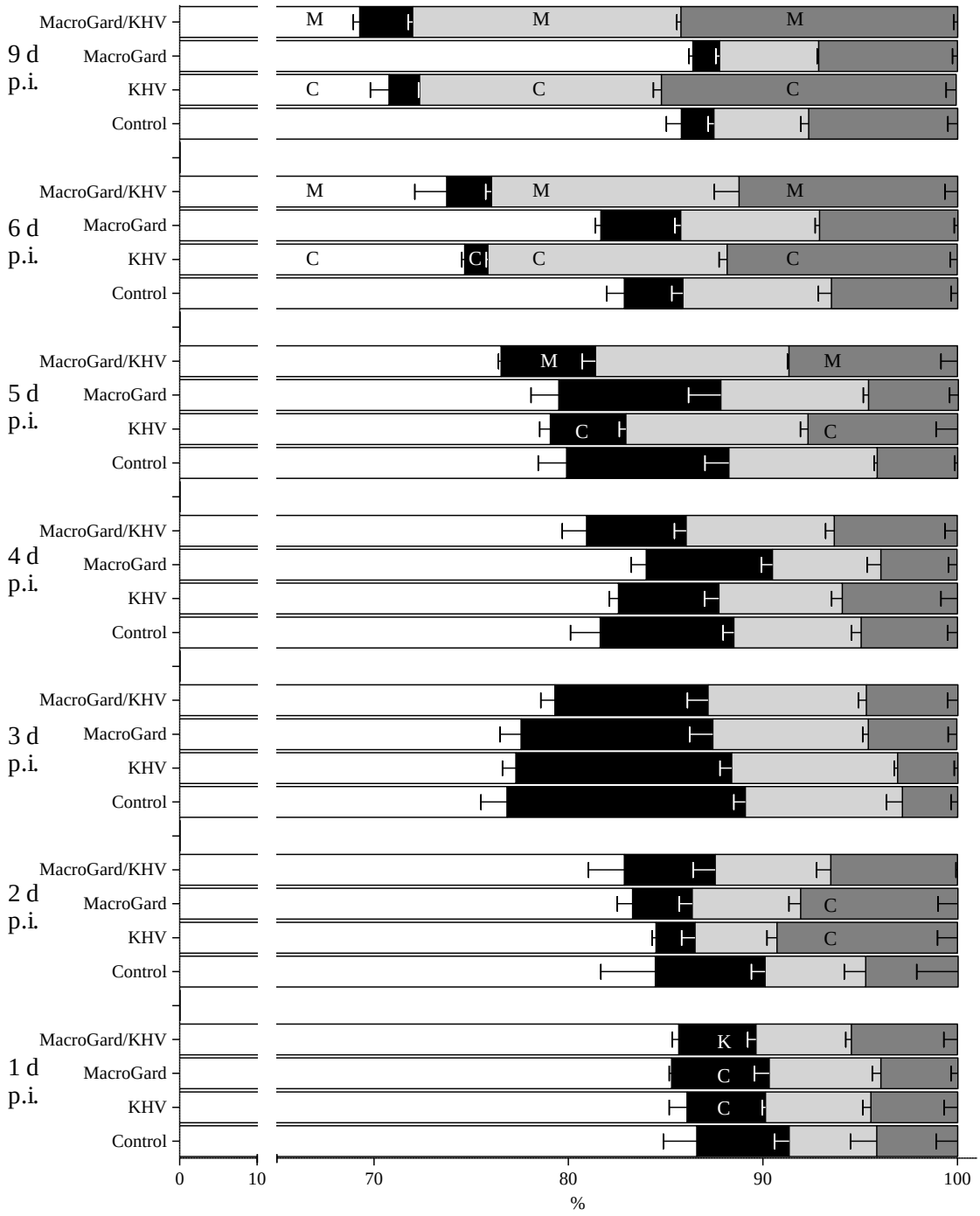

Figure 6.21: Cell morphology during KHV infection study

The proportion of cells with certain morphologies was ascertained by flow cytometry and these proportions are displayed in the above graphic. The graph represents mean ± SEM of n = 3. The bars represent the following: White: small cells with low granularity, black: small cells with high granularity, light grey: large cells with high granularity, dark grey: large cells with low granularity. Statistical significance ($p \leq 0.05$) is indicated by capital letters. C indicates significant difference to control, M = significant difference to MacroGard® group and K = significant difference to KHV infected group.

Propidium iodide staining

The percentage of propidium iodide positive cells (independent of the ability to be stained with CaspaTag) varied with the duration of the experiment ($F = 13.9$, $p < 0.001$), the sampling point ($F = 38.1$, $p < 0.0001$) and the interaction between treatment and time ($F = 4.1$, $p < 0.0001$) (Figure 6.22).

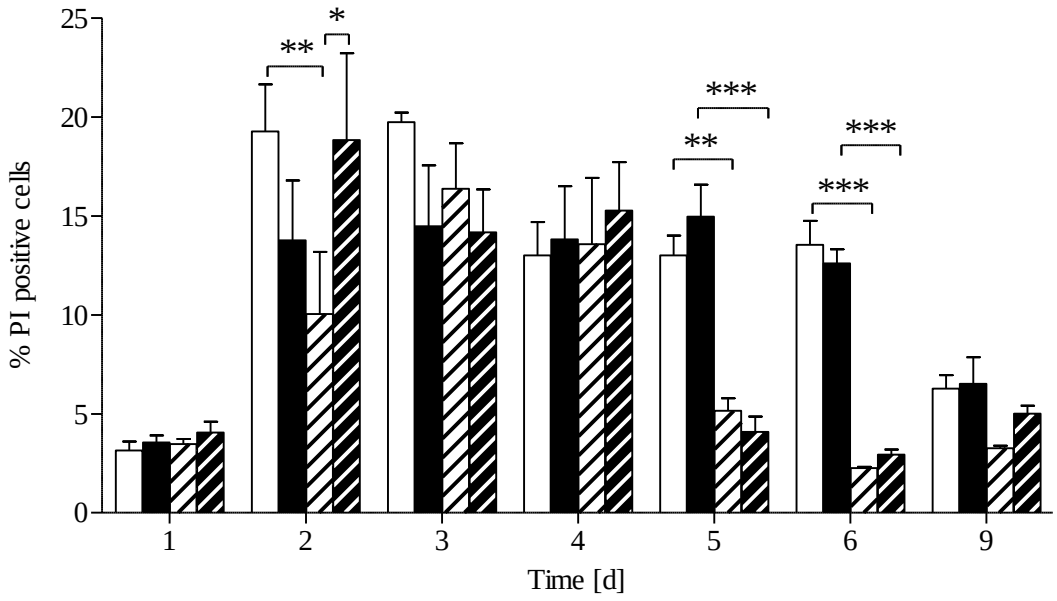

Figure 6.22: Percentage of PI positive cells during KHV infection

CCB cells were incubated with MacroGard® and KHV infected. For the analysis cells were stained with propidium iodide and percentage of PI positive cells was measured by Flow Cytometry. Bars: White: control, black: MacroGard® exposed, white with stripes: KHV infected, black with stripes: MacroGard® exposed and KHV infected. Graph shows mean ± SEM for n = 3. Statistical significance is defined as $p \leq 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

The lowest levels of PI positive cells in all treatment groups were recorded on day 1 (average of all treatments: 3.6 ± 0.2 %) and day 9 p.i. (5.3 ± 0.5 %), and also on day 5 (average of all KHV infected samples: 4.6 ± 0.5 %) and 6 (2.6 ± 0.2 %) in the infection treatments. In the latter days (i.e. day 5 and 6) the levels of PI positive cells were significantly higher in the control (13.0 ± 1.0 and 13.5 ± 1.2 % respectively) and the

MacroGard® treated group (15.0 ± 1.6 % and 12.6 ± 0.7 % respectively) in comparison to the respective infection treatments (all $p \leq 0.001$). The incubation of the CCB cells with MacroGard® did not cause changes in PI staining of non-infected cells and only induced significant difference between the infection treatments on day 2. On day 2 MacroGard® treated cells also showed higher PI positive levels that the non-treated but KHV infected cells.

CaspaTag staining

The levels of CaspaTag positive cells differ significantly with time ($F = 8.4$, $p < 0.0001$), treatments ($F = 13.5$, $p < 0.0001$) and the interaction of these two factors ($F = 4.2$, $p < 0.0001$) (Figure 6.23).

From day 3 onwards very high levels of CaspaTag positive cells are detected in the control group (mean of day 3 – 9: 45.88 ± 5.2 %) with the highest levels on day 3 (61.6 ± 14 %). On day 3 these control levels were approximately 55 % higher than any other treatment ($p \leq 0.001$) and on the last sampling day control levels were circa 30 % higher than in the KHV infected group ($p \leq 0.01$). The KHV infected cells only displayed higher CaspaTag positive levels on day 5 p.i. and not on any other day. On this day 64.5 ± 3.3 % of the KHV infected CCB cells were CaspaTag positive while 36.4 ± 9.1 % of the control and 26.1 ± 1.7 % of the MacroGard® treated and KHV infected cells were positive for this staining.

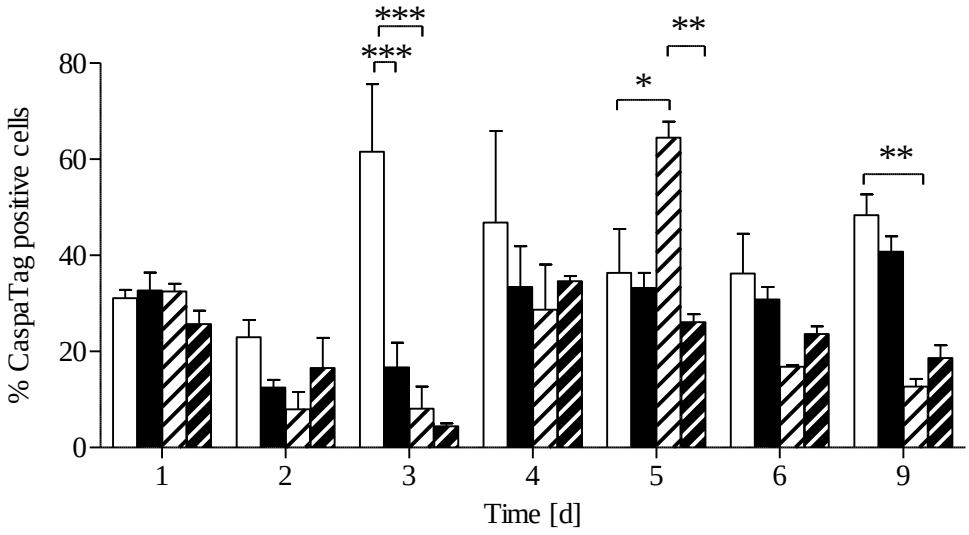

Figure 6.23: Percentage of CaspaTag positive cells during KHV infection

Cells of the CCB cell line were exposed to MacroGard® and were infected with KHV. To ascertain levels of apoptotic cells, samples were stained with CaspaTag and were analysed by Flow Cytometry. Bars: White: control, black: MacroGard® exposed, white with stripes: KHV infected, black with stripes: MacroGard® exposed and KHV infected. Graph shows mean ± SEM for n = 3. Statistical significance is defined as $p \leq 0.05$, *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$.

6.4.4 Gene expression during KHV infection

Gene expression in CLC cells after exposure to KHV

The virus presence was determined by analysing the expression levels of the KHV's thymidine kinase (TK). In the CLC cells (Figure 6.24) there was no significant difference between the infected and non-infected treatments ($F = 3.2$, $p = 0.09$). Due to this lack of virus in the cells solution it was concluded that CLC cells were not infected by KHV.

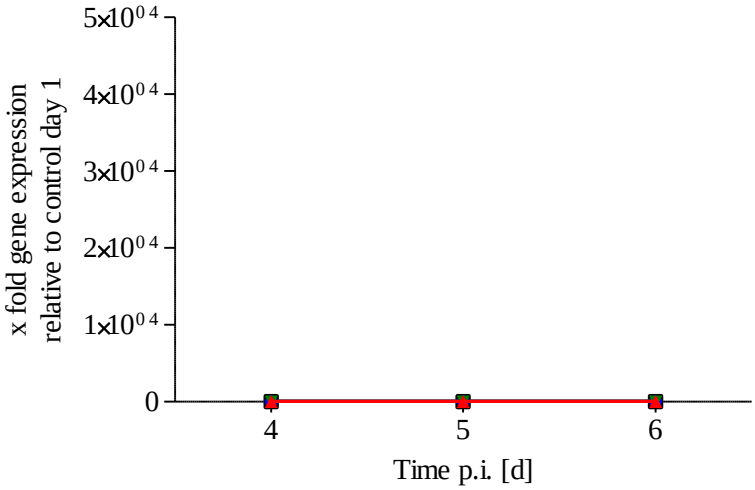

Figure 6.24: KHV gene expression in CLC cells

CLC cells were incubated with 50 µg/ml MacroGard® for 24 h and then infected with 1.5 x 10^2 $TCID_{50}$/ml KHV. Samples were taken on day 4, 5 and 6, and of each treatment three replicates were analysed per time point. The load of the KHV virus in the sample was assessed by measuring expression of virus innate TK (thymidine kinase) relative to time 0 h. □: control, ●: MacroGard®, ▼: KHV, ▲: KHV + MacroGard®. Expression of TK is expressed in relation to the control value on day 4. n = 3, graph shows mean ± SEM.

The gene expression of iNOS was extremely low in the samples analysed and was not detected by real time PCR until the very late cycles (cycles 34 – 40, all other genes: cycles 24 – 33). Hence it was decided to exclude iNOS gene expression from the analysis.

The analysis of the other genes (Table 6.2) showed that even though there is little virus replication, exposure to the infection did influence the pro-and anti-apoptotic genes (Caspase 9, Apaf-1, IAP) and p38 ($F = 3.3 – 20.4$, $p = 0.02 – 0.0001$). In addition, time period since virus exposure influenced the gene expression in all genes studied with the exception of Apaf-1 ($F = 4.5 – 28.3$, $p = 0.02 – 0.0001$), and a significant interaction between the time and treatment was only detected for two of the analysed genes i.e. Caspase 9 ($F = 2.8$, $p = 0.04$) and Bcl-2 ($F = 4.4$, $p = 0.004$).

Table 6.2: 2-way ANOVA analysis of x-fold gene expression in CLC cells exposed to KHV*

CLC	Time		Treatment		Interaction	
KHV	F	p	F	p	F	p
p53	4.5	0.02	0.9	0.4	1.2	0.3
Caspase 9	7.3	0.004	6.4	0.003	2.8	0.04
Apaf-1	0.6	0.6	3.3	0.04	0.7	0.7
Bcl-2	28.3	< 0.0001	2.0	0.1	4.4	0.004
IAP	7.9	0.0023	20.4	< 0.0001	1.6	0.2
p38	5.9	0.008	4.1	0.02	0.9	0.5

*grey background indicates significance

The exposure to KHV caused an up-regulation of the pro-apoptotic p53 and Caspase 9 genes on day 5 p.i. ($p \leq 0.05$ and 0.001 respectively), whilst 24 hour exposure to MacroGard® induced elevated levels of anti-apoptotic genes on day 5 (Bcl-2, IAP, both $p \leq 0.01$) and on day 6 (IAP, $p \leq 0.001$). In addition, a significant difference was detected for the IAP gene on day 4 p.i. between the two infection groups i.e. expression in the combined MacroGard®/KHV treatment was approximately double than with KHV infection alone. The anti-apoptotic Bcl-2 was down-regulated on day 4 by approximately 40 % ($p \leq 0.05$) (Figure 6.25).

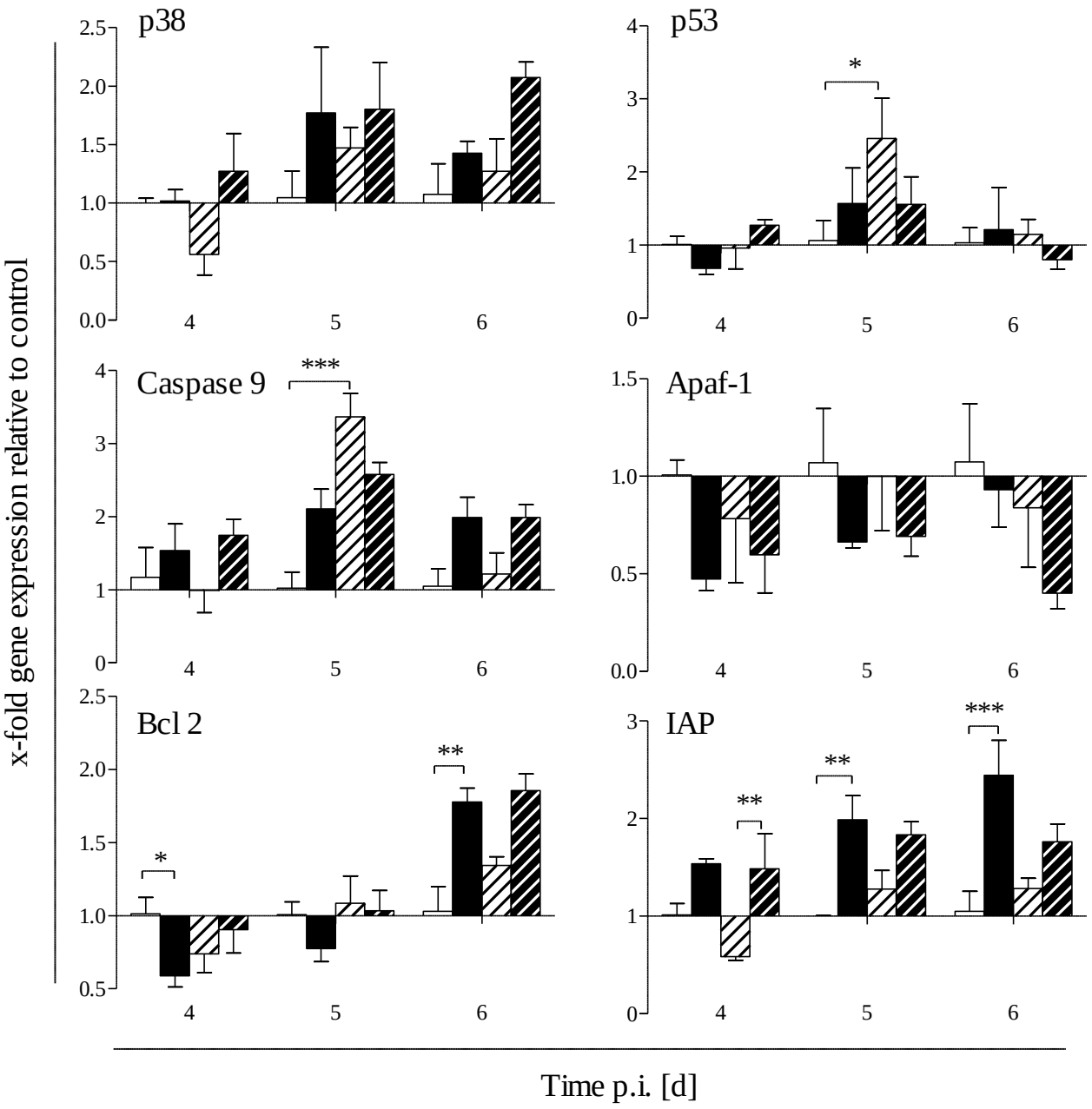

Figure 6.25: Gene expression in CLC cells after KHV exposure

CLC were incubated with 50 µg/ml MacroGard® for 24 h and then exposed to KHV. The x-fold gene expression was calculated as relative to the control of the respective time point. Bars: white = control, black = MacroGard®, white with stripes = KHV, black with stripes = MacroGard® & KHV. Graphs show mean ± SEM, n = 3, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

Gene expression in CCBs after KHV infection

The analysis of the KHV innate gene thymidine kinase in CCB cells confirmed that the infection led to differences in virus load between the treatments ($F = 10.18$, $p = 0.0002$). The non-infected samples were negative for the presence of the virus, i.e. control group 1.9 ± 0.07-fold and MacroGard® group 1.7 ± 0.9-fold. In contrast, the expression pattern of thymidine kinase revealed that the virus load increased steadily in both infection groups from day 3 – 4 onwards reaching a maximum (KHV: 2888 ± 1306-fold and MacroGard®/KHV: 2482 ± 921-fold expression compared to control, $p \leq 0.01$) on the last sampling day (Figure 6.26). No significant difference was found between the two infection treatments.

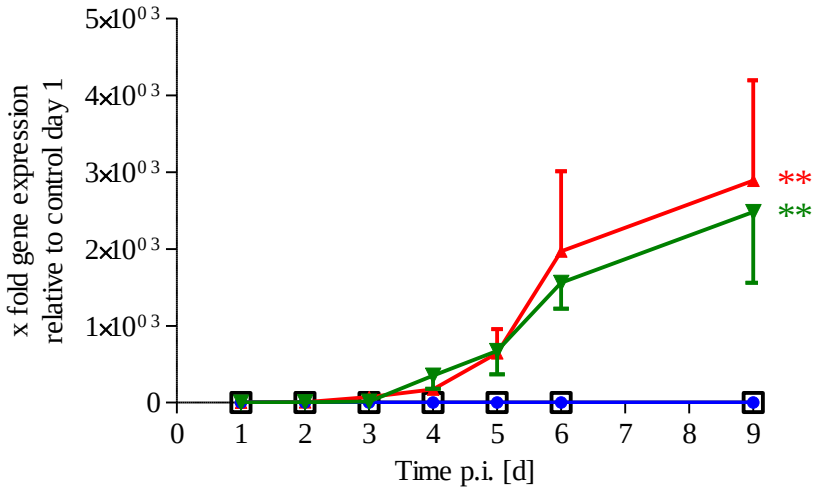

Figure 6.26: KHV expression in CCB cells

CCB cells were exposed to 50 µg/ml MacroGard® and infected with KHV (1.5 x 10^2 $TCID_{50}$/ml) and the x-fold gene expression of thymidine kinase (TK) was analysed relative to time 0 h. Graph shows n = 3 and mean ± SEM. □: control, ●: MacroGard®, ▼: KHV, ▲: KHV + MacroGard®. ** = significantly different to respective control with $p \leq 0.01$.

In all treatments there was a significant effect on gene expression ($F = 2.8 – 10.7$, $p \leq 0.05 – 0.0001$) with the exception of the pro-apoptotic genes Caspase 9 and Apaf-1 (Table 6.3). Apaf-1 was also the only gene studied that did not change over the time period of the experiment, while all other genes were influenced by this factor, which mainly resulted in increased gene expression levels on day 2 p.i. ($F = 4.7 – 8.7$, $p \leq 0.0009$). A significant ($F = 1.9 – 3.0$, $p \leq 0.04$) interaction between the treatment and time was also detected for all genes except Apaf-1.

Table 6.3: 2-way ANOVA analysis of x-fold gene expression during KHV infection in CCB cells*

	Time		Treatment		Interaction	
	F	p	F	p	F	p
iNOS	8.7	< 0.0001	6.5	0.0009	2.8	0.003
p53	5.8	0.0001	10.7	< 0.0001	2.2	0.02
Caspase 9	4.9	0.0006	2.7	0.06	2.1	0.02
Apaf-1	2.0	0.08	1.5	0.2	1.4	0.2
Bcl-2	4.7	0.0009	8.2	0.0002	2.5	0.007
IAP	8.1	< 0.0001	10.3	< 0.0001	3.0	0.002
p38	5.1	0.0004	2.8	0.05	2.0	0.04
Nemo	4.9	0.0006	5.3	0.003	1.9	0.04

*grey boxes highlight significant effects

In comparison with the SVCV infection (see section 6.3.4) KHV infection had little effect on the expression pattern of the genes studied (Figure 6.27). The only significant effects of the KHV infection were detected on day 6 p.i. in the p53 gene (5.2 ± 1.2-fold increase) and day 9 p.i. in the Nemo gene (8.3 ± 0.2-fold increase) (both $p \leq 0.05$). The influence of the MacroGard® exposure is similar to the study involving CCB and SVCV (see section 6.3.4) since none of the analysed genes was affected by this treatment. Thus most effects were seen in the combined KHV/MacroGard® treatment, where a general

increase in gene expression levels was detected on day 2 ($p \leq 0.05 - 0.001$); however this effect was regarded as anomalous due to the similar influence of the treatment on all the genes. Levels of the pro-apoptotic gene p53 also significantly increased due to the KHV infection in the MacroGard® exposed cells on day 5 and 6 p.i. ($p \leq 0.05$ and 0.01 respectively). Expression of the anti-apoptotic gene IAP was elevated in the combined treatment, i.e. KHV/MacroGard®, on day 5 compared to the MacroGard® control ($p \leq 0.05$) and on day 4 when compared to the KHV infection alone ($p \leq 0.01$). The cell signalling factor Nemo was the only other gene that was significantly induced by the MacroGard® treatment in the infected cells. This led to up-regulation of the gene (5.9 ± 0.7-fold) on day 6 in comparison to the MacroGard® control ($p \leq 0.05$).

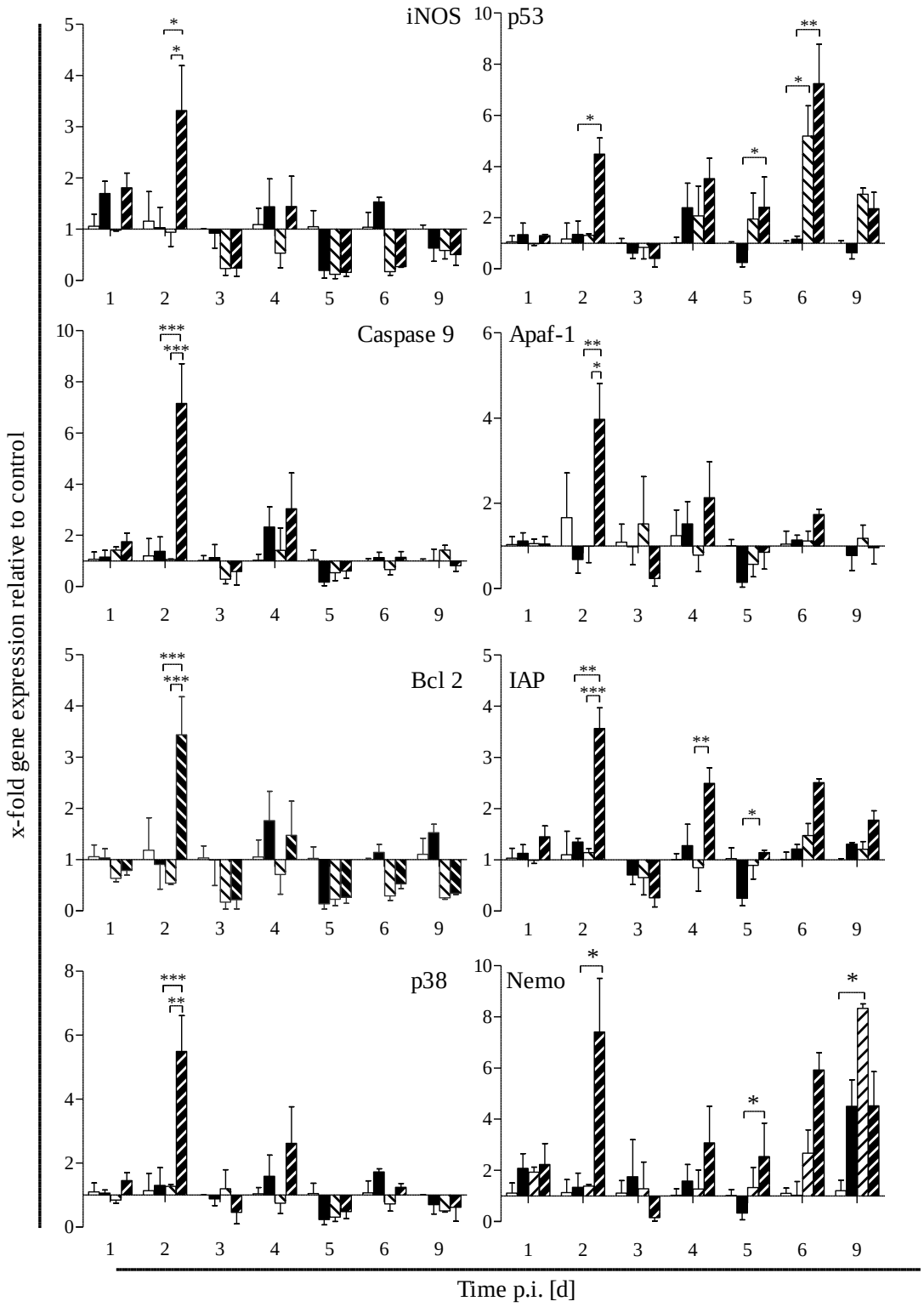

Figure 6.27: Gene expression in CCB cells during KHV infection

CCB cells were incubated with 50 µg/ml MacroGard® for 24 h and then infected with KHV. X-fold gene expression relative to control was analysed. Bars: white = control, black = MacroGard®, white with stripes = KHV, black with stripes = MacroGard® & KHV. Graphs show mean ± SEM, n = 3, * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

6.5 Discussion

In the previous sections the analysis of the apoptosis inducing properties of SVCV, KHV alone and in combination with MacroGard® was described. The results indicate differential effects of the two viruses. Whilst SVCV clearly induced apoptosis no such effect was detected for KHV. Gene expression studied confirmed that apoptosis-related genes were induced due to treatment with SVCV but only little effect on the gene expression was detected during the KHV infection. MacroGard® alone did not induce apoptosis and did not affect apoptosis levels and gene expression in KHV infected cells. In the SVCV infected groups MacroGard® also did not influence apoptosis levels but appeared to heighten the virus induced expression of some genes e.g. p53. In addition differential properties of KHV to infect the CLC and CCB cell line were observed.

In this chapter apoptosis levels ascertained microscopically with acridine orange staining and by flow cytometry with CaspaTag staining were compared. These staining methods detected different levels of apoptosis, which were generally higher in the CaspaTag staining and in addition the pattern of induced apoptosis in response to the various applied treatments differed. These differences might not only be due to the different stages of apoptosis detected (acridine orange: late stage, CaspaTag: early stage) but might also arise from the sample preparation procedure. Whilst acridine orange samples underwent only one centrifugation step and were analysed within 30 minutes of the sampling, CaspaTag samples underwent various washing steps and a 1 hour incubation period. This difference in sample treatment and time between sampling and analysis could have resulted in changes in the cell physiology and morphology and led to higher levels of apoptosis in the CaspaTag treated samples. Due to these factors it was chosen to omit the

results from the CaspaTag staining and to use the results from the acridine orange staining for the analysis of apoptosis in MacroGard® treated and virus infected cells. This would allow direct comparison with other studies carried in this thesis.

6.5.1 Effect of MacroGard® on cell lines

In the CLC cell line the incubation with MacroGard® caused significant changes in the anti-apoptotic genes Bcl-2 and IAP. Generally the effect of MacroGard® resulted in enhanced mRNA levels of these genes. This anti-apoptotic effect was not observed in the CCB cells, which may imply a cell-type dependent MacroGard® effect. However in the observed morphology of the monolayer it was apparent that a higher density was observed in the MacroGard® treated samples. This effect could be due to the partial solubility of MacroGard® but this does not seem likely due to the two wash steps, which the cells experienced during the treatment. More probable is that MacroGard® led to increased proliferation in the cells and this caused the denser cell layer. Such an effect was observed in human L-929 fibroblast cultures, where β-glucan caused proliferation (Son *et al.,* 2005). In addition MacroGard® leads to enhanced wound healing in common carp (Dominika Przybylska, personal communication) and growth factor production in human fibroblasts (Wei *et al.,* 2002). These facts taken together indicate a proliferating effect of MacroGard® in fish fibroblasts. Further research carried out at Keele University will aim to elucidate the mechanisms of this effect.

6.5.2 Differential susceptibility of cell lines to KHV

In section 6.4.1 it was shown that KHV had a differential effect on the two cell lines tested. Whilst KHV caused CPE in CCB cells, no such effect was observed in the CLC cell

line. In addition, antibody staining against KHV and analysis of the relative virus load confirmed that the virus infected CCB cells but not the CLC cells. Similar differences in infectability of CCB and CLC by KHV had been observed previously (Dieter Steinhagen, personal communication). A study conducted by Davidovich *et al.*, (2007) demonstrated that KHV replicates in CCB, KFC (koi fin cells), Tol/FL (silver carp) and goldfish fin (Au) cells but not in EPC (Epithelioma Papulosum Cyprini), FHM (fathead minnow fin cells) and CCO (channel catfish ovary) cells. The authors explain this effect with the specificity of KHV for a very narrow host range and hence its inability to infect fish species outside this host range. However this explanation is problematic in the case of CCB and CLC, since they are both derived from common carp. However, it is possible that the virus displays specificity for the cell type. CCB cells are thought to be fibroblasts (Neukirch *et al.*, 1999), while CLCs have macrophage-like properties (Faisal and Ahne 1990; Weyts *et al.*, 1997a). None of the cell lines tested by Davidovich *et al.* (2007) were leucocytes and in my study it is therefore not possible to draw a conclusion on the ability of KHV to infect immune cells. However *in vivo* KHV has been shown to infect both immune and non-immune organs (Gilad *et al.*, 2004) and hence a cell type specificty of KHV can probably be excluded. Interestingly CLC cells displayed a response to the virus at the gene expression level. However since there was also a trend to enhanced gene levels in the other two treatment groups (i.e. MacroGard® incubation and MacroGard® with KHV incubation), it is possible that this pro-apoptotic effect is due to the handling of the cells rather than the treatments. Visual observation of the cell monolayer showed the formation of cell plagues in the monolayer, which were a result of disturbed cell layers during the treatments. Generally CLCs detached very easily from the 24-well plates, a problem which is also mentioned in the description of the cell line in the HPA culture collections

catalogue (http://hpacultures.org.uk). Thus the detachment of the cells might be due to a short period of subculturing before the start of the experiment. It is also possible that the cells would need an additional matrix such as fibronectin for the attachment which would form the basis of future studies (Ruoslahti *et al.,* 1985).

6.5.3 Viral infection in CCB cells

A successful viral infection in CCB cells was confirmed for SVCV and KHV by positive antibody staining and detection of viral genes in the samples. Throughout the study the KHV load in the CCB cells increased (Figure 6.28), an observation which supports the pattern of infection observed in previous studies (Davidovich *et al.,* 2007). In the case of SVCV infected cells however the virus load reached a maximum at day 2 p.i. and decreased thereafter until it reached values similar to day 1 p.i. Since apoptosis is a host response to eliminate infected cells (Hoole and Williams 2004), the decline in virus load could be associated with the increase in apoptosis levels during the SVCV infection (Figure 6.28). The increased virus load in the KHV samples was thus related to the absence of apoptosis in the cell population.

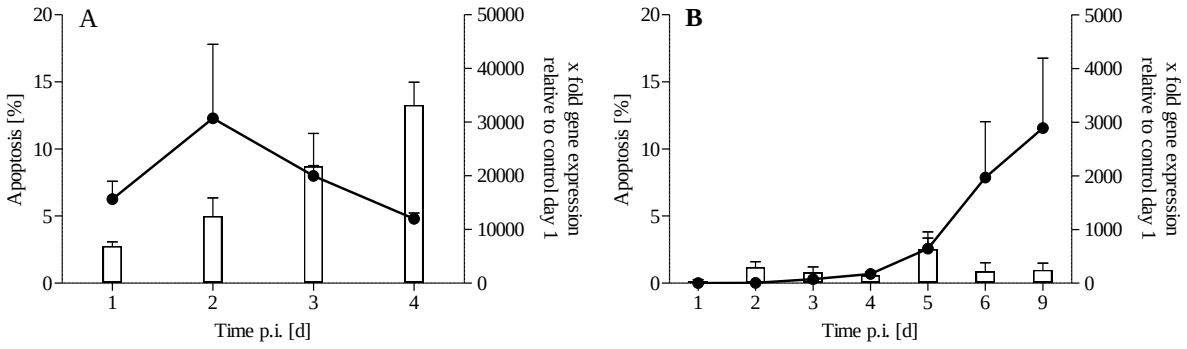

Figure 6.28: Virus load in relation to apoptosis levels

The virus load in the infected but non-MacroGard® exposed samples was plotted against the corresponding level of apoptosis. A) SVCV, B) KHV. The Graph shows mean ± SEM for n = 3. Bars represent apoptosis levels (left x-axis), ● represents virus load (right x-axis).

Viral infection with SVCV led to elevated levels of apoptosis, which was confirmed by acridine orange. This finding is in line with previous reports that indicate that members of the rhabdovirus family (e.g. SVCV, viral haemorrhagic septicaemia (VHSV) and infectious pancreatic necrosis virus (IPNV)) induce apoptosis *in vivo* (Björklund *et al.*, 1997; Eléouët *et al.*, 2001). Flow cytometry detected slight differences in the cell composition between the infected and non-infected groups. This possibly reflects a changing cell composition due to the immune response. The higher values of small cells in the SVCV infected cells most probably reflect the increased amount of apoptotic cells and apoptotic bodies.

Nitric oxide is an anti-viral agent (Croen 1993; Saura *et al.*, 1999) and hence it is not surprising that SVCV infection led to increased levels of the inducible NO synthase (iNOS). Rhabdoviruses in particular are known to induce iNOS and NO, and nitric oxide has been linked to host apoptosis and limitation of RNA-virus replication (Lin *et al.*, 1997; Ubol *et al.*, 2001).

The increased levels of apoptosis during the infection are also reflected in elevated mRNA levels of p53 and a trend to elevated levels of Caspase 9. The pro-apoptotic effects seem to compensate the inhibitory effects of apoptosis inhibitor IAP as apoptosis levels rose throughout the experiment. The execution of apoptosis is also aided by the down-regulation of cytoprotective heme oxygenase-1, which was observed in EPC cell cultures and *in vivo* in carp. This gene is involved in the protection of cells against oxidative damage and thus its down-regulation leaves the cell more vulnerable to damage caused by nitric oxide (Yuan *et al.*, 2012).

In contrast to SVCV infection exposure of CCB cells to KHV did not induce apoptosis but instead caused the formation of giant cells and multinucleated giant cells (MGC). These cells were visualized in the microscopic analysis and were detected in the flow cytometric analysis as large cells with low or high granularity. This effect on cell morphology is a common characteristic of fish and other herpesviruses. Many of the known *Alloherpesviridae* have been reported to induce syncytia, i.e. multinucleated cells, *in vitro*. For example Neukirch *et al.,* (1999) reported formation of syncytia in CCB and CCG (*Cyprinus carpio* Gill) cell lines upon KHV infection. Also IcHV-1 (Ictalurid HV 1), SalHV-1 (Salmonid HV 1) and AngHV-1 (Anguillid HV 1) induce syncytia formation *in vitro* (Wolf and Darlington 1971; Wolf *et al.*, 1978; Ueno *et al.,* 1996). This morphological effect is not limited to fish as syncytia formation due to herpesvirus infection has been noted in AHV-1 virus infection in ducks (Guo *et al.,* 2009), herpes simplex virus infections (Muggeridge 2000) and the human Epstein-Barr virus (EBV) in human cell lines (Miller *et al.*, 1972). These multinucleated cells are thought to be derived from cell fusion induced by viral proteins (Muggeridge 2000). Another possible reason for GMC formation is an inability of the cell to complete mitosis and a deficiency in the p53 mediated control over the cell cycle (Almog and Rotter 1997). The formation of giant cells in a viral infection may provide the viron with a sacred haven to replicate unrecognized by the immune system.

While CPE in SVCV infected cells was clearly induced by apoptosis this appears not to be the case in KHV infected cells. Microscopical analysis revealed that multinucleated giant cells (MGCs) eventually disrupted which may suggest that cell death occurred via necrosis. Necrosis is a hallmark of KHV infection, which is why it had been previously termed “carp interstitial nephritis and gill necrosis virus” (Michel *et al.,* 2010). Thus it can

be assumed that the modulation of apoptosis and alteration of cell morphology contribute to the pathogenicity of the disease.

The finding that KHV infection did not induce iNOS gene levels is clearly in contrast to the observations made during the SVCV infection. This lack of iNOS induction is possibly due to an active influence of the KHV virus on the host cells and/or non-recognition of the virus by the cells. The KHV infection did not involve signalling via the p38 MAPKK pathway but the NF-κB pathway was activated at the last sampling day. Unfortunately due to the diverse involvement of the NF-κB pathway it is impossible to speculate about the cause, function and outcome of its activation at this stage.

In contrast to SVCV infection KHV only induced a limited effect on apoptosis-related genes i.e. an up-regulation of p53 gene on day 6 p.i. and a trend to reduce expression of Caspase 9. The induction of apoptosis observed in the SVCV infection is assumed as the normal antiviral response of the cell. Therefore the non-apoptotic state during the KHV infection might be considered as an interference of the virus on the host cell physiological process. The interference of *Herpesviridae* with host apoptosis is a well-established phenomenon. For example Epstein-Barr virus (EBV) expresses genes, i.e. LMP1 and BHRF1, which inhibit host apoptosis. Although in the present study it is not possible to elucidate the mechanisms of the viral interference with the apoptosis pathway, it is possible to speculate that this is not achieved by an induction of host anti-apoptotic genes, as the expression of the anti-apoptotic genes, Bcl-2 and IAP, remained unchanged. Within the next chapter an attempt is made to identify a possible anti-apoptotic gene within

the KHV genome and in that context the interference of herpesviruses with the host response will be discussed further.

The differential effects of KHV and SVCV on the two fish cell lines used may be due to the properties of the individual virus. RNA viruses such as SVCV have a small genome with less complexity as the much bigger DNA viruses such as KHV. The SVCV genome consists of 5 genes and thus lacks genes that can actively interfere with the host response (Ahne *et al.,* 2002). Reports on apoptosis induced by vesicular stomatitis virus (VSV), another rhabdovirus suggest that apoptosis is induced via two independent pathways. One pathway is via host-induced apoptosis during the immune response whilst the second pathway is associated with the expression of viral M-protein (Kopecky *et al.*, 2001; Gaddy and Lyles 2005). This protein is involved in the shut-off of cellular functions and hence this can result in apoptosis (Ahmed and Lyles 1998). The more complex KHV genome (295 kbp) encodes a much larger number of proteins and throughout its evolutionary association with its hosts has probably acquired a number of host genes which it uses to manipulate the immune response. For example a sequence similar to carp IL-10 and TNF receptor is expressed by KHV (van Beurden *et al.,* 2011) and it is therefore possible that KHV has also acquired host genes that can interfere with the apoptosis pathway.

In general viruses can adopt a range of strategies to escape host apoptosis, either by inhibiting it or by avoiding it through completing replication before the onset of apoptosis (Koyama *et al.*, 2000). The small RNA virus SVCV seems to pursue the latter strategy since high virus loads are already detected 24 hours after the infection whilst the much

larger DNA virus, KHV inhibits apoptosis and increased virus loads are not detected until 3 days after infection.

6.5.4 Effect of MacroGard® on viral infection

MacroGard® had a differential effect on the induced gene expression of apoptosis-related genes. In the SVCV infection the previous exposure to 50 μg/ml MacroGard® seemed to facilitate apoptosis, which resulted in significantly higher levels of gene expressions in the comparison of the two infection treatments. In addition the MacroGard® exposure also led to slightly higher numbers of apoptotic cells in the infected cells when compared to the non-exposed SVCV infected cells; however this was not statistically significant. Since apoptosis is a means of the host to clear infected cells, it can be speculated that these heightened gene levels induced by MacroGard® contribute to the improved resistance to viral diseases, which was observed by other authors (LaPatra *et al.*, 1998; Kim *et al.*, 2009b). The affect of exposure to MacroGard on the gene expression of apoptosis-related genes during KHV infection was negligible.

In summary, the results presented in this chapter demonstrate the differential effects of two pathogenic viruses i.e. RNA virus SVCV and DNA virus KHV on the apoptotic process in two carp cell lines. These effects may reflect their evolutionary background, which will be discussed further in the next chapter.

In addition, whilst MacroGard® enhanced apoptosis induced in the SVCV infection it did not affect the low levels of apoptosis associated with the KHV infection. This leads me to conclude that the effect of immunostimulation during viral infection is dependent on the mechanisms of pathogenicity of the virus. Hence the effect of β-glucan and other

immunostimulating substances on viral diseases cannot be generalized, i.e. differences between RNA and DNA viruses as presented in this chapter, and the viral effect on the immune response has to be considered.

7 Apoptosis in relation to viral infection *in vivo*

An important finding in the preceding chapter (chapter 6) was the differential influence of viruses on the apoptotic process *in vitro* in which the herpes virus KHV was shown to have possible anti-apoptotic properties in the cell model utilised. In addition an enhancing effect on the virus-induced gene expression due to pre-treatment with MacroGard® was observed in the cell line. The present chapter aims to establish if these observed effects of KHV occur *in vivo*. For this purpose a study to investigate the effect of a koi herpes virus infection on control and MacroGard® fed fish was carried out at the National Veterinary Research Institute in Pulawy, Poland. However, as described in this chapter, unfortunately KHV exposure failed to infect the fish. Fortunately however, Mikolaj Adamek, from the Veterinary University Hanover, Germany and a collaborator within the EU Marie Curie ITN NEMO kindly provided samples from a successful KHV infection carried out previously at Pulawy. This experiment forms the second part of this chapter. The results will be discussed not only in respect to the viral influence on apoptosis in fish but also in respect to possible mechanisms involved and the relationship to herpes virus infection in general.

7.1 Introduction

Koi herpes virus (KHV), which causes high mortalities in carp aquaculture (see chapter 6), belongs to the *Herpesvirales*, which is a large virus order whose members are believed to originate from a common ancestor. This order is subdivided into three families: the *Herpesviridae* which contains three subfamilies alpha, beta- and gammaherpesvirus, the *Malacoherpesviridae* and the *Alloherpesviridae* family. The *Herpesviridae* family are viruses that mainly infect mammals, birds and reptiles, whilst the family of *Malacoherpesviridae* usually infects molluscs. The *Alloherpesviridae* family, to which KHV belongs, primarily infects fish and amphibians (Davison *et al.,* 2009), and is divided into two clades. The first clade comprises the cyprinid herpes viruses CyHV-1, CyHV-2, CyHV-3 (i.e. KHV) and the eel herpes virus (AngHV-1) whilst the second clade contains the herpes viruses of channel catfish (IcHV1), white sturgeon (AciHV1 and 2), rainbow trout (SalHV1), salmon (SalHV2), Atlantic cod (GaHV1) and frogs (RaHV1 and 2) (Hanson *et al.*, 2011). Within the clades the virus species are closely related (Figure 7.1) but are more distantly related to viruses of the other clade (Waltzek *et al.,* 2009).

Hence the human herpesviruses and the fish herpesviruses, including KHV, belong to distinct families (*Herpesviridae* and *Alloherpesviridae*) and various studies have shown that they are distantly related (Hanson *et al.,* 2011). However, even though this relationship is only evident in a conserved gene sequence of the ATPase subunit of the terminase, a protein involved in the virion assembly, they have a highly conserved structure (e.g. double stranded linear DNA, icosahedral capsid) (Davison *et al.,* 2009).

The three subdivisions in the *Herpesviridae* (i.e. alpha-, beta-, gammaherpesviruses) diverged over 400 million years ago. McGeoch *et al.,* (2006)

suggested that the divergence of the three herpes families occurred even longer ago, possibly 570 – 505 million years. This indicates that herpesviruses have co-evolved with their host for millions of years and this long adaptation period has led to most members of this virus family inducing only modest pathogenicity within their host. In contrast, the high pathogenicity observed in KHV infections is probably due to an imbalance in the viral/host association caused by human activity, such as the culture of fish at high densities (Davison 2002) or due the virus infecting a new host species that has not adapted to the pathogen (Longdon *et al.*, 2011).

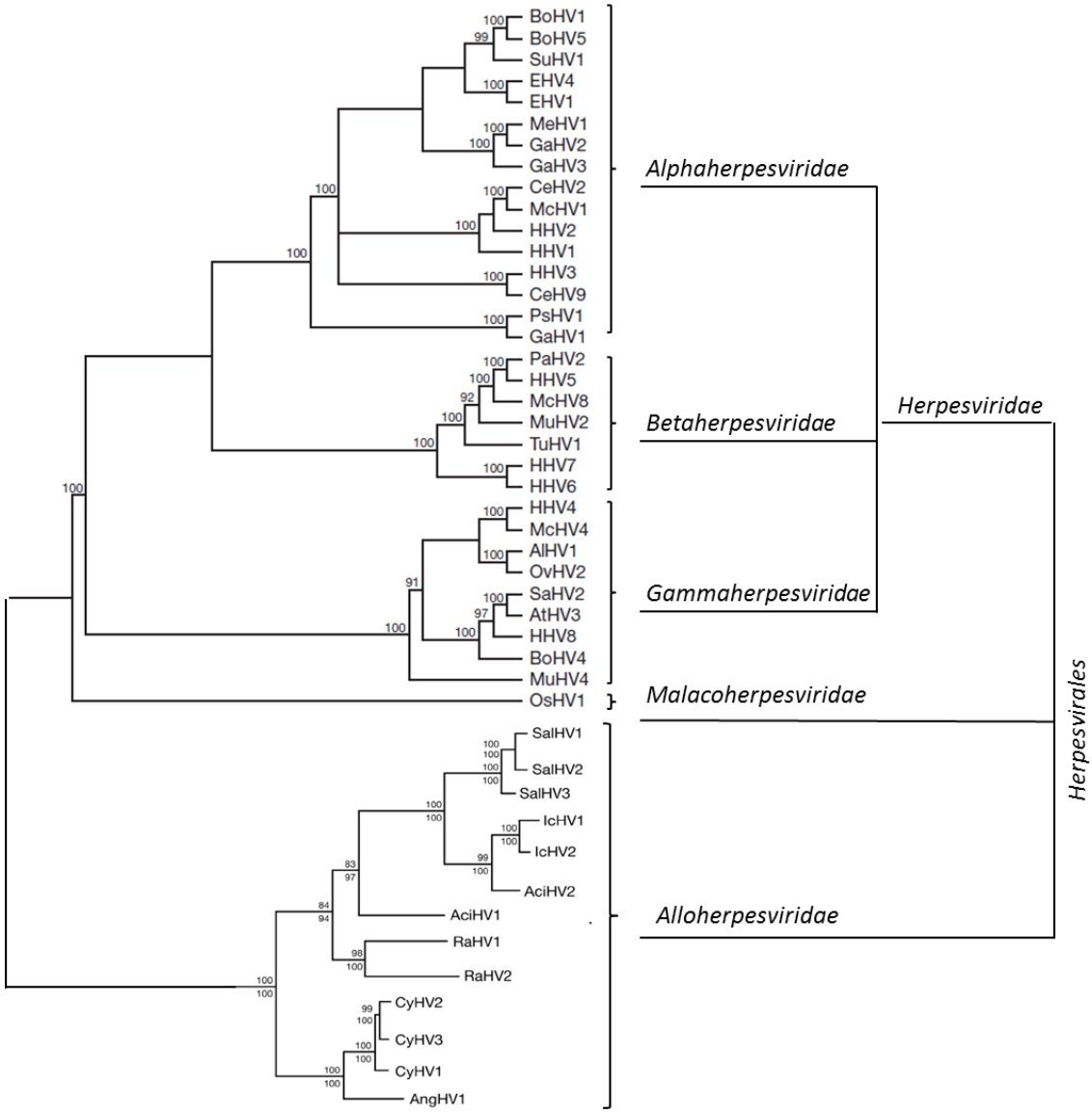

Figure 7.1: Phylogenetic relationship of *Herpesvirales*

The cladogram displays the relationships among members of the order *Herpesvirales* adapted from Waltzek *et al.*, (2009).

Many herpes viruses are known to interfere with the host's immune response and apoptosis pathway by expressing host-like genes. For example Epstein-Barr virus (EBV, HHV4), human cytomegalovirus (CMV, HHV5), Anguillid herpesvirus 1 (AngHV-1) and KHV (CyHV3) express a virus form of IL-10 (Hsu *et al.,* 1990; Kotenko *et al.,* 2000; van Beurden *et al.,* 2011), which has broad immunosuppressive properties (Redpath *et al.,* 2001). These host-like genes were most probably acquired independently several times by horizontal transfer (e.g. gene captured from host DNA) during the co-evolution with the host (van Beurden *et al.,* 2010; 2011).

In a similar manner some viruses are able to influence the host's apoptotic response to a viral infection. As mentioned in the introduction to this thesis (chapter 1) apoptosis is induced by the host as an anti-viral response but several viruses have acquired means to counteract this apoptotic response. Viruses can avoid this immune response by either inducing apoptosis themselves in immune cells or they can inhibit apoptosis to secure replication within the host cell. The latter is an anti-host mechanism that has been adapted by many DNA viruses. DNA viruses, such as herpesviruses, often have a large genome and hence have the capacity to express genes that can modify their environment (Hill and Masucci 1998; Roulston *et al.,* 1999). For instance, all members of the human gammaherpesviruses subfamily express a viral Bcl-2 homologue to counteract host-induced apoptosis in virus infected cells (Hardwick 1998; Ku *et al.,* 2008). Bcl-2 prevents apoptosis by inhibiting pro-apoptotic Bcl-2 family members, for example in zebrafish Bcl-2 has been shown to inhibit Bak (Eimon and Ashkenazi, 2010). As a result cytochrome c, which is a major inducer of apoptosis, is not released from the mitochondria and hence apoptosis does not take place. The Bcl-2 family is characterized by conserved domains named Bcl-2 homology (BH) domains, of which anti-apoptotic family members possess

four (BH1, BH2, BH3, BH4) and pro-apoptotic have three (BH1-3) or only BH3. However in carp only BH1 and BH2 have been identified for the Bcl-2 gene sequence, which suggests that BH3 is not essential for the functionality of this gene (Cheng *et al.*, 1997a; Cols Vidal 2006; Cols Vidal *et al.*, 2008).

KHV is related to human herpesviruses (Figure 7.1) and similarly to the human herpesviruses it has the potential to express genes to modulate the host response (Aoki *et al.,* 2007; van Beurden *et al.,* 2011) it was thus hypothesized that KHV can interfere with the carp's apoptosis process as well. This chapter thus aimed to elucidate if KHV influences the host's apoptotic process in a similar manner to other members of the *Herpesviridae* family. Due to the widespread expression of a viral Bcl-2 homologue it was hypothesized that the evident influence of KHV on the apoptosis process of the host during an infection is due to the expression of viral Bcl-2. Hence an attempt was made to identify an open reading frame (ORF) within the KHV genome that has characteristics of carp Bcl-2. The influence on the host's apoptosis process can result in changes of the cellular morphology since the cell cannot induce cell death in response to dysfunctional cell division. A common characteristic among the *Alloherpesviridae,* which could be related to a dysfunctional apoptosis process, is the formation of syncytia, papillomas, and carcinomas (Elmore, 2007; Hanson *et al.,* 2011). Hence it is most likely that the active influence of the virus on the apoptosis-process, as it was observed in chapter 6 as well as the present chapter, is a common characteristic amongst *Alloherpesviruses*.

In addition studies have shown that the administration of β-glucan can enhance protection against viral infections in fish (LaPatra *et al.,* 1998; Kim *et al.,* 2009b) and

therefore the combined effect of MacroGard® and viral infection on the apoptosis process in carp was studied.

In summary the aim of this present chapter is to establish the *in vivo* effects of KHV on the apoptotic process in carp and how this is modulated by dietary immunostimulation. For this purpose the gene expression of pro-apoptotic (p53, Caspase 9, Apaf-1) and anti-apoptotic genes (Bcl-2, IAP) was analysed.

7.2 Material & Methods

7.2.1 MacroGard® feeding & KHV

This experiment was conducted in collaboration with Mikolaj Adamek (Veterinary University Hanover, Germany), Dr. Marek Matras (National Veterinary Research Institute (NVRI) Pulawy, Poland) and Nicolas Pionnier (Keele University, UK) at the Laboratory of Fish Diseases at the NVRI in Pulawy, Poland. For this study batches of 200 carp (*C. carpio*) were obtained from confidential sources within Poland and transported to the facilities of the NVRI and were tested for KHV by conventional PCR as described by Bercovier *et al.,* (2005). The first two batches of fish tested positive for KHV and thus could not be used for the study. The third batch of carp proved negative for KHV and fish were distributed to two 800 L glass tanks, which were connected to a flow-through system. The water temperature was maintained at 21 ± 1 °C with a submersible aquarium heater. During an acclimation period of 2 weeks fish were fed a 0 % MacroGard® diet at a dose of 1 % bodyweight per day (see section 2.4). At the onset of the experiment the carp had an average size of approximately 5 cm and prior to the infection the experimental group which comprised 60 fish were fed a diet containing 0.1 % MacroGard® (equalling 10 mg/kg bodyweight per day) for 14 days, whilst the control group (n = 60) received the same diet minus the MacroGard®. Both of these feeding regimes were carried out by feeding an amount of 1 % body weight per day. The feed was of the same batch as the feed used in the previous studies (chapter 4 & 5). Prior to the infection the fish were distributed into 4 groups and were marked by fin clipping of the pectoral fin.

Cells of the CCB line were used for the propagation of the virus (see section 6.2.2). The koi herpes virus utilized had been isolated in 2005 (passage 4) from infected common carp at the Laboratory of Fish Disease, NVRI in Pulawy, Poland. CCB cells were inoculated with the virus and incubated for 10 days and the infection was checked by observing the cytopathic effect (CPE). The virus-containing medium was harvested and used for the bath infection (see below). The $TCID_{50}$/ml for the KHV virus was calculated to be 3×10^4.

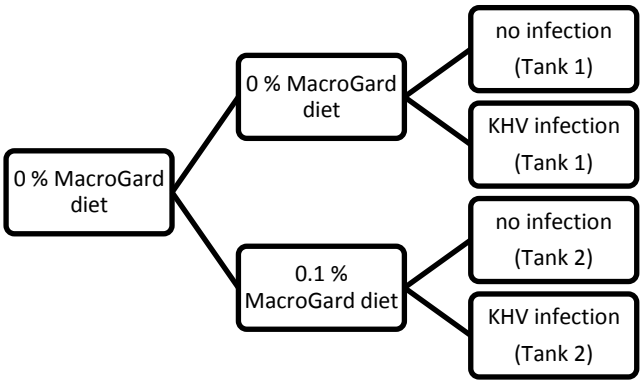

Figure 7.2: Experimental layout MacroGard® and KHV study *in vivo*
Common carp were fed with a MacroGard® free diet (0 %) during the acclimation phase, were divided into 2 feeding groups and were either fed on the 0 % diet or the same diet containing 0.1 % MacroGard® for 2 weeks. After this feeding period fish were bath infected with KHV. 5 fish per groups were sampled at the following time points: 0 h, 6 h, 1, 2, 4, 6 d p.i.

The infection was carried out by bath immersion. For this purpose 40 fish of each feeding group were bathed for 15 minutes in a small plastic container with 5 L of aquarium water and 100 ml of medium containing cells infected with KHV and equal to a virus concentration of 600 $TCID_{50}$/ml. Another 40 fish of each feeding group were mock infected which comprised carp which were bathed at 21 °C for 15 minutes with medium

from non-infected cells. The fish were then distributed into two tanks; one contained the non-infected fish while the second tank was used for the KHV infected fish. Feeding was continued for the rest of the experiment with the 0 % MacroGard® diet. A diagrammatic representation of the experimental design is given in Figure 7.2.

Fish were sampled at 0 hours (i.e. before infection treatment), 6 hours, 1, 2, 4, and 7 days post KHV infection (p.i.). Sampling was carried out by sacrificing the fish with a lethal dose of 0.2 % Prospicin and samples of pronephros, kidney, spleen, liver, mid-gut, tail-fin, skin and gill were excised and all samples were stored in RNAlater. Samples were kept at -20 °C before shipment on ice to Keele University. Kidney, skin and tail-fin were shipped to the Veterinary University Hanover, Germany for analysis of virus load by M. Adamek. This analysis was carried out by real-time PCR utilizing primers specific for thymidine kinase of KHV (Gilad *et al.*, 2004; Rakus *et al.*, 2012).

To establish if the KHV infection was capable of inducing apoptosis in fish cells *in vivo* a small amount of the mid-kidney was used to isolate cells and apoptosis was visualised using acridine orange staining as described in chapter 3.3.4. Mid-kidney was chosen over pronephros, as the size of the fish utilised meant that the pronephros was small and hence all pronephric tissue was required for gene expression analysis. Apoptosis was observed with a Zeiss Axionet 200 microscope with FITC filter.

The results for the acridine orange counting was analysed by 2-way ANOVA and Bonferoni post-hoc test after arcsin transformation. Significance was defined as $p \leq 0.05$ and data are displayed as mean ± SEM.

7.2.2 KHV infection without MacroGard® exposure

Due to the non-infectivity observed in the previous study samples of another KHV infection study were analysed in regard to the expression of genes related to apoptosis in common carp.

The samples for this study were kindly provided by Mikolaj Adamek (Veterinary University Hanover, Germany) and other studies associated with this experiment have been published by Rakus *et al.*, (2012).

Supply and Maintenance of Fish

Common carp of the Polish line K (Irnazarow 1995) were obtained from the Institute of Ichthyobiology & Aquaculture of the Polish Academy of Sciences in Golyz, Poland and were transported to the Laboratory of Fish Disease, National Veterinary Research Institute in Pulawy, Poland. Fish were kept in 800 L tanks at 21 ± 1 °C and prior to the infection animals were divided into 3 groups (control, infection and mortality group) and allowed to acclimate for 4 weeks to the system.

Virus propagation in CCB cells

The proliferation of the virus in CCB cells has been described in section 7.2.1. The harvested virus containing medium had a concentration 8 x 10^4 of $TCID_{50}$/ml and was used as described below.

Bath KHV infection

The infection was carried out by bathing the fish with the virus in 5 L plastic buckets with aquarium water with a virus concentration of 3.2×10^2 $TCID_{50}$/ml in the final volume of water. Fish were bathed for 1 hour at 22 °C and then returned to their respective tanks (see section 7.2.1 for details). Control fish were mock-infected by bathing in aquarium water with medium from uninfected CCB cells. The fish in the mortality group were observed daily for 32 days, the number of dead fish was recorded and these fish were removed from the tank. For gene expression analysis 5 fish of the control and infection group were sampled at 1, 3, 5, and 14 days post infection (p.i.).

Tissue samples of gill, pronephros and spleen were obtained from M. Adamek and were prepared for gene expression analysis at Keele University as described in chapter 2.1. The DNase step described in chapter 6.2.8 was also applied to these samples. The data analysis including statistics was carried out as described in chapter 2. Thus data were subjected to a 2-way ANOVA to test for differences between treatment groups. A Bonferroni Post-hoc test was carried out to test for differences between individual sample groups and significance was defined as $p \leq 0.05$.

7.3 Results

7.3.1 MacroGard® feeding & KHV

The levels of apoptosis measured in the mid-kidney of carp by acridine orange staining showed no statistically significant difference between the four treatment groups (Figure 7.3).

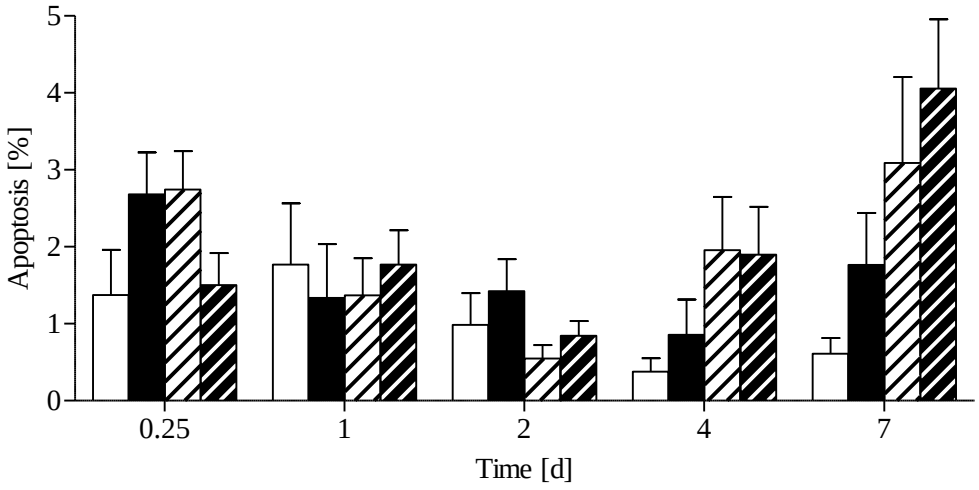

Figure 7.3: Percentage of apoptosis in mid-kidney of MacroGard® and KHV treated carp
Apoptosis was defined as cells with fragmented nucleus detected by acridine orange staining. Fish were fed with a control or a MacroGard® containing diet prior to KHV bath infection. White bars: control diet + control bath, black: MacroGard® diet + control bath, white with stripes: control diet + KHV bath, black with stripes: MacroGard® diet + KHV bath. Bars represent mean ± SEM with n = 5. No significant statistical differences were detected.

Kidney and fin clip samples were analysed by M. Adamek to establish the virus load (Table 7.1 and Table 7.2). A maximum of 15 virus copies were detected in fin clip samples and in kidney most samples were virus free with only one fish displaying virus copies of over 500/250 ng DNA. Due to these findings the analysis of the samples was aborted and instead samples were examined from a KHV infection study carried out previously by M. Adamek (section 7.3).

Table 7.1: Virus copy number in fin clip samples from day 7 p.i.

Sample ID	Ct	Quantity (copies/250 ng DNA)	Fish	Ct	Quantity (copies/250 ng DNA)
Standard curve			**Bath infected samples^{+}**		
10^8	10.51	10^8	1	32.89	14.49
10^7	14.16	10^7	2	36.01	1.57
10^6	17.03	10^6	3	32.94	13.97
10^5	20.72	10^5	4	35.45	2.33
10^4	24.13	10^4	5	32.82	15.23
10^3	26.78	10^3			
10^2	29.92	10^2			
Positive control*					
B1	17.66	7.35×10^5			
B2	18.47	4.14×10^5			
B3	21.88	3.65×10^4			
B4	35	3.22			
B5	19.61	1.84×10^5			

*Positive controls were from a parallel KHV infection carried out by injection, $^{+}$Fish were MacroGard® fed. Ct signifies the threshold cycle of the real time PCR at which the PCR product was detected.

Table 7.2: Virus copy number in kidney

Sampling day	Fish #	Ct	Quantity (copies/250 ng DNA)	Fish #	Ct	Quantity (copies/250 ng DNA)
	Standard curve					
	10^7	14.86	10^7			
	10^6	17.47	10^6			
	10^5	20.91	10^5			
	10^4	24.22	10^4			
	10^3	27.68	10^3			
	10^2	30.56	10^2			
	10^1	34.71	10^1			
	KHV samples			**KHV + MacroGard samples**		
6 hours	1	No CT	0	1	No CT	0
	2	No CT	0	2	No CT	0
	3	No CT	0	3	No CT	0
	4	No CT	0	4	No CT	0
	5	No CT	0	5	No CT	0
Day 1	6	No CT	0	6	No CT	0
	7	No CT	0	7	No CT	0
	8	No CT	0	8	No CT	0
	9	No CT	0	9	No CT	0
	10	No CT	0	10	No CT	0
Day 2	11	No CT	0	11	37	1.48
	12	No CT	0	12	No CT	0
	13	No CT	0	13	No CT	0
	14	No CT	0	14	35.59	3.94
	15	No CT	0	15	No CT	0
Day 4	16	37.4	1.43	16	No CT	0
	17	No CT	0	17	No CT	0
	18	No CT	0	18	No CT	0
	19	No CT	0	19	No CT	0
	20	No CT	0	20	28.51	549
Day 7	21	No CT	0	21	No CT	0
	22	No CT	0	22	No CT	0
	23	No CT	0	23	No CT	0
	24	No CT	0	24	No CT	0
	25	No CT	0	25	No CT	0

7.3.2 KHV infection without MacroGard® exposure

As published in Rakus *et al.,* (2012) mortality started to occur at 6 days p.i. and the last mortalities were observed 18 days p.i. with a cumulative mortality of 76 %. Three days after the infection significantly higher virus levels (i.e. 1000 x higher) were detected by conventional PCR in comparison to fish samples at day 0.

The analysed gene expression differed between treatments (i.e. control and KHV infection) and time post infection (Table 7.3).

Table 7.3: 2-way ANOVA analysis of x-fold gene expression during *in vivo* KHV infection*

	Time		**Treatment**		**Interaction**	
Pronephros	**F**	**p**	**F**	**p**	**F**	**p**
iNOS	2.0	0.1	17.4	0.0003	2.3	0.1
p53	2.3	0.1	1.4	0.2	2.6	0.07
Caspase 9	8.0	0.0004	0.08	0.8	8.5	0.0003
Apaf-1	25.6	< 0.0001	68.3	< 0.0001	26.1	< 0.0001
Bcl-2	1.3	0.3	21.2	< 0.001	2.7	0.2
IAP	11.8	< 0.0001	0.8	0.4	11.5	< 0.0001
Spleen						
iNOS	2.6	0.07	29.4	< 0.0001	4.2	0.01
p53	1.3	0.3	0.1	0.7	1.5	0.24
Caspase 9	6.1	0.002	2.0	0.2	4.5	0.01
Apaf-1	1.8	0.2	3.0	0.09	1.8	0.2
Bcl-2	6.2	0.002	85.9	< 0.0001	6.6	0.001
IAP	5.8	0.003	5.0	0.03	5.6	0.004
Gills						
iNOS	3.1	0.05	14.7	0.0008	2.7	0.07
p53	0.7	0.6	5.8	0.02	0.5	0.7
Caspase 9	1.6	0.2	1.2	0.3	1.7	0.2
Apaf-1	2.3	0.1	5.4	0.03	2.1	0.1
Bcl-2	0.9	0.5	5.9	0.02	0.9	0.5
IAP	2.7	0.06	0.6	0.5	2.7	0.07

*grey boxes indicate significant influence of the tested factor on gene expression

The pattern of the expressed genes seems to differ between organs. For example in spleen the two analysed anti-apoptotic genes (Bcl-2, IAP) and iNOS were differentially regulated due to the infection ($F = 5 – 85.9$, $p \leq 0.03$), whilst in the other analysed organs

both pro- and anti-apoptotic genes showed changes in regulation between control and infection treatment. In the pronephros and gills iNOS expression and expression of pro-apoptotic (pronephros and gill: Apaf-1, only gill: p53) and anti-apoptotic genes (Bcl-2) was significantly influenced by the viral infection ($F = 5.4 – 68.3$, $p \leq 0.03$). The time course of the study also influenced the gene expression in an organ dependent manner. Whilst in the gills only two genes (iNOS, IAP) were significantly modulated due to the time factor ($F = 3.1$ and 2.7 respectively, $p \leq 0.06$) three genes (Caspase 9, Apaf-1, IAP) were influenced by this parameter in pronephros ($F = 8.0 – 25.6$, $p \leq 0.0004$). In the spleen gene expression levels of Caspase 9 and the anti-apoptotic genes (Bcl-2, IAP) changed during the time course ($F = 5.8 – 6.2$, $p \leq 0.003$). No interaction between the treatment and time factor was observed in gill but an interaction effect was observed for many genes in the pronephros (Caspase 9, Apaf-1, IAP; $F = 8.5 – 26.1$, $p \leq 0.0003$) and in the spleen (iNOS, Caspase 9, Bcl-2, IAP; $F = 4.2 – 6.6$, $p \leq 0.01$).

The analysis of the individual effects (i.e. post-hoc analysis) revealed that of the three organs studied the gills were least influenced by the viral treatment (Figure 7.4). The only significant effects were enhanced gene expression levels of iNOS (10.7 ± 7.1-fold expression, $p \leq 0.01$) and Apaf-1 (2.7 ± 0.7-fold expression, $p \leq 0.05$) both occurring at the latest sampling point (i.e. 14 days p.i.).

The gene expression in spleen (Figure 7.5) on the other hand was influenced more strongly by the KHV infection. In this organ iNOS gene expression was enhanced during the intermediate time frame (3 – 5 days p.i.) when iNOS gene levels were increased approximately 10-fold ($p \leq 0.01$ and 0.001 respectively) compared to the non-infected control. During this time frame the gene expression of anti-apoptotic IAP and Bcl-2 was

down-regulated. IAP gene expression was decreased by approximately 55 % on day 3 p.i. ($p \leq 0.001$) and Bcl-2 experienced a general down-regulation due to the infection. The lowest gene levels of Bcl-2 were recorded on day 3 and 5 when they were reduced to 20 % of the non-infected control levels. In contrast the expression of the pro-apoptotic gene Apaf-1 was enhanced on day 14 (1.7 ± 0.2 –fold, $p \leq 0.05$).

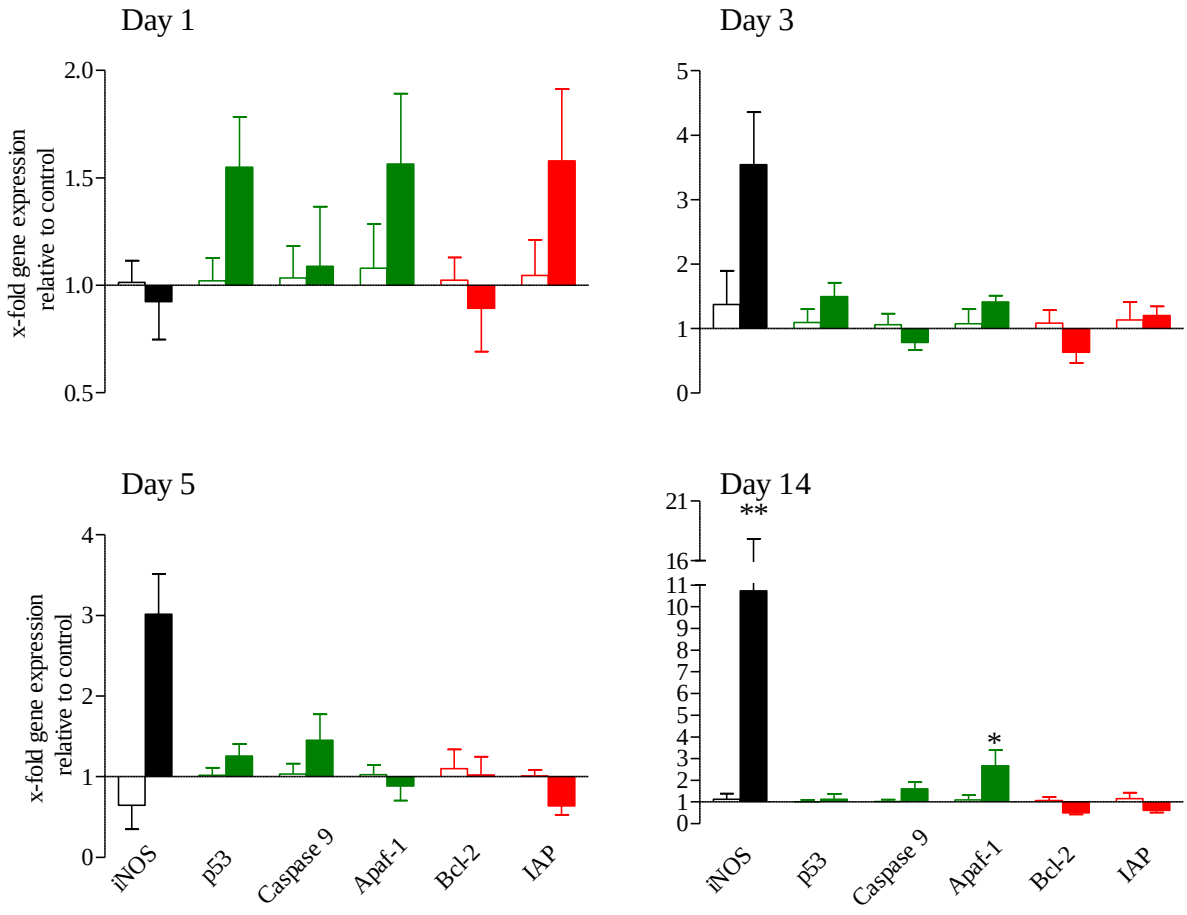

Figure 7.4: Gene expression in gills during KHV infection

Carp were infected with KHV and sampled on day 1, 3, 5, and 14 p.i. and gene expression was analysed with qPCR. Gene expression is displayed as x-fold gene expression relative to the control. Bars represent mean ± SEM with n = 5, blank bars = control (i.e. mock-infection), filled bars = KHV infection, green = pro-apoptotic, red = anti-apoptotic bars, black = iNOS. Significance is defined as $p \leq 0.05$ and represented by asterisks: ** = $p \leq 0.01$

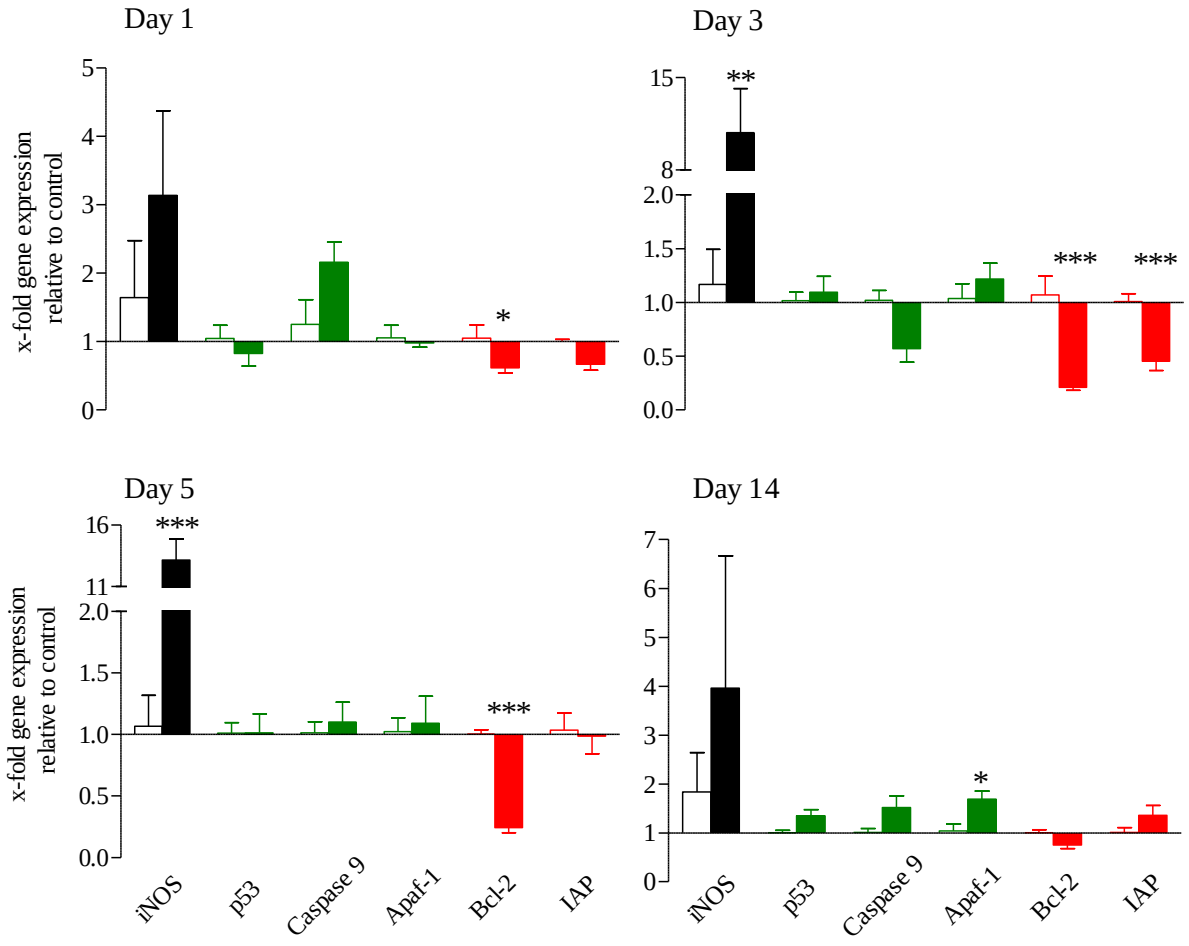

Figure 7.5: Gene expression in spleen during KHV infection

Carp were infected with KHV and sampled on day 1, 3, 5, and 14 p.i. and gene expression was analysed with qPCR. Gene expression is displayed as x-fold gene expression relative to the control. Bars represent mean ± SEM with n = 5, blank bars = control (i.e. mock-infection), filled bars = KHV infection, green = pro-apoptotic, red = anti-apoptotic bars, black = iNOS. Significance is defined as $p \leq 0.05$ and represented by asterisks: * = $p \leq 0.05$, *** = $p \leq 0.001$.

The greatest effect on gene expression in response to the virus was recorded in pronephros (Figure 7.6) since in this organ all analysed genes were influenced by the viral treatment. In addition the time frame of the observed effects differed between the organs as the up-regulation of iNOS gene expression was delayed in pronephros when compared to spleen i.e. spleen iNOS up-regulation occurred at day 3 and 5; pronephros at day 5 and day 14 p.i. ($p \leq 0.01$ and $p \leq 0.05$ respectively). The iNOS mRNA levels were similar between spleen and pronephros since in both organs iNOS was enhanced approximately 10-fold when compared to the control group. All three analysed pro-apoptotic genes (p53, Caspase 9 and Apaf-1) were up-regulated on the last sampling day (day 14 p.i.) when they were all increased by approximately 1.6-fold (p53 = 1.6 ± 0.2, $p \leq 0.05$; Caspase 9 = 1.5 ± 0.2-fold, $p \leq 0.001$; Apaf-1 = 1.7 ± 0.2-fold) compared to the non-infected control. The anti-apoptotic genes (Bcl-2 and IAP) were in general down-regulated: Bcl-2 gene levels were reduced to 30 % of the control values on day 3 and 5 p.i. ($p \leq 0.05$ and $p \leq 0.01$ respectively) and IAP levels were approximately halved at day 3 p.i. ($p \leq 0.05$). However on day 14 IAP gene expression was enhanced 1.9 ± 0.2-fold ($p \leq 0.001$).

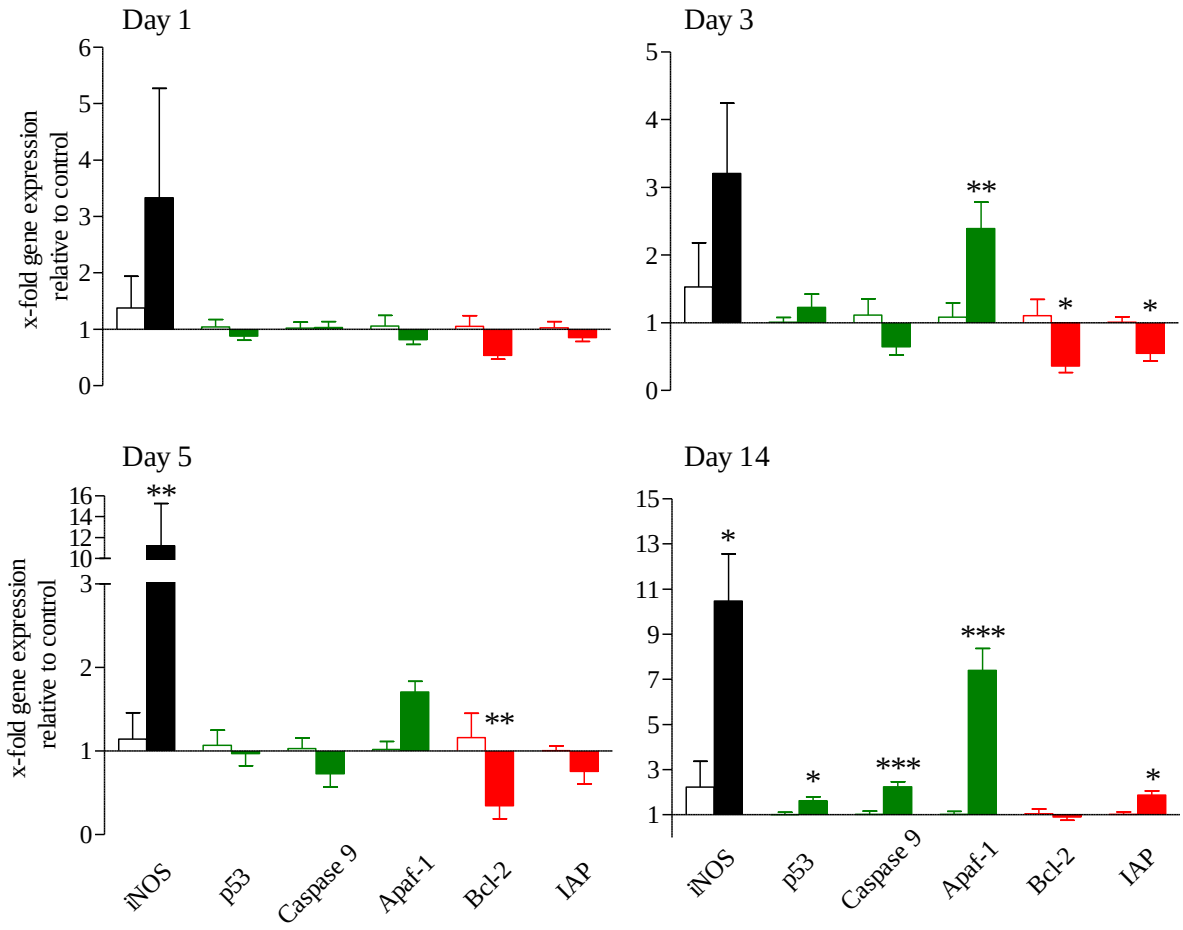

Figure 7.6: Gene expression in pronephros during KHV infection

Carp were infected with KHV and sampled on day 1, 3, 5, and 14 p.i. and gene expression was analysed with qPCR. Gene expression is displayed as x-fold gene expression relative to the control. Bars represent mean ± SEM with n = 5, blank bars = control (i.e. mock-infection), filled bars = KHV infection, green = pro-apoptotic, red = anti-apoptotic bars, black = iNOS. Significance is defined as $p \leq 0.05$ and represented by asterisks: * = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$.

7.3.3 Assessment of viral Bcl-2 as modulator of apoptosis

The KHV genome was analysed for protein sequences similar to the amino acid (AA) sequences of carp Bcl-2 (EU490408) and *Danio rerio* (NM_001030253.2) using BLASTp (Altschul *et al.,* 1997). Two open reading frames (ORF) within the KHV genome were identified, which showed similarity with the two tested fish Bcl-2 sequences: ORF 53 (YP_001096088 / ABG42880) and ORF 17 (YP_001096056 / ABG42848.1). Both these ORFs are currently unnamed hypothetical proteins and their AA sequences were each aligned to the AA sequence of carp Bcl-2 (ccBCl2) with ClustalW2 (Thompson *et al.,* 1994) (Figure 7.7).

a) Alignment of amino acid sequence of ccBcl2 and ORF 53

```
KHVOrf53     RDPKRSLTRAEEDEVRPMTRFGAYDKLALSNALAPESVYPSAVQLLHNALKHTRARWFSE 180
ccBcl2       ------------EEMS--------HQMTFSPSAAQRSFLAVAEELFKDGVRWGRIVPFFE 40
                         :*:         .::::* : * .*. . * :*:::.:: *    * *
                                                                  BH1
KHVOrf53     VGGCGPSDDIDDELA-QLESMARYVDCLLRTPSGDEPVQSLIENRLLNVFAGSRVHLTVA 239
ccBcl2       FGGTMCVESFNREMASQVDNIAHWMTDYLNG-----PLENWIEE-----NGGWDAFVELY 90
             .**    :.:: *:* *:::.:*:::   *.      *::. **:      .*  ..: :
                                                        BH2
KHVOrf53     KPPIDSVYYARRRPPPPSQGPSPPSAHPPSPASPAQPSRSSPSPPSARLGKRKRASSVLN 299
ccBcl2       SQQRDSMF-----------------------------------PPLSYLTKVLGLAALGL 115
             .   **::                                    ** : * *    :::

KHVOrf53     EAVNHGPILEDGLLSKETLVLLPACLSYQLRSPPMGIRIKRSCVKLGVLANGTSALYADA 359
ccBcl2       AGVTIGAFFAQK------------------------------------------------- 127
              .*. *.:: :
```

b) Alignment of amino acid sequence of ccBcl2 and ORF 17

```
KHVorf17     LNKRTRAVVTLPKSKTGPPRPQPPQQQQERSQSQSRSPSSKTASPRQKTVESDCGDTIHA 480
ccBcl2       --------------------------EEMSHQMTFSPSAAQRS---------------- 17
                                      :* *:. : ***:   *

KHVorf17     PVYIQPDIEGMPYAGQPVMIMPFPYGSLRSLLEQNPIKPPKRHEFKDIEKRAWSRTGIKR 540
ccBcl2       ------------------------FLAVAEELFKDGVR--------------WGR----- 34
                                    : :: . * :: ::              *.*
                                           BH1
KHVorf17     SLLALPDSHYELKLFENPAPSTLDEVFVLMVKDTMAGQIEPAYVKLLAEELNRLETNQHT 600
ccBcl2       ----------IVPFFEFGG---------TMCVESFN--------REMASQVDNIAHWMTD 67
                       : :**   .          *  :::         : :*.:::.:

KHVorf17     YRLTQVDYWPKRPNQKGDGFCDVYHMALHMSTYETIPTELSLENCVLRNVRLNSVDFDKH 660
ccBcl2       YLNGPLENWIEE-NGGWDAFVELYSQQR-----DSMFPPLSYLTKVLGLAALG------- 114
             *    :: * :. *   *.* ::*         ::: . **  . **  . *.
                       BH2
KHVorf17     LAHVTSGMCPPAAQ 674
ccBcl2       LAGVTIGAFFAQK- 127
             ** ** *   .
```

Figure 7.7: Amino acid alignment ccBcl2 to KHV ORF 53 & ORF 17

The amino acid sequence of carp Bcl-2 (ccBcl2) was aligned with the sequence of a) KHV ORF 53 and b) KHV ORF 17. Of the ORFs only the part of the sequence is displayed that can be aligned to the ccBCl2 sequence. Numbering of the sequence is indicated on the right. Grey background indicates the conserved BH regions in ccBcl2 according to Cols Vidal (2006). Sequence homology is shown below: * = consensus, : = strong homology and . = weak homology.

The similarity to ccBcl2 was 12 % for ORF 53 and for ORF 17 it is 6 %, which is a very low identity between sequences and in addition the Bcl-2 homologue (BH) domains were not conserved in the analysed ORFs. However even though the AA sequences differ it is possible that the macromolecular structure of the protein is similar and can fulfil the same functions, as demonstrated for KHV IL-10 and KSHV Bcl-2 (Petros *et al.*, 2004; van Beurden *et al.*, 2011). KHV ORF 53 and 17 were therefore analysed for their 3D structure with phyre2 (Kelley and Sternberg, 2009). Phyre2 predicts the hypothetical 3D structure by comparing the AA sequence to known proteins structures from the protein data bank. No structural similarities to apoptosis related proteins were found for ORF 53 and in addition the searched database does not contain any proteins with significant similarity to ORF 53 (Appendix). The analysis of the 3D protein structure of ORF 17 retrieved a 24 % identity (31.8 confidence) with Ced-9 from *C. elegans* (i.e. Bcl-2 like protein) however modelling ORF 17 did not result in a Bcl-2 like structure (Appendix).

In another approach the members of the *Alloherpesviridae* family were analysed for AA sequence similarity with ccBcl-2 using BLASTp. This analysis retrieved several sequences within various members of this family (Table 7.4). However alignment of these viral ORFs with Bcl-2 sequences from zebrafish (*D. rerio*), mouse (*Mus musculus*) and carp (*C. carpio*) revealed that only in the sequence of Anguillid herpesvirus 1 (AngHV 1) the regions characteristic for Bcl-2, i.e. BH domains, are conserved in a similar way as viral Bcl-2 of Kaposi's sarcoma-associated herpesvirus (KSHV). The KHV genome was again analyzed by BLASTp for sequences with similarity to AngHV1 Bcl-2 and KHV ORF 53 was retrieved as the closest match with a maximum identity of 27 % (query

coverage = 23 %) and an E value of 0.083. However alignment using ClustalW found little homology between the two AA sequences (Figure 7.8).

Table 7.4: Alloherpesvirus protein sequences with similarity to ccBcl-2

Virus	I.D.	Accession	Identity	Similarity	Score
Siberian sturgeon herpesvirus	ORF 61	ADC79713	7.1 %	15.8 %	33.0
Acipenserid herpesvirus 2	ORF 61	ACZ55871	7.7	16.0	42.0
Anguillid herpesvirus 1	ORF 33	YP_003358172	16 %	26.2	49.5
Ranid herpesvirus 2	ORF 62	YP_656570	1.7	2.5	26.0
Ictalurid herpesvirus	ORF 2	NP_041093	9.2	11.8	30.0
Cyprinid herpesvirus 3	ORF	YP_001096056	4.5	7.1	23.5

Alignment of AngHV Bcl-2 and KHV ORF 53

```
AngHVorf33   -MEFTYPTTETVARLTQVFG-------VDPRLPPLITKVAGER------DVEAAFVKVLR 46
KHVorf53     MIPYYELETMVAARYQQLLGELGEETVARLQLPNALSTMFTCRSELLKYDLETQYPELMQ 60
             : :     * ..**  *::*       .  :**  ::.:   *      *:*: : ::::

AngHVorf33   NMS---------------------------------------AETPSELILEG------ 60
KHVorf53     VFYNDLHALMGTNVLNGWSTLCDQERVKPLDRDYLRCVPGAVEAGWTAEFISKGEFFNLL 120
              :                                        *  .:*:* :*

AngHVorf33   -DRRSPTLNIRADPTWNWGRL--VVVIMFTELLVAETKNPKLRMLVKPTIEHSIDKWFKS 117
KHVorf53     RDPKRSLTRAEEDEVRPMTRFGAYDKLALSNALAPESVYPSAVQLLHNALKHTRARWFSE 180
              * : .  . . * .    *:     : ::: *..*:  *.   *:: :::*:  :**..

AngHVorf33   VGGWPEPEHRDRDRRWMR-------------AG-------IVGGVIAVVCGVTYYLVK-- 155
KHVorf53     VGGCGPSDDIDDELAQLESMARYVDCLLRTPSGDEPVQSLIENRLLNVFAGSRVHLTVAK 240
             ***   .:. * :   :.             :*       * . :: *..*   :*.
```

Figure 7.8: AngHV1 Bcl-2 alignment with KHV ORF 53

The AA sequences of AngHV Bcl-2 (i.e. ORF 33) and KHV ORF 53 were aligned with ClustalW. Of the ORFs only the part of the sequence is displayed that can be aligned to the ccBcl2 sequence. Numbering of the sequence is indicated on the right. Sequence homology is shown below: * = consensus, : = strong homology and . = weak homology.

7.4 Discussion

7.4.1 Non-infection during the KHV/MacroGard® study

Since in the KHV/MacroGard® study only one of all the tested fish showed considerably increased copy numbers of the KHV virus (i.e. 500 copies/250 ng DNA) it can be concluded that the virus had not infected the fish. In parallel to this study a second study was conducted by other team members during which carp of the same batch were infected with the same virus by injection. All fish from this second study displayed higher virus copy numbers (see "positive control" in Table 7.1) and therefore the crucial factor for the infectivity of the virus seems to be the route of infection. It is possible that the duration of the bath infection (i.e. 15 min) was too short to ensure infection or that the virus concentration was too low in the bath (i.e. 600 $TCID_{50}$/ml). Infection via the natural route, i.e. cohabitation with infected individuals, might have circumvented this problem and could have ensured an infection of the fish (Bergmann *et al.*, 2009). A third possible explanation is that the fish utilised which were specifically selected because they proved negative for KHV infection prior to experimentation may have been immune to infection. Previously infected carp have been shown to have increased resistance against a second KHV infection due to high levels of virus-specific antibodies. In these "immune" fish the viral DNA is not found with conventional PCR methods (Ronen *et al.*, 2003). This possible increased immunity in combination with a weak bath infection might have led to the non-infectivity of the virus in this study.

None of the treatment groups induced apoptosis in the kidney cells and hence it can be concluded that 14 days of MacroGard® feeding did not induce apoptosis. This confirms

results from the study conducted in chapter 5.4.2, where pronephric cells displayed no change in apoptosis levels after 14 days of MacroGard® feeding.

7.4.2 Apoptosis-related gene expression in KHV infected fish

Morphological analyses of the apoptosis levels in the fish were not carried out in this study and therefore conclusions about the state of apoptosis have to be drawn solely from the gene expression analysis. This analysis of the apoptosis-related gene levels clearly showed that all organs displayed a similar response but that the time frame of this response was organ dependent.

The detection of the virus in the tissue and the increased mortalities in the KHV treated fish confirmed the infection in this study (Rakus *et al.,* 2012). An immune response against the virus was observed in spleen from day 3 onwards since elevated levels cytokines (Rakus *et al.,* 2012) and iNOS were observed in this organ. In pronephros cytokine levels were not analysed but iNOS gene expression was influenced in a similar manner as in spleen. Thus the pronephros seems to be involved in the KHV induced immune response. To my knowledge this is the first time that the involvement of iNOS has been demonstrated during infection with KHV. Nitric oxide, which is produced by iNOS, is an important antiviral agent but is often also involved in the pathogenesis of viruses since it can induce tissue damage and T-cell dependent immune responses (Akaike and Maeda 2000). Such tissue damage is often mediated via apoptosis, which would explain the observed up-regulation of pro-apoptotic genes during the late stages of apoptosis, which is most prominent in pronephros on day 14 p.i.

Virus replication and the immune response occurred from day 3 onwards however apoptosis did not occur until day 14. In chapter 6 it was proposed that KHV possesses

apoptosis modulating properties, which is consistent with the absence of apoptosis-related gene expression during the first 5 days p.i. in most of the studied organs. On day 14 p.i. pro-apoptotic genes were up-regulated in pronephros and which indicates the induction of apoptosis in this organ. In the case of Epstein-Barr virus (EBV) apoptosis is inhibited by expression of a viral Bcl-2 homologue as well as by up-regulation of host cell Bcl-2 (Henderson *et al.,* 1991; 1993) (see section 7.4) and it was suggested that in case of EBV apoptosis is delayed rather than prevented. This delay in apoptosis prolongs the life of the infected cell and hence ensures viral replication within this cell (Henderson *et al.,* 1993). The apoptosis induction at the late stage of the infection (i.e. day 14) can be due to a variety of reasons. It is possible that the host induces apoptosis in the infected cells. A study by Perelberg *et al.,* (2008) demonstrated that specific KHV antibodies are produced between day 7 and 14 of the infection. This antibody production indicates the induction of the specific immune response which can lead to apoptosis in viral infected cells mediated by cytotoxic T-cells (Shen *et al.,* 2002; Murphy *et al.,* 2008). This process of T-cell induced apoptosis has been well described in mammals and also appears to exist in fish (Uribe *et al.,* 2011). The finding by Rakus *et al.,* (2012) that the gene expression of various T-cell markers in the spleen of fish were only up-regulated at the very late stage of the infection (i.e. 14 d p.i.) corroborates the assumption that the observed apoptosis is connected to the specific immune response.

On the other hand it is possible that the virus itself induces apoptosis in the infected cell to liberate the progeny as has been observed in members of other virus families e.g. *Rhabdoviridae* (Ammayappan and Vakharia 2011). Virus containing apoptotic bodies are phagocytosed and can then infect the phagocytic cell. This allows the

virus to spread without recognition by the immune system (Roulston *et al.,* 1999; Hay and Kannourakis 2002).

7.4.3 Assessment of viral Bcl-2 as modulator of apoptosis

During this investigation it became apparent that vBcl-2 could not be positively identified in the KHV genome. However eel herpesvirus 1 (AngHV 1), which is closely related to KHV, possesses a viral Bcl-2 (van Beurden *et al.,* 2010). Apart from AngHV1 vBcl-2 conserved domains related to Bcl-2 were not identified within the known *Alloherpesviridae* genomes. Similarly vIL-10 which is present in AngHV 1 and KHV but not in the closely related CyHV-1 and CyHV-2. Therefore it can be speculated that AngHV 1 has acquired its Bcl-2 homologue after the divergence from the common ancestor of the *Alloherpesviridae*, which indicates a separate evolution of anti-apoptotic properties from those observed in human herpesviruses. This is corroborated by the findings regarding vIL-10 which was probably also acquired separately during evolution.

Since a Bcl-2 homologue was not found in the KHV genome it was not possible to ascertain how KHV modulates host apoptosis. Thus it can be speculated that KHV expresses other anti-apoptotic proteins such as for example IAP or it inhibits apoptosis via other pathways. Preliminary results indicate that no characteristic viral IAP exists within the KHV genome but the possible interference mechanisms are manifold and have been reviewed in Roulston *et al.,* (1999). For instance herpes simplex virus 1 and some poxviruses inhibit the interferon response while others inhibit the TNFR and Fas response. A recently published report indicates that KHV inhibits the interferon response of the host (Adamek *et al.,* 2012), which would be in agreement with apoptosis prevention via the inhibition of the interferon response. Various viral products can be involved in this process

and future research is necessary to elucidate which process is involved during a KHV infection.

Within the *Alloherpesviridae* family the genomes of Ranid herpesvirus 1 and 2 (Davison *et al.,* 1999; 2006), Ictalurid herpesvirs 1 (Davison 1992), AngHV 1 (van Beurden *et al.,* 2010) and KHV (Aoki *et al.,* 2007) are currently fully sequenced. With advancing genome sequencing and further identification of *Alloherpesviridae* members a better interpretation of the evolution of anti-apoptotic properties of the *Herpesvirales* order will be possible. In addition a comparison of possible effects of KHV and AngHV 1 on host apoptosis could shed light on the mechanisms involved and, in particular, the involvement of vBcl-2.

7.5 General conclusion

This chapter aimed to investigate the *in vivo* effects of a KHV infection on the apoptotic process of common carp and explored a possible involvement of viral Bcl-2. It can be concluded that KHV does not induce apoptosis during the early stages of the infection but that apoptosis primarily occurs during the later stages. This late apoptosis induction might be connected to the viral replication cycle or the specific immune response.

It was hypothesized that KHV interferes with host apoptosis by expression of a viral Bcl-2 homologue, however this could not be confirmed and future studies will be necessary to elucidate the mechanisms involved. This knowledge will not only contribute

to the understanding of the virus pathology and its evolution but will have application in the treatment of fish and other herpesvirus-induced diseases.

8 General Discussion

The 2010 report by the Food and Agriculture Organisation of the United Nations on fisheries and aquaculture (FAO, 2010) stated that aquaculture will overtake capture fisheries as the primary source of food fish in the future. However the growth of this food-producing sector is impaired by disease outbreaks, which both reduce productivity in fish farms and pose a problem for biosecurity. Immunostimulation is an important tool in aquaculture to increase resistance to pathogens (Sakai 1999) especially since substances such as β-glucan occur naturally in the environment and are thus less likely to raise concerns about residues in food fish and their environmental impact (Gannam and Schrock 2001).

However for many substances used as immunostimulants, including LPS and β-glucan, effectiveness is dose dependent, such that high concentrations often lead to adverse effects, including immunosuppression (Misra *et al.*, 2006a; 2006b; Bonaldo *et al.*, 2007; Nayak *et al.*, 2008). As a result the resistance of the animals to pathogens is not enhanced or is even decreased at high concentrations, and hence it is important for both feed manufacturers and fish farmers that the dietary dose of the immunomodulating substance utilized does not induce such negative effects. Nonetheless, even though reports have shown that β-glucan has dose-dependent immunostimulating and immunosuppressing effects, up to now the mechanisms behind these effects have not been elucidated.

In mammals studies on β-glucan in relationship to tumour biology have revealed that this immunostimulant is able to induce apoptosis (Zhang *et al.*, 2006; Kim *et al.*,

2009a). In chapter 1 it was therefore hypothesized (Figure 1.7) that immunosuppression is possibly mediated via apoptosis of immune cells.

In order to study the effects of immunostimulation and disease on apoptosis, two *in vitro* systems, i.e. primary cell cultures of heterogeneous pronephric leucocytes and cell line cultures of carp fibrocytes (CCB cells), were established. In addition *in vivo* feeding protocols were set up and methods to investigate morphological changes related to apoptosis were established. The investigation of apoptosis-related gene expression in carp has only recently received attention (Chakravarthy, 2003; Cols Vidal, 2006) and in this thesis this previous knowledge was for the first time combined with modern molecular techniques to study the effects of diseases and immunostimulation on apoptosis-related gene expression in carp. As a result a standard protocol to investigate the *in vivo* and *in vitro* regulation of the intrinsic apoptosis pathway in carp was established.

The proposed hypothesis, that β-glucan can cause immunosuppression via induction of apoptosis in leucocytes, was strongly supported by the findings in chapter 3 where it was shown that MacroGard® induces cell death via apoptosis in pronephric leucocytes *in vitro* at concentrations of ≥ 500 µg/ml. This dose dependent induction of apoptosis has important implications for *in vitro* studies on β-glucan since it highlights that cell death has to be monitored in such studies to avoid an effect of apoptosis on the results. This study is the first that reports β-glucan-induced apoptosis in non-mammalian and in non-cancerous cells. The fact that β-glucan-containing MacroGard® induces apoptosis in fish cells has implications for medical science since it could allow the use of fish cancer models (Mione and Trede, 2010) for the study of β-glucan effects. On the other hand the

finding that apoptosis was induced in non-cancerous cells has implications in the treatment of carcinomas as the immunosuppressive effects of β-glucan need to be monitored.

Most importantly the above finding has implications for the aquaculture industry since it highlights the dose dependent effects of β-glucan. This thesis hence aimed to elucidate if MacroGard® induces apoptosis when applied at the manufacturer's recommended dietary dose. In two independent studies (chapter 4 and 5) it was established that feeding of MacroGard® at the recommended dose (10 mg/kg bodyweight per day) over a maximum period of 25 days had no apoptosis-inducing properties on pronephric leucocytes of carp. However this feeding led to an innate immune response after 25 days, when an up-regulation of iNOS was detected. β-Glucan has been previously shown to induce the production of nitric oxide by mammalian macrophages (Ohno *et al.,* 1996; Hashimoto *et al.,* 1997; Ljungman *et al.,* 1998), but to date the same effect has not been studied in detail in fish (El-Boshy *et al.,* 2010; Del Rio-Zaragoza *et al.,* 2011). Nitric oxide, which is produced by iNOS, is an important anti-bacterial and anti-viral agent (Croen 1993; Saura *et al.,* 1999; Campos-Pérez *et al.,* 2000; Murphy *et al.,* 2008) and hence the stimulation of its production can lead to increased disease resistance. However, depending on its concentration in the system, it has anti-apoptotic or pro-apoptotic properties (Dimmeler and Zeiher, 1997), and in the feeding study described in chapter 4 the heightened iNOS gene levels were accompanied by a time and organ dependent influence on expression of pro- and anti-apoptotic genes. It seems that some organs, i.e. spleen, might be more vulnerable to apoptosis induced by a long-term β-glucan administration than others for example mid-gut, pronephros and liver. These pro-apoptotic effects in spleen could explain the reduced activity of some innate immune parameters, e.g. leucocyte cell count, phagocytic activity, and respiratory burst, observed in previous

studies after long-term feeding of β-glucan (Misra *et al.*, 2006a; Lin *et al.*, 2011). Studies involving longer feeding periods (months instead of weeks) could clarify the time frame of induced apoptosis, which would provide the aquaculture industry with better feeding protocols for immunostimulation. The absence of apoptosis in the pronephric leucocytes and the lack of induced apoptosis-related gene expression in most of the analysed organs however indicate that overall MacroGard® feeding does not lead to apoptosis if administered at the recommended dose over a period of two weeks.

In addition to the effects of β-glucan on apoptosis-related genes the effect of the immunostimulant on key cell signalling pathways was also investigated. The mammalian β-glucan receptor Dectin-1, which has not been identified in fish, is known to signal via the NF-κB pathway (Blonska and Lin, 2011). In addition, in mammals NF-κB and the p38 MAPK pathway have been associated with various innate immune factors such as phagocytosis and production of oxygen radicals (NO, ROS), and are linked to apoptosis via p53 (Park 2003; Yang *et al.*, 2008). Hence I chose to target these pathways to elucidate the signalling pathways involved in β-glucan administration and the innate immune response in fish. The identity of the β-glucan receptor in fish is currently under investigation at Wageningen University but to date it is not known which cellular receptor is responsible for the recognition of β-glucan in fish. Interestingly, the results of the investigations in this thesis regarding β-glucan signalling pathways are inconsistent. Whilst *in vivo* feeding of MacroGard® led to the induction of p38 MAPK and NF-κB pathways in spleen (chapter 4) no effect on these signalling pathways were observed during the *in vitro* study in chapter 6. Such differences may arise due to differences in the properties of the cell populations studied: for example heterogeneous leucocytes *in vivo* and homogenous fibrocytes *in vitro*, and hence differences in PAMP recognition receptors on the cellular

surface. The latter would then lead to differences in induced cell signalling pathways. For this reason conclusions about the pathways involved in β-glucan signalling would require further investigation.

In the light of these findings, and taking into account that pathogens can modulate host apoptosis (Hoole and Williams, 2004), it is important to elucidate the influence that MacroGard® has on pathogen induced apoptotic processes. For this purpose the apoptotic properties of the targeted diseases had to be established first and the bacterial pathogen *Aeromonas salmonicida* and the viral pathogens Spring Viremia of Carp Virus (SVCV) and Koi Herpesvirus (KHV) were chosen as models. The investigations clearly showed the different effects of these three pathogens on the apoptotic process. In chapter 5 it was shown that *A. salmonicida* induces apoptosis in pronephric leucocytes of carp, which is consistent with the reported apoptosis-inducing properties of other *Aeromonas* family members *in vitro* (e.g. Shao *et al.*, 2004; Krzyminska *et al.*, 2009). However this is the first time that the *in vivo* apoptosis inducing-properties of *A. salmonicida* have been reported and published (Miest *et al.*, 2012). These observed apoptotic effects could explain the cytotoxicity elicited by this bacterium towards macrophages (Garduño and Kay, 1992) and which are linked to extracellular toxins (e.g. LPS, cytotoxic glycoprotein) (Bernoth *et al.*, 1997). This induced cell death of immune cells probably contributes to the pathogenicity of this bacterium by avoidance of the immune response. By killing macrophages and neutrophils *A. salmonicida* can avoid phagocytosis, and enhance its survival in the host. Enhanced apoptosis of phagocytes also leads to an impaired uptake of apoptotic cells by these cells, which results in secondary necrosis in the dying cells, as discussed for infection with *Photobacterium damselae* by Silva *et al.*, (2008b). In combination with secondary

necrosis the observed apoptosis processes can lead to the massive tissue degeneration observed during this disease. Histological examinations of apoptosis could clarify if and how apoptosis is involved in the clinical signs of *A. salmonicida* infections and would lead to a better understanding of the disease.

The viral pathogen SVCV also causes apoptosis in carp cells (chapter 6) (Björklund *et al.,* 1997; Kazachka *et al.,* 2007). However the genetic mechanisms involved have not been established and hence this is the first time that the involvement of the intrinsic apoptosis pathway was demonstrated for SVCV induced apoptosis. SVCV hence induced apoptosis in fibroblasts (CCB cell line) and epithelial cells (EPC cell line (Björklund *et al.,* 1997; Kazachka *et al.,* 2007)), indicating its ability to infect a wide range of cell types. Other fish rhabdoviruses have been shown to be able to induce apoptosis in a leucocyte cell line (CLC cells) (Du *et al.,* 2004) and hence it is probable that SVCV will also be able to cause apoptosis in immune cells as well. Studies conducted on other members of the same virus family, i.e. rhabdoviruses, suggest that the induced apoptosis is both an anti-viral response of the host and an anti-host mechanism of the virus. In the case of rhabdoviruses the anti-viral response is associated with the extrinsic apoptosis pathway whilst apoptosis induced by the virus is related to the intrinsic host response (Purcell *et al.,* 2012). Due to the conserved genome composition within rhabdoviruses it is likely that these apoptosis-related properties are conserved in SVCV. Hence it can be speculated that the induction of the intrinsic apoptosis pathway observed in chapter 6 is related to the virus modulating the host's apoptosis as a means of immune evasion. The study of both the intrinsic and extrinsic apoptosis pathway (as discussed later) could help to understand the

mechanisms behind the induced apoptosis (anti-host or anti-viral) and its effect on fish immunity.

This induced apoptosis can lead to immune evasion of the virus and could explain why this virus can cause high mortalities in fish farms. In addition in rabies virus infections (a mammalian rhabdovirus) it has been proposed that apoptosis of infected and non-infected bystander cells leads to inflammatory responses that cause extensive tissue damage (Galelli *et al.,* 2000). A similar process might occur during SVCV infections since this disease has been associated with inflammation of the intestine and the peritoneum. It would be interesting to study the apoptotic effects of this virus *in vivo* as done in chapter 7 for KHV to further elucidate the role of apoptosis in the pathogenicity of the virus.

In contrast to both *A. salmonicida* and SVCV, KHV seemed to be able to suppress the antiviral apoptosis response of the host. This effect is well known for some human herpesviruses but has never been demonstrated for a herpesvirus outside the *Herpesviridae* family. Since apoptosis was suppressed but cytopathic effects were still recorded in the cell culture it was proposed in chapter 6 that cell death occurs via necrosis due to lysis of the infected cells. This induced necrosis *in vitro* probably reflects the necrotic tissue damage observed in KHV infected fish. This damage includes necrosis in gill, gut and kidney tissue which leads to osmoregulatory failure and contributes to mortality (Gilad *et al.,* 2004; Negeborn 2009). The inhibition of host induced apoptosis leads to a suppression of the immune response, because infected cells cannot be efficiently eliminated. This immunosuppression is reflected in the often observed secondary bacterial and fungal infections that occur alongside the infection with KHV (Hedrick *et al.,* 2000) and these secondary infections also contribute to the mortality observed during KHV infections.

In chapter 7 it was observed that *in vivo* KHV infection is associated with two stages of apoptosis modulation. During the first stage (day 1 – 5 p.i.) apoptosis-related gene expression is not induced, whilst at the second stage (day 14 p.i.) expression of pro-apoptotic genes is enhanced. Various possible mechanisms behind this effect have been discussed in chapter 7 and further research is required to elucidate the processes involved in the apoptosis-KHV relationship *in vivo*. For example it could be determined if apoptosis is correlated with virus load by PCR or a certain stage during viral replication by electron microscopy. Studies utilizing *in vitro* models to test the ability of lymphocytes to induce apoptosis in infected cells can help to elucidate the involvement of the specific immune response. In addition the characterization of apoptosis-modulating properties of KHV will advance the understanding of the disease caused by this virus and might help to unveil some of the evolutionary background of these mechanisms. In chapter 7 it was speculated that KHV modulates host apoptosis similarly to other members of the *Herpesvirales* order, i.e. expression of a viral Bcl-2 homologue. However no characteristic viral Bcl-2 could be identified within the KHV genome and hence KHV may employ a different strategy to that observed in other herpesviruses such as Epstein-Barr virus and Kaposi's sarcoma-associated herpesvirus. For example KHV could block apoptosis via inhibition of caspase family members or by inhibition of the interferon response (see chapter 7).

Interestingly the closely related eel herpesvirus AngHV 1 appears to express vBcl-2, which suggests that AngHV 1 prevents apoptosis via expression of this anti-apoptotic protein. However nothing is yet known about the influence of AngHV 1 on host apoptosis and this would form an interesting new line of future investigations. This finding of vBcl-2 in AngHV-1 but not in KHV implies the independent evolution of anti-apoptotic

mechanisms in these closely related viruses but it also indicates a general ability of herpesviruses to acquire antiviral mechanisms.

The analysis of the molecular pathways during *A. salmonicida*, SVCV and KHV infections revealed the involvement of iNOS and the intrinsic apoptosis pathway in all three of these infections. This indicates that both bacteria and viruses activate similar immune responses and apoptotic processes, although the recognition of these pathogens depends on different receptors and signalling pathways. Even though the processes involved in pathogen recognition are not fully understood in carp, the results from this study suggest that in fish recognition of bacterial and viral pathogens induce similar signalling pathways.

The knowledge acquired throughout this thesis adds to the current understanding of host-pathogen interactions in fish. All the observations made regarding the relationship of apoptosis and pathogens confirm that bacteria and viruses induce an apoptotic response in the host and that pathogens can modulate this response to ensure their survival. Against this background of pathogen-modulated host apoptosis it was important to evaluate the effect that dietary MacroGard® had on this process. Interestingly MacroGard® feeding enhanced the gene expression induced by both the viral mimic Poly(I:C) and the bacterial mimic LPS. This enhancement indicates cumulative effects of β-glucan and the viral/bacterial Poly(I:C)/LPS. In SVCV-infected CCB cells MacroGard® also led to enhanced gene expression, which corroborates the *in vivo* MacroGard® effect on Poly(I:C)-injected fish. The observed cumulative effect of β-glucan and LPS, which might be expected since the bacterium does produce LPS (Chart *et al.*, 1984), was not reflected during the *A. salmonicida* infection. However there are various factors that influenced the

outcome of these two studies e.g. the different fish strains used, differences in the implementation of these experiments, feeding period and virulence of utilized bacterium/concentration of LPS, and hence comparison between these two studies is difficult. Unfortunately it was not possible to elucidate the *in vivo* effects of MacroGard® feeding on viral infections and hence it is not possible to draw conclusions about the effect of the immunostimulant on virus induced processes *in vivo*.

These cumulative effects that were observed on gene expression level were not reflected in the cellular morphology, since no differences in the percentage of cells undergoing apoptosis were observed between control fed and MacroGard® fed fish in any of the infection treatments. The apoptosis induced by *A. salmonicida* and SVCV was not affected by MacroGard® either *in vivo* or *in vitro*, although the inhibition of apoptosis caused by KHV was also not modulated by the immunostimulant. It is thus difficult to assess if heightened apoptosis-related gene expression levels contribute to increased disease resistance, for example through enhanced killing properties of the innate immune response towards the pathogen. Nevertheless since the influence of MacroGard® on viral modulation of apoptosis was ascertained *in vitro*, future studies are required to determine the *in vivo* effects of dietary immunostimulation on viral-induced apoptosis levels. This knowledge will not only help to understand the mechanisms behind β-glucan enhanced disease resistance but also aid our general understanding of apoptosis in viral diseases.

Throughout this thesis, attempts were made to understand the pathways involved in modulating apoptosis in carp. Currently, few apoptosis related gene sequences have been identified in this species, particularly in regards to the extrinsic apoptosis pathway. The recent sequencing of the carp genome (Henkel *et al.,* 2012) will hopefully aid in the

identification and characterisation of the genes involved in apoptosis, which will in turn facilitate studies of apoptotic processes in carp. Preliminary results from a Blast search have indicated that sequences similar to two main components of the extrinsic pathway, Fadd (Gene bank: EX882196.1) and Tradd (3' start: DW723729.1, 5' end: EC393988.1), exist in the carp genome. Characterisation of these genes will allow the analysis of the interaction of the innate and extrinsic apoptosis pathway during infections as discussed for SVCV infections (see above).

Throughout this thesis (chapter 4 – 7) large-scale feeding and infection studies were conducted in collaboration with other European partners. Such studies, which are conducted within a network, have the advantage of a holistic approach during which a large variety of aspects can be investigated. However due to their complexity such studies are often limited in their reproducibility. In addition observations of individual effects (e.g. affected cellular signalling pathways) in investigations on the interconnectivity of a variety of influences (e.g. feeding and infection) are often difficult and need to be confirmed in separate smaller studies with a higher reproducibility.

In conclusion the work described in this thesis has made a significant contribution to the understanding of the interaction between immunostimulants, infection and the apoptotic process in carp immune cells at both the cellular and the molecular level. Gene expression in relation to apoptosis in fish and especially in carp is not a widely investigated field and the investigations carried out are the first to highlight that immunostimulation on its own or in combination with pathogens does not induce apoptosis but has cumulative effects on the gene expression level and that bacterial and viral pathogens can induce the

intrinsic apoptosis pathway. This has important implications not only for the understanding of the immunological interactions between viral and bacterial pathogens and their fish hosts but also the use of natural immunomodulators in disease prevention.

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Appendix

RNALater Recipe (courtesy of Mikolaj Adamek)

Ingredients:

EDTA disodium, dehydrate

Sodium citrate trisodium salt, dehydrate

Ammonium sulphate (powder)

1 M NaOH

1 M H_2SO_4

Method

Make up stock solutions:

EDTA 0.5 M (18.61 g) in 100 ml – adjust pH to 8.0 with NaOH

Sodium citrate 1 M (29.4 g) in 100 ml

For 1 L RNA later:

40 ml EDTA

25 ml sodium citrate

700 g ammonium sulphate (300 – 1200 g)

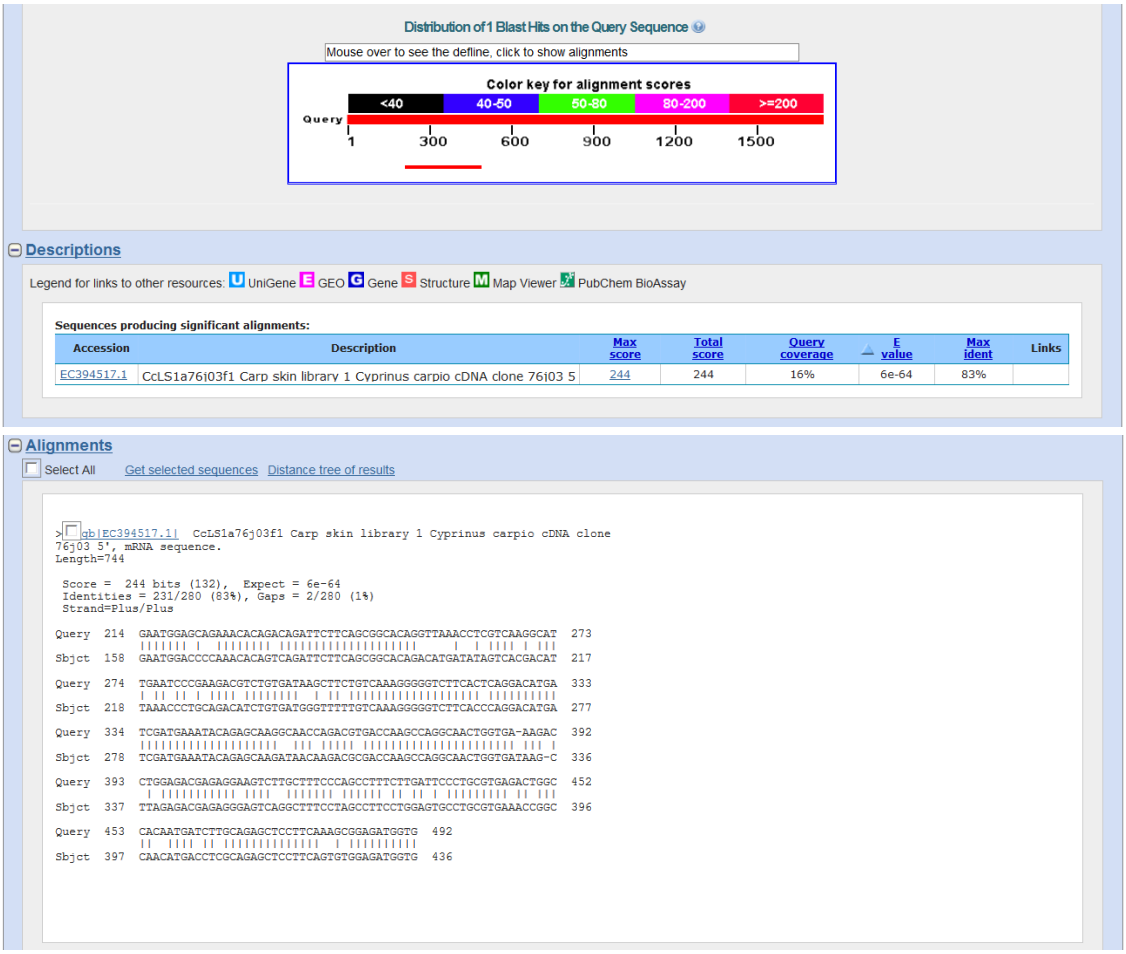
935 ml sterile water

Add soluble ingredients to 800 ml sterile water and stir on a hot plate stirrer on low heat until the ammonium sulphate is completely dissolved. Allow to cool. Adjust to pH 5.2 with 1 M H_2SO_4 and adjust to 1000 ml with sterile water.

Store at room temperature or 4°C.

Identification of Caspase 9 in *Cyprinus carpio*

The Caspase 9 cDNA sequence of *Danio rerio* (NM_001007404.2) was compared to ESTs of common carp using Blast. The EST EC394517.1 was identified as carp homologue of Caspase 9.

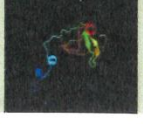


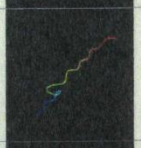
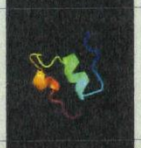
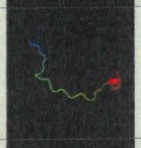
Analysis of possible tertiary structure of KHV ORF 53

Phyre2

Email	rsd76@istm.keele.ac.uk
Description	orf53___
Date	Sun Jul 15 16:22:06 BST 2012
Unique Job ID	2cd6d6ee47978497

Detailed template information

#	Template	Alignment Coverage	3D Model	Confidence	% i.d.	Template Information
1	c3dtpF_	Alignment		39.9	19	**PDB header:**contractile protein **Chain:** F: **PDB Molecule:**myosin regulatory light chain; **PDBTitle:** tarantula heavy meromyosin obtained by flexible docking to2 tarantula muscle thick filament cryo-em 3d-map
2	c2gvsA_	Alignment		36.8	35	**PDB header:**lipid binding protein **Chain:** A: **PDB Molecule:**chemosensory protein csp-sg4; **PDBTitle:** nmr solution structure of cspsg4
3	c2it7A_	Alignment		35.1	59	**PDB header:**plant protein **Chain:** A: **PDB Molecule:**trypsin inhibitor 2; **PDBTitle:** solution structure of the squash trypsin inhibitor eeti-i
4	d2it7a1	Alignment		35.1	59	**Fold:**Knottins (small inhibitors, toxins, lectins) **Superfamily:**Plant inhibitors of proteinases and amylases **Family:**Plant inhibitors of proteinases and amylases
5	c2letA_	Alignment		33.4	59	**PDB header:**proteinase inhibitor(trypsin) **Chain:** A: **PDB Molecule:**trypsin inhibitor ii; **PDBTitle:** an 1h nmr determination of the three dimensional structures2 of mirror image forms of a leu-5 variant of the trypsir inhibitor ecballium elaterium (eeti-ii)
6	d1mcvi_	Alignment		31.0	50	**Fold:**Knottins (small inhibitors, toxins, lectins) **Superfamily:**Plant inhibitors of proteinases and amylases **Family:**Plant inhibitors of proteinases and amylases
7	c1mcvI_	Alignment		31.0	50	**PDB header:**hydrolase **Chain:** I: **PDB Molecule:**hei-toe i; **PDBTitle:** crystal structure analysis of a hybrid squash inhibitor in2 complex with porcine pancreatic elastase
8	d1kx9b_	Alignment		30.4	30	**Fold:**alpha-alpha superhelix **Superfamily:**Chemosensory protein Csp2 **Family:**Chemosensory protein Csp2
9	d1zvfa1	Alignment		20.5	42	**Fold:**Double-stranded beta-helix **Superfamily:**RmlC-like cupins **Family:**3-hydroxyanthranilic acid dioxygenase-like
10	c1m1cB_	Alignment		19.7	26	**PDB header:**virus **Chain:** B: **PDB Molecule:**major coat protein; **PDBTitle:** structure of the l-a virus
11	c1oz3C_	Alignment		18.6	19	**PDB header:**transcription **Chain:** C: **PDB Molecule:**lethal(3)malignant brain tumor-like protein; **PDBTitle:** crystal structure of 3-mbt repeats of lethal (3) maligna brain tumor2 (native-i) at 1.85 angstrom

12	d1m1ca_	Alignment		17.8	26	**Fold:**L-A virus major coat protein **Superfamily:**L-A virus major coat protein **Family:**L-A virus major coat protein
13	c3lydA_	Alignment		16.8	36	**PDB header:**structural genomics, unknown function **Chain:** A: **PDB Molecule:**uncharacterized protein; **PDBTitle:** crystal structure of putative uncharacterized protein from jonesia2 denitrificans
14	c2ljyA_	Alignment		16.8	25	**PDB header:**metal binding protein **Chain:** A: **PDB Molecule:**protein e6; **PDBTitle:** haddock model structure of the n-terminal domain dimer of hpv16 e6
15	c3pghD_	Alignment		15.6	29	**PDB header:**oxidoreductase **Chain:** D: **PDB Molecule:**cyclooxygenase-2; **PDBTitle:** cyclooxygenase-2 (prostaglandin synthase-2) complexed with a non-2 selective inhibitor, flurbiprofen
16	c2rqrA_	Alignment		14.5	26	**PDB header:**protein binding **Chain:** A: **PDB Molecule:**engulfment and cell motility protein 1, linker, dedicator **PDBTitle:** the solution structure of human dock2 sh3 domain - elmo1 peptide2 chimera complex
17	d2fi0a1	Alignment		14.0	13	**Fold:**SP0561-like **Superfamily:**SP0561-like **Family:**SP0561-like
18	d1h9hi_	Alignment		13.8	53	**Fold:**Knottins (small inhibitors, toxins, lectins) **Superfamily:**Plant inhibitors of proteinases and amylases **Family:**Plant inhibitors of proteinases and amylases
19	c1ddxA_	Alignment		13.8	29	**PDB header:**oxidoreductase **Chain:** A: **PDB Molecule:**protein (prostaglandin h2 synthase-2); **PDBTitle:** crystal structure of a mixture of arachidonic acid and prostaglandin2 bound to the cyclooxygenase active site of cox-2: prostaglandin3 structure
20	d2e9xd1	Alignment		13.4	27	**Fold:**GINS helical bundle-like **Superfamily:**GINS helical bundle-like **Family:**SLD5 N-terminal domain-like
21	d1n8va_	Alignment	not modelled	13.4	22	**Fold:**alpha-alpha superhelix **Superfamily:**Chemosensory protein Csp2 **Family:**Chemosensory protein Csp2
22	d1ynjk1	Alignment	not modelled	13.2	48	**Fold:**RPB6/omega subunit-like **Superfamily:**RPB6/omega subunit-like **Family:**RNA polymerase omega subunit
23	c2kpvA_	Alignment	not modelled	13.0	22	**PDB header:**allergen **Chain:** A: **PDB Molecule:**major pollen allergen art v 1; **PDBTitle:** solution structure of the major allergen of artemisia vulgaris (art v2 1)
24	d1yfua1	Alignment	not modelled	13.0	26	**Fold:**Double-stranded beta-helix **Superfamily:**RmlC-like cupins **Family:**3-hydroxyanthranilic acid dioxygenase-like
25	d1wjra_	Alignment	not modelled	12.8	19	**Fold:**SH3-like barrel **Superfamily:**Tudor/PWWP/MBT **Family:**MBT repeat
26	d2czra1	Alignment	not modelled	12.6	83	**Fold:**Restriction endonuclease-like **Superfamily:**TBP-interacting protein-like **Family:**TBP-interacting protein-like
27	c3ut1A_	Alignment	not modelled	12.1	25	**PDB header:**transcription **Chain:** A: **PDB Molecule:**lethal(3)malignant brain tumor-like protein 3; **PDBTitle:** crystal structure of the 3-mbt repeat domain of l3mbtl3
28	d1oi1a2	Alignment	not modelled	11.5	35	**Fold:**SH3-like barrel **Superfamily:**Tudor/PWWP/MBT **Family:**MBT repeat
29	d1oz2a3	Alignment	not modelled	11.5	35	**Fold:**SH3-like barrel **Superfamily:**Tudor/PWWP/MBT

30	c2xgvA_	Alignment	not modelled	11.4	29	**PDB header:**viral protein/isomerase **Chain:** A: **PDB Molecule:**relik capsid n-terminal domain; **PDBTitle:** complex of rabbit endogenous lentivirus (relik)capsid with2 cyclophilin a
31	d1vffa1	Alignment	not modelled	10.9	36	**Fold:**TIM beta/alpha-barrel **Superfamily:**(Trans)glycosidases **Family:**Family 1 of glycosyl hydrolase
32	d1m9fd_	Alignment	not modelled	10.8	24	**Fold:**Retrovirus capsid protein, N-terminal core domain **Superfamily:**Retrovirus capsid protein, N-terminal core domain **Family:**Retrovirus capsid protein, N-terminal core domain
33	d1ffva1	Alignment	not modelled	10.6	14	**Fold:**CO dehydrogenase ISP C-domain like **Superfamily:**CO dehydrogenase ISP C-domain like **Family:**CO dehydrogenase ISP C-domain like
34	c2vytA_	Alignment	not modelled	10.4	50	**PDB header:**transcription **Chain:** A: **PDB Molecule:**sex comb on midleg-like protein 2; **PDBTitle:** the mbt repeats of human scml2 bind to peptides containing2 mono methylated lysine.
35	d1wjsa_	Alignment	not modelled	10.3	38	**Fold:**SH3-like barrel **Superfamily:**Tudor/PWWP/MBT **Family:**MBT repeat
36	c4e0eB_	Alignment	not modelled	10.1	32	**PDB header:**unknown function **Chain:** B: **PDB Molecule:**putative uncharacterized protein; **PDBTitle:** crystal structure of a hypothetical protein (bt_4147) from bacteroides2 thetaiotaomicron vpi-5482 at 2.90 a resolution
37	d1oz2a2	Alignment	not modelled	10.0	41	**Fold:**SH3-like barrel **Superfamily:**Tudor/PWWP/MBT **Family:**MBT repeat
38	c2k5eA_	Alignment	not modelled	10.0	10	**PDB header:**structural genomics, unknown function **Chain:** A: **PDB Molecule:**uncharacterized protein; **PDBTitle:** solution structure of putative uncharacterized protein2 gsu1278 from methanocaldococcus jannaschii, northeast3 structural genomics consortium (nesg) target gsr195
39	d1k81a_	Alignment	not modelled	9.9	75	**Fold:**Zinc-binding domain of translation initiation factor 2 beta **Superfamily:**Zinc-binding domain of translation initiation factor 2 beta **Family:**Zinc-binding domain of translation initiation factor 2 beta
40	c3ceyA_	Alignment	not modelled	9.9	38	**PDB header:**transcription regulator **Chain:** A: **PDB Molecule:**lethal(3)malignant brain tumor-like 2 protein; **PDBTitle:** crystal structure of l3mbtl2
41	d1wjqa_	Alignment	not modelled	9.8	44	**Fold:**SH3-like barrel **Superfamily:**Tudor/PWWP/MBT **Family:**MBT repeat
42	d1oi1a1	Alignment	not modelled	9.5	47	**Fold:**SH3-like barrel **Superfamily:**Tudor/PWWP/MBT **Family:**MBT repeat
43	c2r58A_	Alignment	not modelled	9.4	38	**PDB header:**transcription **Chain:** A: **PDB Molecule:**polycomb protein scm; **PDBTitle:** crystal structure of the two mbt repeats from sex-comb on midleg (scm)2 in complex with di-methyl lysine
44	c2om2B_	Alignment	not modelled	9.3	44	**PDB header:**signaling protein **Chain:** B: **PDB Molecule:**regulator of g-protein signalling 14 goloco **PDBTitle:** crystal structure of human g[alpha]i1 bound to the goloco2 motif of rgs14
45	c1r48A_	Alignment	not modelled	9.1	64	**PDB header:**transport protein **Chain:** A: **PDB Molecule:**proline/betaine transporter; **PDBTitle:** solution structure of the c-terminal cytoplasmic domain2 residues 468-497 of escherichia coli protein prop
46	d1zj8a2	Alignment	not modelled	9.0	24	**Fold:**Ferredoxin-like **Superfamily:**Nitrite/Sulfite reductase N-terminal domain-like **Family:**Duplicated SiR/NiR-like domains 1 and 3
47	c3zxqA_	Alignment	not modelled	8.7	19	**PDB header:**transferase **Chain:** A: **PDB Molecule:**hypoxia sensor histidine kinase response regulator dosr; **PDBTitle:** crystal structure of the atp-binding domain of mycobacterium2 tuberculosis dosr
48	c3cw2M_	Alignment	not modelled	8.5	31	**PDB header:**translation **Chain:** M: **PDB Molecule:**translation initiation factor 2 subunit beta; **PDBTitle:** crystal structure of the intact archaeal translation2 initiation factor 2 from sulfolobus solfataricus .
49	d1q4ga1	Alignment	not modelled	8.2	25	**Fold:**Heme-dependent peroxidases **Superfamily:**Heme-dependent peroxidases **Family:**Myeloperoxidase-like
50	c2dcuB_	Alignment	not modelled	8.1	54	**PDB header:**translation **Chain:** B: **PDB Molecule:**translation initiation factor 2 beta subunit; **PDBTitle:** crystal structure of translation initiation factor aif2betagamma2 heterodimer with gdp
51	c3gj7B_	Alignment	not modelled	8.1	100	**PDB header:**transport protein **Chain:** B: **PDB Molecule:**nuclear pore complex protein nup153; **PDBTitle:** crystal structure of human rangdp-nup153znf12 complex
52	c2i5oA_	Alignment	not modelled	8.1	100	**PDB header:**transferase **Chain:** A: **PDB Molecule:**dna polymerase eta; **PDBTitle:** solution structure of the ubiquitin-binding zinc finger2 (ubz) domain of the human dna y-polymerase eta
53	c3zrhA_	Alignment	not modelled	8.0	29	**PDB header:**hydrolase **Chain:** A: **PDB Molecule:**ubiquitin thioesterase zranb1; **PDBTitle:** crystal structure of the lys29, lys33-linkage-specific trabid otu2 deubiquitinase domain reveals an ankyrin-repeat ubiquitin binding3 domain (ankubd)
54	d2ggea1	Alignment	not modelled	8.0	100	**Fold:**Rubredoxin-like **Superfamily:**Ran binding protein zinc finger-like

55	c1neeA_	Alignment	not modelled	8.0	54	**PDB header:**translation **Chain:** A: **PDB Molecule:**probable translation initiation factor 2 beta **PDBTitle:** structure of archaeal translation factor aif2beta from2 methanobacterium thermoautrophicum
56	d1oz2a1	Alignment	not modelled	7.9	35	**Fold:**SH3-like barrel **Superfamily:**Tudor/PWWP/MBT **Family:**MBT repeat
57	d1nbaa_	Alignment	not modelled	7.4	11	**Fold:**Isochorismatase-like hydrolases **Superfamily:**Isochorismatase-like hydrolases **Family:**Isochorismatase-like hydrolases
58	c2d9sA_	Alignment	not modelled	7.2	33	**PDB header:**ligase **Chain:** A: **PDB Molecule:**cbl e3 ubiquitin protein ligase; **PDBTitle:** solution structure of rsgi ruh-049, a uba domain from mouse2 cdna
59	c1ht8B_	Alignment	not modelled	7.1	29	**PDB header:**oxidoreductase **Chain:** B: **PDB Molecule:**prostaglandin h2 synthase-1; **PDBTitle:** the 2.7 angstrom resolution model of ovine cox-1 complexed with2 alclofenac
60	c1pggB_	Alignment	not modelled	7.1	29	**PDB header:**oxidoreductase **Chain:** B: **PDB Molecule:**prostaglandin h2 synthase-1; **PDBTitle:** prostaglandin h2 synthase-1 complexed with 1-(4-iodobenzoyl)-5-2 methoxy-2-methylindole-3-acetic acid (iodoindomethacin), trans model
61	c3feoB_	Alignment	not modelled	7.1	33	**PDB header:**metal binding protein **Chain:** B: **PDB Molecule:**mbt domain-containing protein 1; **PDBTitle:** the crystal structure of mbtd1
62	c1fvyA_	Alignment	not modelled	7.1	58	**PDB header:**hormone/growth factor **Chain:** A: **PDB Molecule:**parathyroid hormone; **PDBTitle:** solution structure of the osteogenic 1-31 fragment of the2 human parathyroid hormone
63	c3gi8D_	Alignment	not modelled	7.1	67	**PDB header:**transport protein **Chain:** D: **PDB Molecule:**nuclear pore complex protein nup153; **PDBTitle:** crystal structure of human rangdp-nup153znf34 complex
64	c2wlvA_	Alignment	not modelled	7.1	18	**PDB header:**virus protein **Chain:** A: **PDB Molecule:**gag polyprotein; **PDBTitle:** structure of the n-terminal capsid domain of hiv-2
65	c3gi4B_	Alignment	not modelled	7.0	50	**PDB header:**transport protein **Chain:** B: **PDB Molecule:**nuclear pore complex protein nup153; **PDBTitle:** crystal structure of human rangdp-nup153znf3 complex
66	c2w0tA_	Alignment	not modelled	7.0	50	**PDB header:**transcription **Chain:** A: **PDB Molecule:**lethal(3)malignant brain tumor-like 2 protein; **PDBTitle:** solution structure of the fcs zinc finger domain of human2 lmbl2
67	c2luvA_	Alignment	not modelled	7.0	58	**PDB header:**cell adhesion **Chain:** A: **PDB Molecule:**integrin alpha-x; **PDBTitle:** structure and binding interface of the cytosolic tails of axb22 integrin
68	d1c8sa_	Alignment	not modelled	6.9	64	**Fold:**Family A G protein-coupled receptor-like **Superfamily:**Family A G protein-coupled receptor-like **Family:**Bacteriorhodopsin-like
69	c3am6C_	Alignment	not modelled	6.7	64	**PDB header:**transport protein **Chain:** C: **PDB Molecule:**rhodopsin-2; **PDBTitle:** crystal structure of the proton pumping rhodopsin ar2 from marine alga2 acetabularia acetabulum
70	c2jagA_	Alignment	not modelled	6.7	45	**PDB header:**membrane protein **Chain:** A: **PDB Molecule:**halorhodopsin; **PDBTitle:** l1-intermediate of halorhodopsin t203v
71	d1e12a_	Alignment	not modelled	6.7	45	**Fold:**Family A G protein-coupled receptor-like **Superfamily:**Family A G protein-coupled receptor-like **Family:**Bacteriorhodopsin-like
72	d1wgga_	Alignment	not modelled	6.7	26	**Fold:**beta-Grasp (ubiquitin-like) **Superfamily:**Ubiquitin-like **Family:**Ubiquitin-related
73	d2gqba1	Alignment	not modelled	6.7	25	**Fold:**RPA2825-like **Superfamily:**RPA2825-like **Family:**RPA2825-like
74	c3gioA_	Alignment	not modelled	6.7	38	**PDB header:**dna binding protein **Chain:** A: **PDB Molecule:**putative uncharacterized protein; **PDBTitle:** crystal structure of the tnf-alpha inducing protein (tip2 alpha) from helicobacter pylori
75	c3v9oA_	Alignment	not modelled	6.7	19	**PDB header:**lyase **Chain:** A: **PDB Molecule:**dihydroneopterin aldolase; **PDBTitle:** crystal structure of dihydroneopterin aldolase (bth_i0291) from2 burkholderia thailendensis bound to guanine.
76	c3tekA_	Alignment	not modelled	6.4	33	**PDB header:**dna binding protein **Chain:** A: **PDB Molecule:**thermodbp-single stranded dna binding protein; **PDBTitle:** thermodbp: a non-canonical single-stranded dna binding protein with a2 novel structure and mechanism
77	c2k25A_	Alignment	not modelled	6.3	22	**PDB header:**unknown function **Chain:** A: **PDB Molecule:**ubb; **PDBTitle:** automated nmr structure of the ubb by fapsy
78	d1m9dc_	Alignment	not modelled	6.3	28	**Fold:**Retrovirus capsid protein, N-terminal core domain **Superfamily:**Retrovirus capsid protein, N-terminal core domain **Family:**Retrovirus capsid protein, N-terminal core domain
79	c3ktbD_	Alignment	not modelled	6.2	29	**PDB header:**transcription regulator **Chain:** D: **PDB Molecule:**arsenical resistance operon trans-acting repressor; **PDBTitle:** crystal structure of arsenical resistance operon trans-acting2 repressor from bacteroides vulgatus atcc 8482
						PDB header:oxidoreductase/oxidoreductase inhibitor

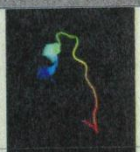
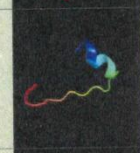
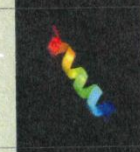
80	c3tq8A_	Alignment	not modelled	4.4	14	**Chain:** A: **PDB Molecule:**dihydrofolate reductase; **PDBTitle:** structure of the dihydrofolate reductase (fola) from coxiella burnetii2 in complex with trimethoprim
81	d1tgxa_	Alignment	not modelled	4.3	37	**Fold:**Snake toxin-like **Superfamily:**Snake toxin-like **Family:**Snake venom toxins
82	d1qlma_	Alignment	not modelled	4.2	29	**Fold:**Methenyltetrahydromethanopterin cyclohydrolase **Superfamily:**Methenyltetrahydromethanopterin cyclohydrolase **Family:**Methenyltetrahydromethanopterin cyclohydrolase
83	c3a7kD_	Alignment	not modelled	4.2	45	**PDB header:**membrane protein **Chain:** D: **PDB Molecule:**halorhodopsin; **PDBTitle:** crystal structure of halorhodopsin from natronomonas2 pharaonis
84	d2pxrc1	Alignment	not modelled	4.1	24	**Fold:**Retrovirus capsid protein, N-terminal core domain **Superfamily:**Retrovirus capsid protein, N-terminal core domain **Family:**Retrovirus capsid protein, N-terminal core domain
85	c2e9hA_	Alignment	not modelled	4.0	23	**PDB header:**translation **Chain:** A: **PDB Molecule:**eukaryotic translation initiation factor 5; **PDBTitle:** solution structure of the eif-5_eif-2b domain from human2 eukaryotic translation initiation factor 5
86	c1u4fD_	Alignment	not modelled	4.0	31	**PDB header:**allergen **Chain:** D: **PDB Molecule:**inward rectifier potassium channel 2; **PDBTitle:** crystal structure of cytoplasmic domains of irk1 (kir2.1)2 channel
87	d2odqc1	Alignment	not modelled	4.0	38	**Fold:**LEM/SAP HeH motif **Superfamily:**LEM domain **Family:**LEM domain
88	c3ch5B_	Alignment	not modelled	3.9	100	**PDB header:**transport protein **Chain:** B: **PDB Molecule:**fragment of nuclear pore complex protein nup153; **PDBTitle:** the crystal structure of the rangdp-nup153znf2 complex
89	d1b8qa_	Alignment	not modelled	3.9	11	**Fold:**PDZ domain-like **Superfamily:**PDZ domain-like **Family:**PDZ domain
90	c2do6A_	Alignment	not modelled	3.8	33	**PDB header:**ligase **Chain:** A: **PDB Molecule:**e3 ubiquitin-protein ligase cbl-b; **PDBTitle:** solution structure of rsgi ruh-065, a uba domain from human2 cdna
91	d1a0ea_	Alignment	not modelled	3.7	17	**Fold:**TIM beta/alpha-barrel **Superfamily:**Xylose isomerase-like **Family:**Xylose isomerase
92	d1u5ta2	Alignment	not modelled	3.7	31	**Fold:**DNA/RNA-binding 3-helical bundle **Superfamily:**"Winged helix" DNA-binding domain **Family:**Vacuolar sorting protein domain
93	c2l8eA_	Alignment	not modelled	3.7	75	**PDB header:**dna binding protein **Chain:** A: **PDB Molecule:**polyhomeotic-like protein 1; **PDBTitle:** solution nmr structure of fcs domain of human polyhomeotic homolog 12 (hph1)
94	c2qixC_	Alignment	not modelled	3.7	35	**PDB header:**metal transport **Chain:** C: **PDB Molecule:**inward rectifier potassium channel 2; **PDBTitle:** cytoplasmic domain structure of kir2.1 containing2 andersen's mutation r218q and rescue mutation t309k
95	d1kxia_	Alignment	not modelled	3.6	29	**Fold:**Snake toxin-like **Superfamily:**Snake toxin-like **Family:**Snake venom toxins
96	c3anwB_	Alignment	not modelled	3.5	19	**PDB header:**replication **Chain:** B: **PDB Molecule:**putative uncharacterized protein; **PDBTitle:** a protein complex essential initiation of dna replication
97	d1ig8a2	Alignment	not modelled	3.5	30	**Fold:**Ribonuclease H-like motif **Superfamily:**Actin-like ATPase domain **Family:**Hexokinase
98	d1yaca_	Alignment	not modelled	3.4	14	**Fold:**Isochorismatase-like hydrolases **Superfamily:**Isochorismatase-like hydrolases **Family:**Isochorismatase-like hydrolases
99	d1smye_	Alignment	not modelled	3.4	60	**Fold:**RPB6/omega subunit-like **Superfamily:**RPB6/omega subunit-like **Family:**RNA polymerase omega subunit

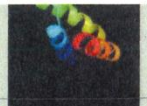
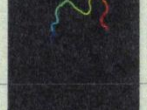
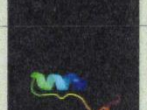
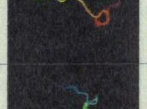
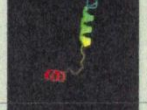
Analysis of possible tertiary structure of KHV ORF 53

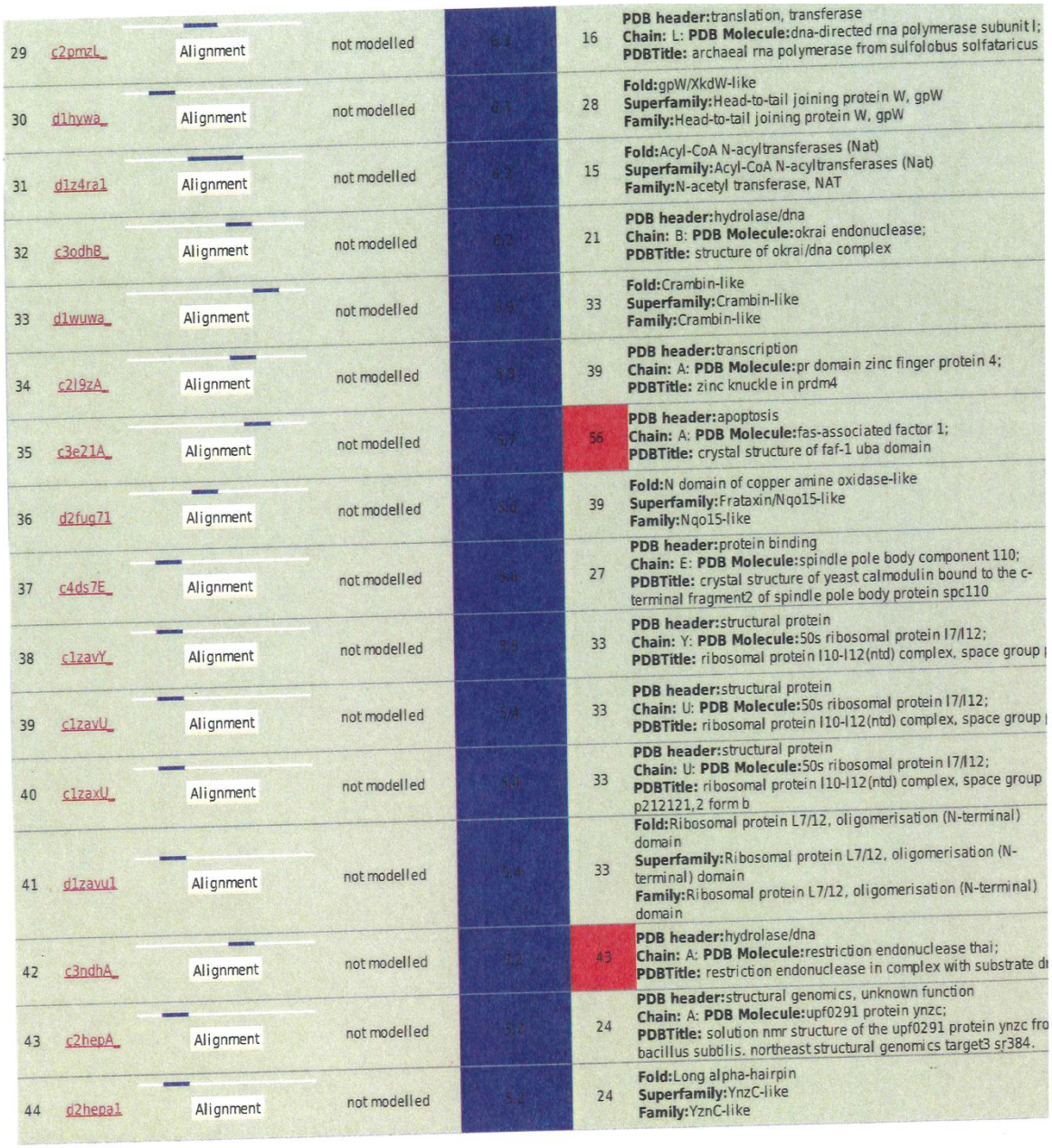
Phyre2

Email	rsd76@istm.keele.ac.uk
Description	orf74___
Date	Sun Jul 15 16:20:49 BST 2012
Unique Job ID	41c20f72a8d48071

Detailed template information

#	Template	Alignment Coverage	3D Model	Confidence	% i.d.	Template Information
1	d1ftra1	Alignment		66.3	26	**Fold:**Ferredoxin-like **Superfamily:**Formylmethanofuran:tetrahydromethanopterin formyltransferase **Family:**Formylmethanofuran:tetrahydromethanopterin formyltransferase
2	d1iyjb1	Alignment		57.3	24	**Fold:**BRCA2 helical domain **Superfamily:**BRCA2 helical domain **Family:**BRCA2 helical domain
3	d1m5sa1	Alignment		57.1	21	**Fold:**Ferredoxin-like **Superfamily:**Formylmethanofuran:tetrahydromethanopterin formyltransferase **Family:**Formylmethanofuran:tetrahydromethanopterin formyltransferase
4	c2fhjD_	Alignment		55.8	28	**PDB header:**transferase **Chain:** D: **PDB Molecule:**formylmethanofuran--tetrahydromethanopterin **PDBTitle:** crystal structure of formylmethanofuran:2 tetrahydromethanopterin formyltransferase in complex with3 its coenzymes
5	d1m5ha1	Alignment		53.3	16	**Fold:**Ferredoxin-like **Superfamily:**Formylmethanofuran:tetrahydromethanopterin formyltransferase **Family:**Formylmethanofuran:tetrahydromethanopterin formyltransferase
6	c1m5sC_	Alignment		47.6	21	**PDB header:**transferase **Chain:** C: **PDB Molecule:**formylmethanofuran--tetrahydromethanopterin **PDBTitle:** formylmethanofuran:tetrahydromethanopterin2 fromyltransferase from methanosarcina barkeri
7	c1m5hF_	Alignment		43.8	16	**PDB header:**transferase **Chain:** F: **PDB Molecule:**formylmethanofuran--tetrahydromethanopterin **PDBTitle:** formylmethanofuran:tetrahydromethanopterin2 formyltransferase from archaeoglobus fulgidus
8	d1miua1	Alignment		27.1	25	**Fold:**BRCA2 helical domain **Superfamily:**BRCA2 helical domain **Family:**BRCA2 helical domain
9	c3ajbB_	Alignment		19.4	47	**PDB header:**protein transport **Chain:** B: **PDB Molecule:**peroxisomal biogenesis factor 19; **PDBTitle:** crystal structure of human pex3p in complex with n-terminal pex19p2 peptide
10	c3mk4B_	Alignment		16.9	73	**PDB header:**protein transport **Chain:** B: **PDB Molecule:**peroxisomal biogenesis factor 19; **PDBTitle:** x-ray structure of human pex3 in complex with a pex19 derived peptide
11	c1iyjB_	Alignment		16.3	25	**PDB header:**gene regulation/antitumor protein **Chain:** B: **PDB Molecule:**breast cancer susceptibility; **PDBTitle:** structure of a brca2-dss1 complex

12	c3qsvD_	Alignment		14.9	20	**Chain:** D: **PDB Molecule:**mothers against decapentaplegic homolog 4; **PDBTitle:** structural basis for dna recognition by constitutive smad4 mh1 dimers
13	d1vk0a_	Alignment		14.3	10	**Fold:**Ribonuclease H-like motif **Superfamily:**Ribonuclease H-like **Family:**DnaQ-like 3'-5' exonuclease
14	d1cfea_	Alignment		11.6	35	**Fold:**PR-1-like **Superfamily:**PR-1-like **Family:**PR-1-like
15	d2ieaa3	Alignment		11.0	33	**Fold:**TK C-terminal domain-like **Superfamily:**TK C-terminal domain-like **Family:**Transketolase C-terminal domain-like
16	c2z5bB_	Alignment		10.9	27	**PDB header:**chaperone **Chain:** B: **PDB Molecule:**uncharacterized protein ylr021w; **PDBTitle:** crystal structure of a novel chaperone complex for yeast2 20s proteasome assembly
17	d1rp0a1	Alignment		10.3	14	**Fold:**FAD/NAD(P)-binding domain **Superfamily:**FAD/NAD(P)-binding domain **Family:**Thi4-like
18	c1lq7A_	Alignment		10.1	39	**PDB header:**de novo protein **Chain:** A: **PDB Molecule:**alpha3w; **PDBTitle:** de novo designed protein model of radical enzymes
19	d2a1ja1	Alignment		9.6	19	**Fold:**SAM domain-like **Superfamily:**RuvA domain 2-like **Family:**Hef domain-like
20	d2aq0a1	Alignment		9.4	18	**Fold:**SAM domain-like **Superfamily:**RuvA domain 2-like **Family:**Hef domain-like
21	c3d35A_	Alignment	not modelled	8.9	39	**PDB header:**transferase **Chain:** A: **PDB Molecule:**regulator of ty1 transposition protein 109; **PDBTitle:** crystal structure of rtt109-ac-coa complex
22	c1n20A_	Alignment	not modelled	7.6	14	**PDB header:**isomerase **Chain:** A: **PDB Molecule:**(+)-bornyl diphosphate synthase; **PDBTitle:** (+)-bornyl diphosphate synthase: complex with mg an 3-aza-2 2,3-dihydrogeranyl diphosphate
23	d1pbya5	Alignment	not modelled	6.9	57	**Fold:**Streptavidin-like **Superfamily:**Quinohemoprotein amine dehydrogenase A chain, domain 3 **Family:**Quinohemoprotein amine dehydrogenase A chain, domai
24	c3h4cA_	Alignment	not modelled	6.9	67	**PDB header:**transcription **Chain:** A: **PDB Molecule:**transcription factor tfiib-like; **PDBTitle:** structure of the c-terminal domain of transcription fac iib from2 trypanosoma brucei
25	d1dd3a1	Alignment	not modelled	6.2	38	**Fold:**Ribosomal protein L7/12, oligomerisation (N-terminal) domain **Superfamily:**Ribosomal protein L7/12, oligomerisation (N-terminal) domain **Family:**Ribosomal protein L7/12, oligomerisation (N-terminal) domain
26	c1zawY_	Alignment	not modelled	6.2	38	**PDB header:**structural protein **Chain:** Y: **PDB Molecule:**50s ribosomal protein l7/l12; **PDBTitle:** ribosomal protein l10-l12(ntd) complex, space group p212121,2 form a
27	c1zawX_	Alignment	not modelled	6.2	38	**PDB header:**structural protein **Chain:** X: **PDB Molecule:**50s ribosomal protein l7/l12; **PDBTitle:** ribosomal protein l10-l12(ntd) complex, space group p212121,2 form a
28	c2gezE_	Alignment	not modelled	6.1	18	**PDB header:**hydrolase **Chain:** E: **PDB Molecule:**l-asparaginase alpha subunit; **PDBTitle:** crystal structure of potassium-independent plant asparaginase



29	c2pmzL_	Alignment	not modelled	6.1	16	**PDB header:**translation, transferase **Chain:** L: **PDB Molecule:**dna-directed rna polymerase subunit l; **PDBTitle:** archaeal rna polymerase from sulfolobus solfataricus
30	d1hywa_	Alignment	not modelled	6.1	28	**Fold:**gpW/XkdW-like **Superfamily:**Head-to-tail joining protein W, gpW **Family:**Head-to-tail joining protein W, gpW
31	d1z4ra1	Alignment	not modelled	6.2	15	**Fold:**Acyl-CoA N-acyltransferases (Nat) **Superfamily:**Acyl-CoA N-acyltransferases (Nat) **Family:**N-acetyl transferase, NAT
32	c3odhB_	Alignment	not modelled	6.2	21	**PDB header:**hydrolase/dna **Chain:** B: **PDB Molecule:**okrai endonuclease; **PDBTitle:** structure of okrai/dna complex
33	d1wuwa_	Alignment	not modelled	5.9	33	**Fold:**Crambin-like **Superfamily:**Crambin-like **Family:**Crambin-like
34	c2l9zA_	Alignment	not modelled	5.8	39	**PDB header:**transcription **Chain:** A: **PDB Molecule:**pr domain zinc finger protein 4; **PDBTitle:** zinc knuckle in prdm4
35	c3e21A_	Alignment	not modelled	5.7	56	**PDB header:**apoptosis **Chain:** A: **PDB Molecule:**fas-associated factor 1; **PDBTitle:** crystal structure of faf-1 uba domain
36	d2fug71	Alignment	not modelled	5.6	39	**Fold:**N domain of copper amine oxidase-like **Superfamily:**Frataxin/Nqo15-like **Family:**Nqo15-like
37	c4ds7E_	Alignment	not modelled	5.6	27	**PDB header:**protein binding **Chain:** E: **PDB Molecule:**spindle pole body component 110; **PDBTitle:** crystal structure of yeast calmodulin bound to the c-terminal fragment2 of spindle pole body protein spc110
38	c1zavY_	Alignment	not modelled	5.5	33	**PDB header:**structural protein **Chain:** Y: **PDB Molecule:**50s ribosomal protein l7/l12; **PDBTitle:** ribosomal protein l10-l12(ntd) complex, space group
39	c1zavU_	Alignment	not modelled	5.4	33	**PDB header:**structural protein **Chain:** U: **PDB Molecule:**50s ribosomal protein l7/l12; **PDBTitle:** ribosomal protein l10-l12(ntd) complex, space group
40	c1zaxU_	Alignment	not modelled	5.4	33	**PDB header:**structural protein **Chain:** U: **PDB Molecule:**50s ribosomal protein l7/l12; **PDBTitle:** ribosomal protein l10-l12(ntd) complex, space group p212121,2 form b
41	d1zavu1	Alignment	not modelled	5.4	33	**Fold:**Ribosomal protein L7/12, oligomerisation (N-terminal) domain **Superfamily:**Ribosomal protein L7/12, oligomerisation (N-terminal) domain **Family:**Ribosomal protein L7/12, oligomerisation (N-terminal) domain
42	c3ndhA_	Alignment	not modelled	5.2	43	**PDB header:**hydrolase/dna **Chain:** A: **PDB Molecule:**restriction endonuclease thai; **PDBTitle:** restriction endonuclease in complex with substrate d
43	c2hepA_	Alignment	not modelled	5.2	24	**PDB header:**structural genomics, unknown function **Chain:** A: **PDB Molecule:**upf0291 protein ynzc; **PDBTitle:** solution nmr structure of the upf0291 protein ynzc fro bacillus subtilis. northeast structural genomics target3 sr384.
44	d2hepa1	Alignment	not modelled	5.2	24	**Fold:**Long alpha-hairpin **Superfamily:**YnzC-like **Family:**YznC-like