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Cell-pathogen interactions in common carp (*Cyprinus carpio* L.):
Studies on cell membranes and neutrophil responses

THESIS

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To my parents
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The effect of β-glucan on formation and functionality of neutrophil extracellular traps in carp (Cyprinus carpio L.). To be submitted


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Cholesterol-depletion in human blood-derived neutrophils by methyl-β-cyclodextrin leads to formation of neutrophil extracellular traps. To be submitted

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Isolation and analysis of membrane lipids and lipid rafts in common carp (Cyprinus carpio L.). Submitted

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The Cyprinid herpesvirus-3 (CyHV-3) uses lipid rafts as a mode of entry into carp cells. Submitted
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Chapter 1

Introduction
1 Introduction
The growing worldwide demand for protein has led to a boom in the aquaculture industry over the last few decades. The rapid expansion of aquaculture has simultaneously led to a rise in challenges including disease outbreaks. In common carp (Cyprinus carpio L.) aquaculture, bacterial and viral infections can be controlled with antibiotics or vaccines respectively, however a reduction in the occurrence of infections is more preferable due to the potential for antibiotic resistance developing and the associated costs. To reduce the frequency and severity of disease outbreaks, more research needs to be conducted in the basic biology of microbial infections in carp. Since host-pathogen interactions at the cellular level are critical in determining the course of an infection, a more detailed understanding of the mechanisms regulating carp-pathogen interactions can aid in vaccine development, characterisation of new therapeutic targets or identification of prophylactic strategies e.g. by using immunomodulating feed additives. Therefore, the aim of this thesis was to investigate the effect of the feed additive β-glucan on cell-pathogen interaction in carp by focusing on neutrophil responses as the first line of defence against infections and on the cellular membrane as central signalling platform of a host cell. To investigate bacteria-neutrophil interactions in carp, a pathogenic strain of the Gram-negative bacterium Aeromonas hydrophila was used as a prototype infection model, which has been shown to cause hemorrhagic septicaemia and skin ulcers in carp. Furthermore, the herpes virus, CyHV-3 was used as an additional virus infection model to study host-viral interactions in carp due to its economic importance in carp aquaculture where mortality can reach 100% in CyHV-3-infected populations.

1.1 Aquaculture
As the world’s population grows exponentially, so does our demand for reliable, sustainable sources of protein. Catching popular wild fish species is seen as unsustainable and has led to population crashes in large numbers of species such as Atlantic cod (Gadus morhua), witch flounder (Glyptocephalus cynoglossus), redfish (Sebastes fasciatus), Atlantic halibut (Hippoglossus hippoglossus) and haddock (Melanogrammus aeglefinus) (FAO 2012). Therefore, aquaculture is seen as one of the answers to provide a sustainable and reliable source of protein to an ever growing population. Out of the top 5 farmed fish species worldwide four of them belong to the cyprinid family; Silver carp (Hypophthalmichthys molitrix), Indian carp (Labeo rohita), grass carp (Ctenopharyngodon idella) and the common
carp (*Cyprinus carpio*), with the fifth species being tilapia (*Oreochromis niloticus*) (FAO 2012). In 2010, 71.9% (24.2 million tonnes) of freshwater fishes produced by aquaculture were species of carp. Of these carp varieties, the common carp, which is cultured across Asia and Central Eastern Europe, is both economically and socially important. Furthermore coloured koi carp varieties are kept as pets worldwide and are often worth several thousand euros.

![Figure 1](image.png)

Figure 1. The production of common carp has grown almost exponentially over the last 60 years (FAO 2012).

Common carp are farmed in semi-intensive ponds and production has grown rapidly over the last 60 years, including a tripling of production in the last 20 years from below 1.1 million T to over 3.7 million T. Most of this rapid growth came from China, whereas Europe has seen sluggish growth in the last 20 years. As production levels and transport of carp (and other fish species) increases, so do challenges including sourcing alternative feed ingredients and disease outbreaks, which cost millions of US dollars annually (FAO 2010). To reduce the occurrence and severity of disease outbreaks, higher value fish could be vaccinated or treated with antibiotics, however vaccines are not yet available for several fish pathogens and the large scale use of antibiotics in aquaculture is prohibited due to the strong possibility of bacteria gaining resistance to the particular antibiotic (DEFOIRDT et al. 2011).

To reduce the dependence on antibiotics, more and more research is being conducted into the possible use of immunomodulants as a method of reducing the frequency and severity of disease outbreaks, increasing growth rates and consequently profit for the fish farmer.
1.2. Viral and bacterial challenges affecting carp aquaculture

One of the main challenges facing aquaculture is the losses associated with pathogens such as parasites, bacteria and viruses (FAO 2010). Bacterial and viral infections are responsible for reducing growth rates of fish through energy used by the immune system to fight the infection, loss of appetite and even death of the fish, both of which will lead to monetary losses for the farmer. Depending on the country, some viruses such as Cyprinid Herpesvirus 3 (CyHV-3) are listed as notifiable diseases, which can potentially lead to the killing of all the fish on the farm and severe losses incurred to the farmer.

In England alone, 24 separate cases of CyHV-3 have been detected since 2010 (www.defra.gov.uk/aahm/disease/khv) and CyHV-3 has been listed as one of the major threats to aquaculture (FAO 2010). As well as viral infections, bacterial infections also cause a severe burden on aquaculture. In common carp aquaculture, *Aeromonas hydrophila* has been associated with primary and secondary (SIWICKI et al. 2012) infections leading to skin damage and red open sores on the surface of the skin, rendering the effected fish unsellable (HEDRICK et al. 2000).

1.2.1 CyHV-3

Cyprinid Herpesvirus 3 (CyHV-3), also known as koi herpesvirus (KHV), is responsible for causing koi herpesvirus disease (KHVD), which can result in a mortality of up to 100% in common and koi carp (*Cyprinus carpio*) populations. The virus was first identified in the late 1990’s, and through the trade of latently infected, outwardly healthy looking carp, the virus has spread rapidly worldwide (CHENG et al. 2011), with the virus now detected on a yearly basis in Europe, Asia and America, with Australasia remaining the only clear inhabited continent (NEUKIRCH et al. 1999; HEDRICK et al. 2000; PERELBERG et al. 2003; HAENEN et al. 2004). The importance of the virus and the impact it has had on carp aquaculture has led to the Food and Agriculture Organisation to include the virus as a threat to carp aquaculture and food safety resources. Therefore CyHV-3 was included on the list of notifiable diseases (FAO 2010) in both the UK and Germany (http://www.defra.gov.uk/aahm/forms/guidelines-list/, www.fli.bun.de).

The virus is responsible for causing KHVD (koi herpesvirus disease), with common symptoms including apathy, skin discolouration, gathering at the water inlet, rough texture of the skin, gill necrosis and sunken eyes (HEDRICK et al. 2000).
CyHV-3 has recently been classified as being a member of the *Alloherpesviridae* family in the *Herpesvirales* order. The family is comprised of both piscine and amphibian herpesviruses and is evolutionary distinct from the other families of the order Herpesvirales (DAVISON et al. 2009; WALTZEK et al. 2009; HANSON et al. 2011). Members of the *Alloherpesviridae* family are being increasingly recognised as pathogens in aquaculture. One important pathogen is the Cyprinid herpesvirus-3 (CyHV-3), a novel herpesvirus from the *Cyprinivirus* genus which infects common carp, *Cyprinus carpio* and its coloured variety, the koi (WALTZEK et al. 2009). CyHV-3 infections may cause severe outbreaks of the so called koi herpesvirus disease (KHVD) leading to up to 100% mortalities in infected populations, which consequently causes a severe negative impact on carp aquaculture and koi ornamental trade. In surviving carp, the virus has been shown to have a latency phase, where it has been publicised to reside in leukocytes at low copy numbers (EIDE et al. 2011). From the latency phase, the virus can be reactivated under stress situations such as netting or transportation (BERGMANN u. KEMPTER 2011) and then later released into ponds containing previously uninfected populations.

The virus has been characterised as consisting of a large 295 kbp long linear genome with 156 potential ORFs (AOKI et al. 2007), which encode for at least 40 proteins building the mature viron (MICHEL et al. 2010). The viral envelope has been shown in Herpesviruses to be required for the critical steps of fusion and fission to the host’s cell (METTENLEITER 2002). The CyHV-3 virion has an icosahedral capsid, an amorphic protein tegument and a lipid envelope containing virus glycoproteins (DISHON et al. 2005; HUTORAN et al. 2005) which it acquires during the budding step from infected cells. Studies investigating the assembly of CyHV-3 in infected cells have shown that nucleocapsids appear to bud from the inner nuclear membrane into the perinuclear space. The budding and transport process of CyHV-3 follows a similar mechanism observed for mammalian herpesviruses (METTENLEITER 2002). As in mammalian herpesviruses, the primary envelope is lost as the nucleocapsids cross the outer nuclear membrane into the cytoplasm and a second lipid envelope is acquired through budding into cytoplasmic vesicles (MIWA et al. 2007; HANSON et al. 2011).

Herpesvirus replication has been studied extensively in mammals, however at the time of writing no research has been published studying aquatic herpesviruses. Human herpesviruses have been shown to bind to the plasma membrane and require cholesterol
rich lipid raft microdomains (BENDER et al. 2003b; G. E. LEE et al. 2003a). The virus is then pulled by dynein along tubulin microtubules towards the nucleus (DOHNER et al. 2002) where RNA polymerase II is phosphorylated and the virus DNA can be replicated (LONG et al. 1999).

Therefore, as lipid rafts have been implicated in the replication cycle of mammalian herpesviruses, we wanted to investigate if the role of lipid rafts in the replication cycle of CyHV-3 in carp cells has been preserved in piscine herpesviruses. The data is presented in chapter 5.

1.2.2 Aeromonas hydrophila

*Aeromonas hydrophila* is a Gram-negative rod shaped bacterium which can replicate at a wide range of temperature from as low as 4°C in fish to 37°C in mammals. *A. hydrophila* is ubiquitous and commonly found in water bodies and depending on the strain can be present in fish without leading to an infection (VAN IMPE 1977) and some strains also play an important role in aquaculture as natural biofilters (KOMPANETS et al. 1992). However *A. hydrophila* has also been attributed to causing enterotoxin associated morbidity in fish (BOULANGER et al. 1977), and has also been shown to have zoonotic potential being able to infect humans leading to open wounds and diarrhoea (HUYS et al. 2002). *A. hydrophila* has been shown to contain several virulence factors including: aerolysin, cytotoxic enterotoxin, cytotoxic enterotoxin, temperature-sensitive protease and serine protease (HU et al. 2012).

In fish, aeromonands have been shown to cause haemorrhagic septicaemia and skin ulcers (KOZINSKA u. PEKALA 2012). Isolated *A. hydrophila* has been shown to account for most than 50 % of the isolated stains of aeromonands in crucian carp (*Carassius carassius*) showing symptoms of haemorrhagic septicaemia (NIELSEN et al. 2001).

Healthy carp showing no symptoms of disease also contain a variety of pathogenic *Aeromonas* subspecies present in the kidney, gills and skin, which were shown to have haemolytic and proteolytic activity in challenge tests performed in healthy carp (KOZINSKA 2007). Furthermore, Hedrick and Siwicki (HEDRICK et al. 2000; SIWICKI et al. 2012) also showed that during CyHV-3 infection, carp and koi frequently showed signs of a secondary bacterial infection from *A. hydrophila, A. sobria, Flavobacterium* sp and *Pseudomonas* sp. These results suggest that pathogenic strains of *Aeromonas hydrophila* can cause secondary
infections as a result of stresses such as poor water quality, feed quality or primary infections from CyHV-3, which will lead to the skin or gut barrier being compromised, allowing the bacterium to pass through this physical barrier (SYAKURI et al. 2013a; SYAKURI et al. 2013c).

1.3 Immunomodulators and beta glucan in aquaculture
One of the main limiting factors in the sustainable production of fish in the aquaculture industry is the occurrence of infectious diseases e.g. infections by A. hydrophila and CyHV-3 as described above. A successful way of limiting the frequency and severity of disease is through the inclusion of immomodulaters into the feed. There are a large number of feed additives from a broad range of sources currently used in aquaculture. One of the most successful and commonly used feed additives is β-glucan (RAA 1996; DALMO u. BOGWALD 2008). Beta glucan was first shown to have immunostimulatory properties in mammals by increasing the resistance to infectious pathogens (LUZIO 1985). However, recently attention has turned to the effects of β-glucan on fish in aquaculture, where studies have shown that it is beneficial against pathogens (SELVARAJ et al. 2005), growth rates (KUHLWEIN et al. 2013b) and as a vaccine adjuvant (SKOV et al. 2012).

Beta glucans are naturally occurring polysaccharides and are important components of a wide range of plant cell walls (oat, wheat, rye and barley), yeast (baker’s and brewer’s yeast) and fungi (TOKUNAKA et al. 2000; ZECHNER-KRPAN et al. 2010). β-glucans consist of D-glucose monomers linked by β-glycosidic bonds. The glucose hexagons can be joined to each other in a variety of ways giving rise to β-1,3-1,6 and β-1,3-1,4 glycosidic bonds.

The form of β-glucan used throughout this thesis is yeast (Saccharomyces cerevisiae) derived β-1,3-1,6 glucan in the form of the feed additive MacroGard® (Biorigin, Norway), which contains a minimum of 60 % purified β-1,3-1,6 glucan. MacroGard® has been used in salmon and trout aquaculture for over 15 years and is also incorporated into livestock and pet feed (www.Biorigin.net).

β-glucan was first used in Atlantic salmon (Salmo Salar) aquaculture, where yeast derived β 1,3-1,6 glucan was used (RORSTAD et al. 1993) and it is currently added to feeds for a wide range of cultured species, including; black tiger shrimp (Penaeus monodon), common carp (Cyprinus carpio), Nile tilapia (Oreochromis labrax).
In mammalian cells, β-glucan is detected by immune cells by Dectin-1 and Toll Like Receptor 2 (TLR 2) receptors and it can be up-taken and transported through the gut via M cells (GANTNER et al. 2003). However, at the time of writing, dectin-1 has not been found in fish, but β-glucan has been shown to bind to TLR receptors, but also non-TLR receptors (PIETRETTI et al. 2013b). Furthermore, conclusive evidence of M cells in fish is also lacking, however, the presence of immature M-like cells has been found in a salmonids (FUGLEM et al. 2010). However, further work needs to be conducted to determine the receptors responsible for β-glucan binding and if and how β-glucan can pass through the gut epithelial membrane.

The effect of feeding yeast derived β-glucan to common carp was investigated by Kühlwein et al. (KUHLWEIN et al. 2013b). Mirror carp (Cyprinus carpio) fed a diet supplemented with 1 or 2 % MacroGard® showed a significant increase in the growth rate and feed conversion rate compared to fish fed a diet without β-glucan. Furthermore, in another study Kühlwein et al (KUHLWEIN et al. 2013a) noted that intraepithelial leucocytes numbers increased.

The inclusion of β-glucan in feed has also been shown to lower the blood cholesterol level in mammals by preventing the absorption of cholesterol from food in the gut (RAHAR et al. 2011). Human patients receiving 3 g doses of oat derived beta glucan per day showed lower levels of blood cholesterol (OTHMAN et al. 2011). This and similar studies show how feed additives can lower the cholesterol reservoir, from which cells can uptake cholesterol via an LDL receptor (BROWN u. GOLDSTEIN 1986). At the time of writing, no similar studies have been conducted in fish, however cholesterol has been shown to be present in the cell membrane, with high concentrations in lipid rafts (ZEHER u. HAZEL 2003) and that these cholesterol rich membrane regions are functionally active in fish (GARCIA-GARCIA et al. 2012).

Apart from increasing growth rates and improving the blood parameters of the organism by reducing cholesterol levels, a relatively large amount of research has been conducted into the beneficial effects of β-glucan on the immune system of fish. Isolated carp macrophages treated with MacroGard® showed increased levels of oxidative burst and nitric oxide production (PIETRETTI et al. 2013a). In isolated head kidney cell, MacroGard® has also been shown to Increase ROS production (VERA-JIMENEZ u. NIELSEN 2013). Furthermore, carp fed
a diet enriched in 1% MacroGard® induced higher levels of C-reactive protein after *Aeromonas salmonicida* infection Pionnier N *et al.* (PIONNIER et al. 2013).

However, in some instances, an inflammatory response in the gut could lead to enteritis and therefore be negative for the fish (URAN et al. 2008). Falco *et al.* (FALCO et al. 2012) showed that fish fed a β-glucan supplemented diet and intraperitoneally infected with *Aeromonas salmonicida* significantly down-regulated a range of pro-inflammatory cytokines in the gut including IL 1 beta and IL 6. Importantly, levels of TNF α1 and α2 were significantly higher, which also correlated with lower levels of specific antibodies, indicating that the feeding of β-glucan both reduced infection levels and gut inflammation (FALCO et al. 2012). In contrast, Atlantic cod (*Gadus morhua*) fed a diet of β-glucan and infected with *Vibrio anguillarum* also showed a modulation in the expression of pro- and anti-inflammatory cytokines in the rectum. Cod fed a diet enriched in β-glucan and infected with *V. anguillarum* showed an increase in the expression levels of the pro-inflammatory cytokines il1b and il8 increase, and a decrease in the anti-inflammatory il10 compared to non-β-glucan fed fish (LOKESH et al. 2012). The results presented in carp under an *Aeromonas* infection (FALCO et al. 2012; SYAKURI et al. 2013b) and Atlantic cod under a *Vibrio anguillarum* infection, show that the effects of β-glucan on host-pathogen interactions in the gut are not fully understood, as differences between fish species and bacteria species have been shown.

As well as β-glucan modulating the expression of pro- and anti-inflammatory genes, β-glucan has also been shown to influence the quantity of immune related cell types. The supplementation of β-glucan to red snapper (*Lutjanus guttatus*) diet resulted in an increase in the percentage of monocytes and neutrophils in the peripheral blood. Furthermore, the associated functions, respiratory burst and nitric oxide release, were also increased (DEL RIO-ZARAGOZA et al. 2011). However in carp, Kühlwein et al. (KUHLWEIN et al. 2013b) showed that feeding β-glucan increased the percentage of monocytes, however, granulocytes and monocytes remained unaltered in the peripheral blood.

Furthermore, injecting zebrafish with immunomodulators such as β-glucan can enhance the immune response against *Aeromonas hydrophila*. Rodriguez and colleagues showed that zebrafish injected first with β-glucan and 2, 4 or 6 days later with *A. hydrophila* showed decreased mortality, which was linked to an increase in the number of neutrophils in the kidney (RODRIGUEZ et al. 2009). Here in this study, the effect of beta glucan on carp immune
system, with special focus on neutrophil extracellular traps and their role during an infection with *Aeromonas hydrophila* was investigated. We showed in chapter 5 that β-glucan is able to protect DNA-protein based NETs against *A. hydrophila* associated nuclease. Additionally, in chapter 7 we showed that NETs are able to entrap, but not kill *A. hydrophila*.

### 1.4 The fish immune system
The fish immune system is tasked with protecting the organism against a wide range of pathogens. The fish immune system, as in mammals, is comprised of an innate and an acquired component. An effective immune response must be able to rapidly determine self from non-self and induce a proportional response to the pathogen. The first line of defence consists of the surface barrier which is in constant contact with the environment. In fish, the barrier consists of the skin, gills and gut, which are coated in a layer of mucus containing antimicrobial peptides (MAREL et al. 2012).

However, once the physical barrier is breached the pathogen must first be identified by pattern recognition receptors (PRRs). PRRs recognise pathogen associated molecular patterns (PAMPs) and damage associated molecular patterns (DAMPs) and are located either on the membrane or in the cytoplasm of the cell (BIANCHI 2007). There is a wide range of receptors present in fish and more are being discovered. However, there are large differences between mammalian and fish and between fish species (PALTI 2011). There are currently 21 Toll like receptors (TLRs) identified in fish, with 7 unique to teleosts (TLR 5s, 14, 19, 20, 21, 22 and 23). Each receptor is responsible for detecting a specific group of pathogens, for instance mammalian TLR 4 has been shown to bind to ligands such as LPS found on bacteria such as *Aeromonas hydrophila* in rare minnow (*Gobiocypris rarus*) (SU et al. 2009; AOKI et al. 2013). TLR 3 and TLR 9 have been described in carp and have been shown to detect virus associated dsRNA and dsDNA respectively in teleosts (YANG u. SU 2010; AOKI et al. 2013).

Once a potential pathogen has been identified, specific cells and organs are tasked with inducing a specific response. In mammals, antigens will be presented in the lymphoid tissues and new immune competent cells are produced in the bone marrow. However, as fish lack bone marrow, the head kidney (also known as pronephros), trunk kidney and the thymus are the main sites of pluripotent stem cell production (ZAPATA et al. 2006).
The pronephros plays a major role in the acquired immune system, where macrophages phagocytose antigens and thus plays an important role in immunological memory (URIBE et al. 2011). Similarly the thymus also plays an important role and has been described as a site for T cell production (URIBE et al. 2011).

Fish also have an active and important acquired immune system. Antibody producing B cells are produced in the kidney (URIBE et al. 2011) and thus fish can utilise antibodies in their immune repertoire against pathogens. Natural antibodies are present in significant concentrations in teleost plasma and IgM and IgD immunoglobulins. Recently IgT has been identified in teleost mucus (ZHANG et al. 2010).

1.4.1 Innate immune system

The teleost innate immune system is, like in mammals, comprised of cellular and humoral components. However, as fish are evolutionary precursors to mammals, the acquired immune system does not appear to have developed to the same level as seen in mammals. Although fish have the equivalent immune organ homologues to mammals, they exhibit lower levels of structural complexity, which potentially limits the capability of their acquired immune response (L. TORT 2003).

The innate immune system comprises of a cellular and humoral response. Fish, like mammals, contain a large repertoire of cytokines, which act as signal conductors within the immune system (URIBE et al. 2011) and are divided into pro- and anti-inflammatory cytokines. Antimicrobial peptides (AMPs) have been shown to be very important in the immune defence against bacteria and viruses (ELLIS 2001; MAIER et al. 2008). AMPs such as defensins have shown to be involved in the immune defence of Chinese loach (Paramisgurnus dabryanus) against bacteria, including Aeromonas hydrophila (CHEN et al. 2013). Furthermore, β defensins have been described in carp and they have also been shown to be tissue specific and up-regulated by β-glucans (MAREL et al. 2012).

Fish have a strong arsenal of immuno-competent cell types including macrophages, mast cells and polymorphonuclear (PMNs) leukocytes. Macrophages are able to phagocytose bacteria, and along with neutrophils are the two most prominent cell types which are involved in phagocytosis in fish (SECOMBES u. FLETCHER 1992). Furthermore, phagocytosis is even more important to poikilotherms as this process is relatively not so adversely
affected by changes in temperature (BLAZER 1991; LANGE u. MAGNADOTTIR 2003; MAGNADOTTIR et al. 2005).

Mast-like cells have been identified in zebrafish (S. DA'AS et al. 2011; S. I. DA'AS et al. 2012), however, there is still some controversy due to differing tissue distribution and staining profiles compared to their mammalian counterparts (REITE u. EVENSEN 2006).

Finally, PMNs such as neutrophils are a critical component of the innate immune system and can perform four main functions; degranulation (PALIC et al. 2005), cytokine release (KASAMA et al. 2005), phagocytosis (W. L. LEE et al. 2003b) and the production of neutrophil extracellular traps (BRINKMANN et al. 2004).

1.4.2 Neutrophil extracellular traps

Neutrophils are an important component of the innate immune system in fish. Neutrophil extracellular traps (NETs) have recently been identified as a novel important host innate immune defence mechanism against pathogens in mammals such as human (BRINKMANN et al. 2004), mice (BUCHANAN et al. 2006), cats (WARDINI et al. 2010) and zebrafish (PALIC et al. 2007b). NETs consist of a nuclear DNA backbone associated with antimicrobial peptides and stabilising proteins such as histones, which are released during a kind of programmed cell death, known as NETosis. NETs have been shown to be responsible for the extracellular entrapment and in some cases killing of invading pathogens (FUCHS et al. 2007).

NETs in fish have been described in zebrafish and fathead minnow (PALIC et al. 2007a; JOVANOVIC et al. 2011). Kidney derived neutrophils were shown to produce NETs when incubated in vitro with β-glucan. However, fish NETs have not as yet been shown to be functionally active. Therefore, the role of carp derived NETs in response to the bacterium Aeromonas hydrophila was investigated, with focus on the entrapment and killing ability (chapter 2) and the host evasion strategies employed by the bacterium and how β-glucan is able to stabilise and protect against the host evasion strategy employed (chapter 3).
Chapter 1 Introduction

Figure 1. Diagram showing the process of NET formation. Annotations in blue have been described in fish (PALIC et al. 2007a). Black annotations have only been described in mammals at the time of writing (FUCHS et al. 2007; VON KOCKRITZ-BLICKWEDE u. NIZET 2009).

As this new immune mechanism has only recently been discovered, large areas in the understanding of NETosis still remain unanswered. NETosis was first described as a programmed cell death mechanism which involved the release of DNA fibres bound to...
specific antimicrobial proteins, however, recent research has shown that after neutrophils have released their DNA, they are still able to simultaneously function by crawling towards a stimulus and phagocytising bacteria (YIPP et al. 2012).

The mechanism of NET formation is a complicated and not fully understood process. Firstly a neutrophil must be activated by a cytokine such as IL-8 or IFNα/γ, or a PAMP such as LPS from bacteria such as *Staphylococcus aureus* (PILSCZEK et al. 2010). Stimulation of neutrophils will lead to the activation of NADPH oxidases which will catalyse the conversion of NADPH to NADPH\(^+\) +H\(^+\)N and the release of reactive oxygen species (ROS). ROS signalling is commonly described as a prerequisite with the start of NETosis, although ROS independent NETosis has also been observed (MARCOS et al. 2010) and also presented in this thesis in chapter 4. The next step is the disruption of the nuclear membrane and decondensation of the chromatin leading to the mixing of nuclear DNA and proteins, mostly histones. Interestingly the majority of the proteins comprising the NETs do not originate in the cytoplasm, with the greatest percentage of proteins originating from the nucleus, including histones 2A (26.29 %), 2B (23.95 %) and H3 (14.50%) (URBAN et al. 2009). Finally, the DNA strands encrusted with stabilising proteins is released into the external environment and thus producing an extracellular trap cable of entrapping and in some cases killing bacteria.

Interestingly, the role of lipids in NET formation has only tentatively been explored. Firstly, Oh H *et al.* demonstrated that higher levels of cholesterol led to slower rolling behaviour, which was attributed to an increase in the length of tethers produced and an increase in cell deformity leading to an increase in contact surface area (OH et al. 2009). Furthermore, Chow *et al.* (CHOW et al. 2010), demonstrated that treatment of isolated neutrophils with the cholesterol synthase inhibitor Mevastatin induced the formation of NETs. These results show that cholesterol plays an important role in neutrophils function. However, detailed insight into the mechanism underlying NET-formation is still missing, e.g. it is still completely unclear how a membrane remodelling is involved in the process.

**1.5 The cell membrane**

Cell membranes act as an interface between the cell and its external environment. Singer and Nicolson (SINGER u. NICOLSON 1972) first described the cell membrane as existing in a
fluid mosaic lipid bilayer, containing a random distribution of lipids and proteins packed together which facilitates the diffusion of molecules in and out of the cell.

The plasma membrane consists of two layers of lipids, with the hydrophilic polar heads on the outside and the hydrophilic lipid tails pointing inwards. This bilayer consists of a high concentration of different phospholipids and also sterols such as cholesterol which adds stability (EVANS u. WAUGH 1977). This bilayer is present on all living cells and even some enveloped viruses, such as the common carp infecting CyHV-3. The lipid content of a cell reflects the role of the cell, as packing of lipids or increasing the content of cholesterol can reduce the flexibility and permeability of the membrane (EVANS u. WAUGH 1977).

An important task of the cell membrane is to maintain the homeostasis of the cell by regulating molecules and ions that enter and leave the cell. The plasma membrane utilises several different mechanisms to transport molecules in and out of the cell.

Endocytosis is a process whereby molecules can enter a cell without passing through the cell membrane. The mechanism plays an important role in regulating the lipid and protein composition of the plasma membrane and thus influences how the cell interacts with its surrounding environment. There are several different independent mechanisms identified such as clathrin mediated, phagocytosis and caveolae dependent (DOHERTY u. MCMAHON 2009). Transport of molecules can also occur by passive or ATP dependent active transport.

Apart from facilitating transcellular transport through the plasma membrane, another important function of the cell membrane is to facilitate cell-cell contact through tight junction proteins such as claudins. These tight junctions between cells form a barrier by preventing transport of liquids, molecules or pathogens between the cells (GUNZEL u. YU 2013). A range of claudins have also been identified in carp, whereby it was shown that some carp claudins are tissue specific and that claudin-2, -3c, -11 and -23 are up-regulated in the intestine during a CyHV-3 infection (SYAKURI et al. 2013a).

Furthermore, the cell membrane is rich in a wide variety of receptors capable of binding to specific molecules which will initiate a signal cascade resulting in a reaction from the cell, such as the transport of substances in or out of the cell, or the triggering of an immune response. β-glucan has been shown to act as a PAMP and would therefore bind to cell membrane based receptors.
For some receptors to function, or for signal cascades to be initiated, specific proteins need to be localised within close proximity of each other on the plasma membrane. It has been shown that certain proteins cluster together in specific ordered microdomains called lipid rafts (Simons and Ikonen 1997).

1.5.1. Lipid rafts

1.5.1.1 Structure

Since 1982, evidence was gathering suggesting that the cell membrane is not composed of a random distribution of lipids and proteins, but Karnovsky (1982) and others discovered that lipids and proteins are not uniformly distributed throughout the membrane, but clustered in ordered gel-like microdomains called lipid rafts (Simmons 1997). Lipid rafts are sterol and sphingomyelin rich microdomains enriched in certain proteins. Cholesterol consists of a hydrophobic and a hydrophilic portion and has been shown to be important for membrane stability (Evans and Waugh 1977). The hydroxyl group interacts with the polar head of the phospholipids and the bulky head is imbedded in the membrane bilayer between the phospholipids providing rigidity. Lipid rafts are believed to exist in a range of sizes from 10 – 100 nm and ‘superrafts’ of up to 200 nm in artificially created membranes (Braccia et al. 2003). Besides sterols and sphingolipids, lipid rafts also contain high concentrations of certain proteins, such as glycosylphosphatidylinositol (GPI) anchored proteins, doubly acylated proteins, cholesterol-linked and palmitoylated transmembrane proteins (Simons and Toomre 2000).

Analysis of lipids has been conducted predominately in vitro, although some studies have used ex vitro and synthetic membranes to study lipid organisation (Jacob and Naim 2001). A range of detergents can be used to isolate specific types of lipid rafts including; primarily plasma membrane rafts isolated using Triton X-100, mannose rich lipid rafts isolated using either Tween 20 or Brij 98, or post golgi apparatus using Lubrol (Schuck et al. 2003).

The presence and functionality of fish lipid rafts has also been recently investigated. Zehmer et al. (Zehmer et al. 2003, 2004, 2005) described the presence and effects of thermal acclimation on lipids rafts in rainbow trout. These results also revealed that fish and mammalian lipid rafts share similar characteristics such as significant concentrations of cholesterol and sphingolipids. More recently, Garcia-Garcia et al. (Garcia-Garcia et al.
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2012 described the presence of lipid rafts in a second species, goldfish (*Carassius auratus*), and furthermore showed that the lipid rafts are functionally important.

1.3.1.2 Function

Lipid rafts have been shown to perform several important functions within a cell, such as protein and receptor trafficking, signalling and have also been shown to be utilised by certain pathogens such as viruses and bacteria as a method of entering a cell (SIMONS u. IKONEN 1997; SHIN et al. 2000; ONO u. FREED 2001). Lipid rafts enable high concentrations of proteins to exist in close proximity to each other, which enables signalling cascades to be initiated.

Lipid rafts have been implicated in protein vesicular trafficking. Li N et al. (LI et al. 2003) showed that lipid rafts are present along the endosome pathway, with the lipid raft marker proteins flotillin 1 and 2 present in Triton X-100 isolated rafts. The late and early endosome trafficked proteins, Rab-1 and Rab-7 were also present with the late endosomal LIMPII protein, providing further evidence that lipid rafts are present throughout the endosome maturation (LI et al. 2003).

Signalling via cell surface receptors has also been shown to occur via lipids rafts. TLR 2 has been shown to form heterodimers with TLR 1 and TLR 6, however, these heterodimers only migrate to lipid rafts post stimulation where the signalling cascade via NF-κB can be initiated (TRIANTAFILOU et al. 2011).

Lipid rafts are important portals utilised by the cell to facilitate transport of molecules and proteins from the golgi apparatus, the site of synthesis, through sorting channels in the endoplasmic reticulum and to the cell membrane where they can function. The protein clustering environment created by the long chain phospholipids and stabilising sterols facilitate trafficking of proteins, signalling cascades and promoting cell-cell communication through the high concentration of receptors. However, lipid rafts have also been implicated to have a role in diseases, such as bacteria invasion and lipid raft mediated virus entry (ONO u. FREED 2001).

In infection, Lu DY et al. (LU et al. 2012) showed that lipid rafts play an important role in *Helicobacter pylori* infections in the gut. Infection of AGS cells resulted in increased levels of
TLR4 protein in isolated lipid raft fractions, which induced a signalling cascade resulting in inflammation in the gastric epithelial cells. Additionally, lipid rafts have been shown as a portal of entry for both bacteria (SHIN et al. 2000) and viruses (ONO u. FREED 2001). There are several routes a virus can use to enter a cell including lipid raft or caveolin mediated entry (MARSH u. HELENIUS 2006). Human immunodeficiency virus, Ebola virus and herpesviruses have been shown to use lipid rafts to enter cells (CAMPBELL et al. 2001; BENDER et al. 2003a; FREITAS et al. 2007). Furthermore, human simplex virus is able to modulate lipid rafts during infection by mobilising glycolipid B into lipid rafts which can, thereafter, serve as a platform for virus entry and cell signalling (BENDER et al. 2003a).

Finally it can be summarised that cellular membranes and in particular its lipid rafts play a central role in cellular signalling and during infectious processes. Thus, this study will in part focus on carp cell membranes/lipid rafts involved in host-pathogen interactions during microbial and viral infections.

1.6 Aims
Immunomodulators, such as β-glucan, have been used in aquaculture for several years as a method of reducing the frequency and severity of disease outbreaks, increasing the speed of growth and quality of fish and thereby increasing profit for the fish farmer. Recent results have shown that beta glucan is able to positively influence host-pathogen interactions in several fish species. However, the influence of beta glucan on carp kidney and pronephros derived neutrophils, and specifically the novel immune defence mechanism described by Brinkmann et al. (BRINKMANN et al. 2004) as NETs, remains to be investigated. Therefore, it was my aim to investigate the effect of β-glucan on the formation of NETs (chapter 2), antimicrobial activity of NETs (chapter 2) and the stabilisation of NETs against bacterial nuclease degradation (chapter 3). The cell membrane acts as an interface between the host cell and pathogens such as viruses and bacteria. There is a large amount of evidence showing the importance and role of lipid rafts in infection in mammals, however, unfortunately research in this field in fish is lacking. Therefore, a human neutrophil model was used to investigate the role of lipid rafts and cholesterol in the formation of NETs (chapter 4).

Lipid rafts have been well described in mammals, where they have been shown to play important physiological roles in trafficking and cell signalling. Also the lipid composition
influences the function of lipid rafts and has been shown to be modulated by pathogens, surrounding environment and even by feed additives. Therefore, a methodology for the isolation and characterisation of the lipid content of carp lipid rafts and DSM fractions from several tissues and a cell line was optimised. This methodology will be used for range of experiments investigating the modulations in lipid rafts (chapter 5).

Finally, research has shown the presence of lipid rafts in fish and they have also recently been shown to be functionally active in goldfish (*Carassius auratus*) (GARCIA-GARCIA et al. 2012). To investigate if carp lipid rafts play a role in virus entry, CCB cells were depleted of cholesterol and infected with CyHV-3. The progression of infection, with or without lipid raft depletion was evaluated using RT-PCR and immunocytochemistry (chapter 6).
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Chapter 2

The effect of β-glucan on formation and functionality of neutrophil extracellular traps in carp (*Cyprinus carpio* L.).

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Foreword: The feed additive β-glucan has been shown to have immunomodulatory effects and is able to enhance the immune response against *Aeromonas hydrophila*. However, the effect of β-glucan on carp neutrophil functions is still mainly unclear. Here we focused on the effect of β-glucan on the formation and functionality of neutrophil extracellular traps (NETs). NETs have recently been described as a novel innate immune response shown to act against bacterial infection by entrapment and killing of bacteria. An immunoboosting effect of β-glucan on formation of NETs might have a positive effect in the immune defence against infections with *A. hydrophila* in carp.

The extent of Graham Brogden’s contribution to the article is evaluated according to the following scale:

A. has contributed to collaboration (0-33%).
B. has contributed significantly (34-66%).
C. has essentially performed this study independently (67-100%).

1. Design of the project including design of individual experiments: B
2. Performing of the experimental part of the study: B
3. Analysis of the experiments: B
4. Presentation and discussion of the study in article form: B
Short communication

The effect of β-glucan on formation and functionality of neutrophil extracellular traps in carp (Cyprinus carpio L.)

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ABSTRACT

The formation of neutrophil extracellular traps (NETs) has been characterised as a novel antimicrobial host defence strategy of neutrophils besides phagocytosis and degranulation, which may lead to entrapment and subsequent immobilisation and/or killing of bacterial pathogens. Here we studied the effect of the feed additive β-glucan, namely MacroGard®, on the formation and functionality of NETs in carp. Therefore, common carp (Cyprinus carpio) head kidney and kidney cells were isolated and treated with or without β-glucan over time. The formation of NETs was analysed by immunofluorescence microscopy and revealed a distinct increase of NET-formation with β-glucan. Furthermore the subsequent entrapment of Aeromonas hydrophila, an important fish pathogen, was increased after stimulating the cells with β-glucan. However, β-glucan did not lead to a stimulation of antimicrobial activity of neutrophils against A. hydrophila. In conclusion, the data underline the fact that the feed additive β-glucan is able to modulate carp neutrophil functions.

Keywords: Neutrophils, neutrophil extracellular traps (NETs), β-glucan, Aeromonas hydrophila, carp

Highlights

- β-glucan augments formation of neutrophil extracellular traps in carp head kidney- and kidney-derived neutrophils.
- β-glucan increases entrapment of Aeromonas hydrophila by carp head kidney- and kidney-derived neutrophils.
- β-glucan does not stimulate antimicrobial activity of carp head kidney- and kidney-derived neutrophils against Aeromonas hydrophila.

Abbreviations: Neutrophil extracellular traps (NETs)
1. Introduction

Neutrophils are an important component of the carp (Cyprinus carpio) innate immune defence against a range of invading pathogens [1]. They mediate their antimicrobial activities by intracellular uptake and killing (phagocytosis), degranulation of antimicrobial substances and/or release of neutrophil extracellular traps (NETs). NET-formation has recently been characterised as a strategy to entrap and subsequently immobilise and/or kill microbial pathogens in fish and mammals [2]. They consist of nuclear DNA fibres linked to antimicrobial peptides and stabilising proteins such as histones, and are released upon stimulation by proinflammatory signals or pathogens themselves [3]. Interestingly the feed additive β-glucan has been shown to induce NET formation in zebrafish kidney cells [4]. However, until now, the functionality of NETs in fish remains to be investigated and only little is known about β-glucan-dependent NET-formation in carp [5].

Our own previous work has already shown that the feed additive β-glucan is able to stabilise the formation of NETs against degradation by Aeromonas hydrophila. Aeromonas hydrophila is a ubiquitous Gram-negative bacterium associated with mass mortalities in carp aquaculture [6]. Recently, we demonstrated that A. hydrophila is able to degrade NETs via nuclease activity [5] and that β-glucan is able to stabilise NETs against this nuclease degradation [5]. However it was still unclear if β-glucan is able to induce the formation of NETs and subsequently increase entrapment and/or killing of bacteria in carp. Thus, here we investigated the effect of β-glucan on NET formation and functionality of NETs against A. hydrophila derived from carp kidney and head kidney cells.
2. Material and methods

2.1 Isolation of neutrophils from head kidney and kidney of carp

For all experiments, head kidneys and kidneys were isolated from minimum of n=5 common carp (Cyprinus carpio), which were kept at 22°C (±1°C) at the University of Veterinary Medicine Hannover, Germany. All experiments have been approved by the respective Committee on the Use and Care of Animals and have been performed using internationally accepted veterinary standards and federal guidelines. The fish were euthanized by narcosis with MS-222 (Tricaine Methane Sulphonate Powder, Pharmaq, United Kingdom) and exsanguination. Isolated head kidneys and kidneys were kept in RPMI (PAA, Germany) on ice. The tissues were processed by being passed through a 100 µm cell strainer, centrifuged at 250 x g for 15 min at 4°C and resuspended and kept in unsupplemented RPMI without phenol red. The viability of the cells was tested using trypan blue exclusion and confirmed more than 95% to be viable. Flow cytometry using an Epics XL (Beckman Coulter, USA) was used to quantify the percentage of neutrophils in each individual tissue based on granularity and cell size and revealed an average of 42.66 ± 1.34% of the cells to be neutrophils in both head kidney and kidney samples.

2.2 Visualisation of NETs

For immunofluorescent microscopic visualisation of NETs, 2 *10^6 of the total cells were seeded on poly-L-lysine coated glass slides in a 24 well-plate. To allow attachment of cells to glass slides, the cells were incubated for 30 min at 26 °C. Then, the cells were incubated in the presence or absence of β-glucan (MacroGard®, Biorigin) at indicated concentrations. For immunostaining, cells were fixed with 4% PFA and kept at 4 °C. Immunostaining of NETs was done as previously described [7]. Briefly, fixed and washed cells were treated with mouse anti H2A-H2B- DNA complex antibody [8] at a concentration of 0.95 mg/ml overnight at 4 °C followed by an Alexa 488-conjugated secondary goat anti
mouse antibody (1:500-diluted; Invitrogen, Germany) for 45 min at room temperature. After washing, samples were embedded in Dapi-Prolong-Gold (Invitrogen, Germany) to stain all nuclei blue. Microscopic evaluation of NETs was done using a Leica DMI6000CS confocal microscope with a HCXPLAPO 40x 0,75 – 1,25 oil objective oil objective. The gain settings used remained constant throughout each individual experiment. A minimum of 5 random images were taken from each sample (individual fish) and the number of neutrophils showing histone protrusions were counted. The number of positive NET cells was then adjusted to the percentage of neutrophils in each tissue, which was determined by flow cytometry, to standardise differences between individual fish and tissues.

2.3 Entrapment assay

*A. hydrophila* subsp. *Hydrophila* (Chester 1901; DSMZ No. 30187) was grown in LB medium at 26°C shaking at 150 rpm and was harvested during the log-phase of growth (at an optical density at 600 nm of 0.5) by centrifugation at 3736 x g for 10 min. The supernatant was removed and the bacteria were washed with PBS. The bacteria were centrifuged again and resuspended in 5 ml PBS and adjusted to an OD<sub>600</sub>=0.5. After this, 15 ml of the bacterial solution was labelled with 0.33 mg/ml of FITC (Invitrogen) and incubated for 30 min on ice in the dark. After incubation the bacteria were centrifuged again at 3000xg for 10min. After removing the supernatant and washing with PBS, the bacteria were centrifuged again and finally resuspended in RPMI without phenol red.

For the entrapment assay the cells of the head kidney and kidney of the fish were isolated as described above. 4 x 10<sup>5</sup>/ml cell in 100 µl were seeded into a 96 well plate and incubated for 30 min in the presence or absence of 200 µg/ml MacroGard® at room temperature. Then, FITC-labelled bacteria were added to the cells at a multiplicity of infection of 20 or 200. The cells and bacteria were incubated for 30 min in the dark after centrifugation once at 370 x g for 5 min. After this, the wells were washed twice with RPMI and centrifuged again to remove unbound bacteria. Finally, entrapment of FITC-labelled bacteria was measured using green fluorescence at 485/538 nm (exc./em.). Percentage of entrapment...
was calculated compared to total fluorescence of total bacteria per well (incubated under the same conditions but in the absence of cells).

2.4 Visualisation of bacterial entrapment

The kidney and head kidney cells were isolated as described above. The amount of 2 *10^6 of the total cells were seeded on poly-L-lysine coated glass slides in a 24 well-plate. To allow attachment of cells to glass slides, the cells were incubated for 30 min at 26 °C. Then, the cells were incubated in the presence or absence of 200 µg/ml β-glucan (MacroGard©, Biorigin) and subsequently infected with FITC-labelled *A. hydrophila* at a multiplicity of infection of 20 or 200 bacterium per cell for 30 min. For immunostaining, cells were fixed with 4% PFA and kept at 4 °C. Immunostaining of NETs was done as previously described [10]. Briefly, fixed and washed cells were treated with mouse anti H2A-H2B-DNA complex antibody [8] at a concentration of 0.95 mg/ml overnight at 4 °C followed by an Alexa 633-conjugated secondary goat anti mouse antibody (1:500-diluted; Invitrogen, Germany) for 45 min at room temperature. After washing, samples were embedded in Dapi-Prolong-Gold (Invitrogen, Germany) to stain all nuclei blue. Microscopic evaluation of NETs was done using a Leica DMI6000CS confocal microscope with a HCXPLAPO HCXPLAPO 40x 0,75 – 1,25 oil objective.

2.5 Bacterial killing assay

A colony forming units (cfu) plating assay was used to quantify the bactericidal ability of carp NETs. *A. hydrophila* was cultured as described above. Head kidney and kidney cells were isolated as previously described and seeded in 96 well plates at a density of 5 *10^6 in a total volume of 250 µl. The cells were then stimulated with or without 200 µg/ml β-glucan for 30 min at 23°C. Bacteria at MOI 2 was added, centrifuged for 5 min at 370 x g and incubated for a further 25 min at 23°C. After incubation, the extracellular surviving bacteria 50 µl of 0.25% Triton X-100 in PBS were collected, plated and enumerated on LB agar plates. Colonies were counted after 24 hr incubation at 37°C.
2.6 Statistical analysis

Statistical analysis was performed using GraphPad (GraphPad Prism 5, 2011). Data are presented as means +/- standard error of the mean (SEM) of a minimum of 5 individual fish. Statistical testing was done using paired one-tailed Student’s t-test. Results with p ≤ 0.05 were considered as significant different.
3. Results and discussion

Fig. 1. Effect of β-glucan on NET formation over time in neutrophils isolated from head kidney (A) and kidney (B). Total cells were incubated in the absence or presence of β-glucan (MacroGard®) at concentrations of 2, 20 and 200 µg/ml. The presence of NET positive cells were visualised by immunofluorescence microscopy using an antibody against H2A-H2B-DNA complexes combined with the cell nuclei staining Dapi. The percentage of NET positive cells was then adjusted to the amount of neutrophils present in each sample as determined by flow cytometry. Data shown derived from n=5 fish.
Fig 2. Carp head kidney (A) and kidney (B) derived neutrophils are able to entrap *A. hydrophila*. Presence of NETs was visualised by immunofluorescence microscopy using an antibody against H2A-H2B-DNA complexes (red) in combination with Dapi to stain all cell nuclei (blue). FITC labelled *A. hydrophila* (green) can be seen in close proximity to the extracellular H2A-H2B-DNA strands (arrows). Representative image (A) was obtained from kidney incubated with 200 µg/ml β-glucan plus *A. hydrophila* at an MOI of 100, and image (B) was obtained from head kidney derived cells incubated with 200 µg/ml β-glucan plus *A. hydrophila* at an MOI of 50. (C-F). Effect of 200 µg/ml β-glucan on entrapment of FITC-
labelled *A. hydrophila*: Addition of β-glucan led to a significant increase in the percentage of FITC labelled *A. hydrophila* entrapped by the NETs in both the kidney (C, D) and the head kidney (E, F). Data shown derived from *n*=5 fish. Significance given at *p*<0.05 * and *p*<0.01 **.

**Fig. 3.** Effect of β-glucan on antimicrobial activity of carp neutrophils against *A. hydrophila*. *A. hydrophila* was incubated with either control media with or without 200 μg/ml β-glucan, head kidney (HK) derived cells with or without 200 μg/ml β-glucan or kidney (K) derived cells with or without 200 μg/ml β-glucan. Bacteria concentrations were quantified by plating dilutions and counting surviving cfu after 30 min of coincubation. There was no difference between all groups detected. The data are displayed as mean ± SEM of surviving cfu log(10). Data shown are from *n*=5 fish.
3.1 β-glucan augments the formation of NETs in kidney and head kidney-derived neutrophils

Since Brinkmann et al. first discovered NETs in 2004 [11], they have been additionally found in a wide range of vertebrate animals including 3 species of fish: zebrafish [4] fat head minnow [12] and common carp [5]. Here in this study we investigated the NET formation from both carp kidney and head kidney derived neutrophils and the effects of β-glucan on this process. Cells were incubated with 3 different concentrations of β-glucan, 2, 20 and 200 µg/ml plus a control medium without the addition of β-glucan. Cells were analysed at 15, 30, 60, 120 and 240 min and NET formation was quantified by immunofluorescence microscopy using an antibody against histone-DNA-complexes. As shown previously by Brogden et al. [5], isolated cells cultured in control medium showed high levels of spontaneous NET production. Interestingly, the addition of a high concentration of β-glucan (200 µg/ml) resulted in elevated levels of NET-formation in head kidney and kidney derived neutrophils. These results are in accordance with data published by Palic et al [4], who showed that a similar high concentration of 200 µg/ml induces NET formation in zebrafish-derived kidney neutrophils.

Furthermore, our results also show that NET production is time dependent, with more individual neutrophils extruding DNA at later time points compared to earlier time points. This has also previously been shown in mouse and human [13, 14]. Interestingly, our findings additionally show that carp NET-formation can be produced very rapidly within 15 to 30 min of stimulation. This result is supported by recent data published using a mouse model by Yipp et al [15], which described NET-formation as a rapid response to an invading pathogen. Thus it can be hypothesised that this feature of neutrophils is evolutionary conserved amongst vertebrate organisms, similar to other neutrophil or immune cell functions [16, 17].
3.2 β-glucan increases entrapment of *A. hydrophila* by kidney and head kidney-derived neutrophils

NETs have been shown to act antimicrobial by mediating entrapment of microbes and subsequent immobilisation of invading pathogens. Besides entrapment, some pathogens e.g. *E. coli* and *S. pyogenes* have been shown to be directly killed within NETs [11, 18]. Other pathogens e.g. *Streptococcus pneumoniae* [19], *Staphylococcus aureus* and *Candida albicans* [20] were shown to be entrapped by NETs but not killed. So far, little is known about the functionality of NETs in fish. As shown in Figure 2A and B, head kidney and kidney derived cells were able to entrap FITC-labelled *A. hydrophila*. The presence of NETs was visualised by using an antibody against H2A-H2B-DNA complexes (red) in combination with DAPI, which stained the nuclei blue. The FITC labelled bacteria appeared as green rod-like structures, which can be seen in close contact to the NETs as indicated by arrows (2 A and B).

To quantify the effect of β-glucan on entrapment capacity of cells, total cells were isolated from head kidney and kidney, and either stimulated with β-glucan (200 µg/ml) or incubated with untreated RPMI medium. After incubation, FITC labelled *A. hydrophila* was added to the cells. The percentage of bacteria entrapped was determined by measuring the fluorescence emitted from FITC labelled bacteria using a spectrophotometer. Compared to the negative controls, cells derived from head kidneys and kidneys were able to entrap up to 16 % of bacteria. In both tissues analysed, a higher percentage of bacteria were entrapped at an MOI of 200 relative to 20. Interestingly, the addition of β-glucan significantly increased the percentage of bacteria entrapped by extracellular traps in both head kidney and kidney derived neutrophils (Figure 2). This goes in line with the data, that β-glucan augments NET formation as shown in Figure 1, and with recent data published by our group, which showed that β-glucan stabilises NETs against *A. hydrophila*-mediated NET-degradation [5].
3.3 β-glucan does not boost the antimicrobial activity of head kidney and kidney derived neutrophils

Finally, the antimicrobial effect of kidney and head kidney derived neutrophils against *A. hydrophila* was tested using a cfu plating assay, which quantified surviving bacteria after 30 min of co-incubation with kidney or head kidney cells. As shown in Figure 3, both cell populations did not exhibit any antimicrobial activity against *A. hydrophila*. Furthermore, beta-glucan was not able to boost the antimicrobial activity of the cells. These data lead to the suggestion that β-glucan-dependent NET-formation has no bactericidal effect against *A. hydrophila*. These data go in line with previous publications indicating that NETs do not have any bactericidal activity [20].

4. Conclusions

The feed additive β-glucan is well known for its immunomodulatory effects and its ability of increasing immunity protection against pathogens (Damlo et al 2008). Recent data have also shown that β-glucan is able to modulate formation of NETs in fish and human: In human derived peripheral blood neutrophils, the recognition of β-glucan by the complement receptor 3 resulted in rapid NET formation [21]. Furthermore, Palic et al [4] showed that 200 µg/ml β-glucan induces the release of extracellular DNA in kidney neutrophils derived from zebrafish after 90 min of stimulation. Here we have shown that β-glucan is also able to augment NET formation in kidney and head kidney derived neutrophils from carp. At the same time the entrapment, but not the killing, of *A. hydrophila* was increased after treatment of cells with β-glucan. These data go in line with our previous data, which revealed that β-glucan is able to protect NETs against degradation by *A. hydrophila* [5].

Interestingly, comparisons of NET formation and entrapment of *A. hydrophila* by kidney versus head kidney-derived neutrophil revealed significant differences (Supplemental Figure 1). After 30 min incubation, kidney derived neutrophils produced more NETs (p=0.05)
and entrapped a significantly higher percentage of bacteria (p<0.05) than head kidney derived NETs. Joernik et al. [22] described the head kidney as the site of pluripotent cell production by polarising head kidney derived macrophages. Our results support this theory and suggest that there are also potentially two differing populations of neutrophils in fish. A naive population may be present in the head kidney as immature neutrophils may not be as immunologically active, whereas the neutrophil population isolated from the kidney are significantly more effective against a bacterial challenge and are therefore considered to be the mature population [23]. Future work will focus on differences of NET-formation in kidney versus head kidney neutrophils and on the underlying mechanisms mediating β-glucan-dependent NET formation in kidney versus head-kidney derived neutrophils from carp and their impact on the course of infection in vivo.
References

Chapter 2 Effects of β-glucan on carp NETs


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Supplemental Figure 1

A

**% of neutrophils producing NETs**

![Bar chart showing comparison between carp head kidney (HK) and kidney (K) derived neutrophils. A significantly higher percentage of kidney derived cells produced a NET compared to head kidney derived cells after 30 min incubation (A). After 30 min incubation with *A. hydrophila*, a greater percentage of *A. hydrophila* was entrapped by kidney derived NETs compared to head kidney derived NETs. Data shown derived from n=5 individual fish. Significance given at p<0.05 * and p<0.01.](image)

B

**Percent entrapment**

![Bar chart showing comparison between carp head kidney (HK) and kidney (K) derived neutrophils. After 30 min incubation with *A. hydrophila*, a greater percentage of *A. hydrophila* was entrapped by kidney derived NETs compared to head kidney derived NETs. Data shown derived from n=5 individual fish. Significance given at p<0.05 * and p<0.01.](image)

Sup 1.

Comparison between carp head kidney and kidney derived neutrophils. A significantly higher percentage of kidney derived cells produced a NET compared to head kidney derived cells after 30 min incubation (A). After 30 min incubation with *A. hydrophila*, a greater percentage of *A. hydrophila* was entrapped by kidney derived NETs compared to head kidney derived NETs. Data shown derived from n=5 individual fish. Significance given at p<0.05 * and p<0.01.
Chapter 3

β-glucan protects neutrophil extracellular traps against degradation by *Aeromonas hydrophila* in carp (*Cyprinus carpio*).

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Foreword: In chapter 2, we have shown that formation of carp NETs and subsequent entrapment of *A. hydrophila* can be induced by treatment of kidney and head-kidney-derived neutrophils with β-glucan. However, some Gram-positive bacterial pathogens have been shown in literature to avoid entrapment by NETs via release of bacterial nucleases that are able to degrade NETs. Nothing is known about *A. hydrophila*-mediated NET-degradation so far. Thus, in this chapter we investigated the ability of *A. hydrophila* to degrade NETs and, furthermore, the effect of β-glucan on NET stabilisation against degradation by *A. hydrophila*.

The extent of Graham Brogden’s contribution to the article is evaluated according to the following scale:

A. has contributed to collaboration (0-33%).
B. has contributed significantly (34-66%).
C. has essentially performed this study independently (67-100%).

1. Design of the project including design of individual experiments: B
2. Performing of the experimental part of the study: B
3. Analysis of the experiments: B
4. Presentation and discussion of the study in article form: B
Short communication

β-glucan protects neutrophil extracellular traps against degradation by *Aeromonas hydrophila* in carp (*Cyprinus carpio*)

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ABSTRACT

A novel host innate immune defence mechanism against invading pathogens, namely the formation of neutrophil extracellular traps (NETs), has recently been discovered. These NETs are described as DNA fibres released by dying neutrophils, which are able to entrap and kill various microbes. Here we studied the effect of the feed additive β-glucan, namely MacroGard®, on the degradation of NETs by the important fish pathogen Aeromonas hydrophila. Therefore, common carp (Cyprinus carpio) head kidney cells consisting of approximately 45% neutrophils were isolated and treated with or without β-glucan. The degradation of NETs after co-incubation with A. hydrophila was analysed by immunofluorescence microscopy. The data show that A. hydrophila is able to degrade NETs and that treatment of cells with β-glucan significantly protects the NETs against bacterial degradation. Control experiments revealed that β-glucan augments nuclease activity of the bacteria at the same time while protecting the NETs against its degradation. In conclusion the data indicate that β-glucan might affect the composition and stabilisation of NETs and thereby protecting them against degradation by A. hydrophila nuclease.

Keywords: Neutrophils, neutrophil extracellular traps (NETs), β-glucan, Aeromonas hydrophila, carp

Highlights

- Carp head kidney derived neutrophils are able to produce NETs.
- Aeromonas hydrophila is able to degrade NETs.
- β-glucan protects against degradation of NETs by Aeromonas hydrophila.

Abbreviations: Neutrophil extracellular traps (NETs)
1. Introduction

Neutrophil extracellular traps (NETs) have recently been identified as a novel important host innate immune defence mechanism of mammalian and fish neutrophils [1],[2]. They consist of nuclear DNA associated with antimicrobial peptides and stabilising proteins such as histones, and are released by dying neutrophils as a kind of suicide mechanism, called NETosis, to rescue other immune cells [3]. NETs have been shown to be responsible for the trapping and extracellular killing of invading pathogens and thereby play a protective role during the course of infection with bacteria or fungi (reviewed by von Köckritz-Blickwede and Nizet, 2008) [4]. Some bacteria such as Streptococcus pyogenes have been shown to efficiently avoid NET-entrapment and killing by the production and release of NET-degrading nucleases [5]. An isogenic nuclease-deficient bacterial mutant showed reduced virulence and infection-associated mortality using a mouse model of infection [5]. Thus, it was discussed in literature whether stabilisation of NETs or inhibition of bacterial nucleases might improve the host defence against an infection [4]. So far, nothing is known about possible drugs or feed additives to protect NETs against bacterial degradation. MacroGard® (Biorgin, Norway) is a feed material containing a minimum of 60% purified β-1,3/1,6-glucan. It is well known for its ability to modulate the immune system and thereby increasing protection against bacterial infections [6]. Recently, MacroGard® has been shown to induce the formation of NETs in neutrophils from zebrafish [7]. Here we have investigated the effect of β-glucan, in the form of MacroGard®, on its ability to protect NETs released from common carp (Cyprinus carpio) derived neutrophils against degradation by Aeromonas hydrophila nuclease.
2. Material and methods

2.1 Isolation of neutrophils from head kidney of carp

For the experiments, head kidneys were isolated from \( n = 6 \) specific pathogen free common carp (Cyprinus carpio), which were kept at 23°C ± 1 at the University of Veterinary Medicine Hannover, Germany. Fish were fed a diet for 2 weeks prior of experiments consisting of the following ingredients (prepared by Tetra, Germany): 45% fish protein concentrate, 40% wheat starch, 4.5% soybean oil, 4.5% fish oil, 2.565% cellulose, 2.06% mineral-premix, 1% MacroGard\textsuperscript{®}, 0.25% vitamin premix, 0.11% stabilized vitamin C, and 0.015 ethoxyquin. All experiments have been approved by the respective Committee on the Use and Care of Animals and have been performed using internationally accepted veterinary standards and federal guidelines. Isolated head kidneys were kept in RPMI (PAA, Germany) on ice. The tissues were processed by being passed through a 100 \( \mu \)m cell strainer, centrifuged at 250 x g for 15 min at 4°C and resuspended and kept in unsupplemented RPMI without phenol red. The viability of the cells was tested using trypan blue exclusion and confirmed more than 95% to be viable. Flow cytometry using an Epics XL (Beckman Coulter, USA) was used to quantify the percentage of neutrophils in each individual population based on granularity and cell size and revealed an average of 43.33 ±1.05% of the cells to be neutrophils.

2.2 Visualisation of NETs and its degradation

For immunofluorescent microscopic visualisation of NETs, \( 1 \times 10^6 \) of the total cells were seeded on poly-L-lysine coated glass slides in a 24 well-plate. To allow attachment of cells to glass slides, the cells were incubated for 30 min at 26°C. Then, the cells were incubated in the presence or absence of \( \beta \)-glucan (200 \( \mu \)g/ml MacroGard\textsuperscript{®}, Biorigin) and/or \textit{A. hydrophila} subsp. hydrophila (Chester 1901; DSMZ No. 30187; harvested and frozen in brain heart infusion medium at an optical density of 0.8) \[8\] at a multiplicity of infection of 0.01 bacterium
per cell. For immunostaining cells were fixed with 4% PFA for 15 min at room temperature and kept at 4°C. Immunostaining of NETs was done as previously described [9]. Briefly, fixed and washed cells were treated with mouse anti H2A-H2B-DNA complex antibody [10] at a concentration of 0.95 µg/ml overnight at 4°C followed by an Alexa 488-conjugated secondary goat anti mouse antibody (1:500-diluted; Invitrogen, Germany) for 1 h at room temperature. After washing, samples were embedded in Dapi-Prolong-Gold (Invitrogen, Germany) to stain all nuclei blue. Microscopic evaluation of NETs was done using a Leica DMI6000CS confocal microscope with a HCXPLAPO 40 x 0.75-1.25 oil objective. The gain settings used remained constant throughout each individual experiment. A minimum of n = 5 random images were taken from each sample (individual fish) and percentage of area covered with NETs was quantified by assigning a number for each image relating to the total percentage area of NET coverage. The following arbitrary scale was used: 0 = 0 – 9%, 1 = 10 – 19% NETs, 2 = 20-29% up until 9 = 90-100%. This value was then related to the amount of each individual’s neutrophil population as determined by flow cytometry.

2.3 Quantification of cell death

For quantification of cell death, 2 x 10^5 cells were seeded into a black 96-well plate using identical conditions as described above. Then, without any prior fixation, cells were treated with 5 µM Sytox Green (Invitrogen, Germany) and incubated for 10 min at room temperature in the dark. Sytox Green is a high-affinity nucleic acid stain that easily penetrates cells with compromised plasma membranes and can not cross the membranes of live cells. Therefore, Sytox Green is a marker of general cell death. Fluorescence level as a marker for cell death was quantified using a fluorometer (FLUOstar OPTIMA, BMG Labtech, Germany) at 485 nm excitation and 520 nm emission (Gain 800). The background fluorescence of control wells (without neutrophils) containing pure medium, medium with bacteria and/or medium with beta-glucan was subtracted from the respective values for final data analysis.
2.4 Bacterial growth curve and determination of bacterial nuclease activity

To study the effect of β-glucan on bacterial growth, a bacterial overnight-culture was diluted 1:100 in LB medium alone or LB medium containing β-glucan (200 µg/ml MacroGard®). Then, bacterial growth at 26°C (shaking at 150 rpm) was analysed by measuring the optical density at 600 nm hourly. In a second approach, bacteria at an optical density of 0.8 were diluted in RPMI and incubated in a 24 well-plate (non-shaking) for 1 h in the presence or absence of β-glucan (identical as used for the NET degradation assay). To quantify the production and release of nuclease by A. hydrophila, bacteria in 24 well-plate were centrifuged at 1753 x g for 10 min and the supernatant was harvested. Then, 75 µl of the supernatant was incubated in the presence of 10 µg/ml calf thymus DNA for 1 h in a black 96 well-plate. DNA-degradation was quantified using Quant-iT™ PicoGreen® dsDNA Reagent (Invitrogen) as recommended by the manufacturer. For visualization of nuclease activity, a volume of 10 µl of the supernatant was incubated with 7.5 µl calf thymus DNA (1 mg/ml, Sigma) and 40 µl DNase buffer (3 mM MgCl₂, 3 mM CaCl₂, 300 mM Tris; pH 7.4) for 60 min at 37°C. The nuclease reaction was stopped by putting the samples on ice, then 12.5 µl 6x loading dye was added and 20 µl of each sample was run on a 1% agarose gel for visual examination of DNA degradation.

2.5 Statistical analysis

Data were analysed by using GraphPad Prism 5.0 (GraphPad Software). Differences between groups were analysed by using a two-tailed paired Student's t-test. Normal distribution of data was verified by D’Agostino Kolmogorov-Smirnov normality test (GraphPad software) prior to statistical analysis.
3. Results and discussion

Fig. 1.

A

B

C

D
**Fig. 1.** Effect of β-glucan on degradation of NETs by *A. hydrophila*. (A) Total cells were isolated from carp head kidney and incubated in the presence or absence of *A. hydrophila* (bacteria; MOI 0.01) and/or β-glucan (BG; 200 µg/ml MacroGard®). Presence of NETs was visualised by immunofluorescence microscopy using an antibody against H2A-H2B-DNA complexes (green) in combination with Dapi to stain all cell nuclei (blue): Control cells show spontaneous NET release (Control). Co-incubation of the cells with *A. hydrophila* led to the degradation of NETs (Bacteria). Treatment of the cells with β-glucan (200 µg/ml) significantly protected the NETs against degradation by *A. hydrophila* (Bacteria + BG). (B) Higher magnification of Fig.1A, Bacteria. Scoring of the presence of NETs is shown in (C): The presence of NETs was graded based on the following scale: 0 = 0 – 9% of area covered with NETs, 1 = 10 – 19% NETs, 2 = 20-29% etc. up until 9 = 90-100%. (D) Quantification of cell death using the Sytox Green DNA intercalating dye (Invitrogen, Germany). Data are shown as mean ± SEM of n=6 individual fish.
Fig. 2.

A. Growth of *A. hydrophila*

![Graph showing growth of *A. hydrophila* with optical density at 600 nm over time. The graph includes two lines, one for + BG and one for - BG, indicating the growth of the bacterium with and without background growth.]

B. Growth of *A. hydrophila*

![Bar graph showing x-fold cfu compared to initial inoculum. The graph includes two bars, one for - BG and one for + BG, indicating the growth with and without background growth.]

C. Nuclease activity of *A. hydrophila*

![Bar graph showing U/ml nuclease activity. The graph includes two bars, one for - BG and one for + BG, indicating the nuclease activity with and without background growth.]

**Note:** The graphs illustrate the degradation of carp NETs by *Aeromonas hydrophila* over time, with measurements of growth and nuclease activity under different conditions.
Fig. 2. Effect of β-glucan on bacterial growth and bacterial nuclease activity. (A) Bacterial growth in LB (shaking at 150 rpm, 26°C) in the presence or absence of β-glucan (BG; 200 µg/ml MacroGard®). Data in (A) are shown as mean ± SEM of n=3 independent experiments. (B) Bacterial growth in RPMI in the presence or absence of β-glucan (BG; 200 µg/ml MacroGard®) after 1 h incubation in a 24 well plate (non-shaking, 26°C). The data (mean ± SEM of n=6 independent experiments) are shown as x-fold cfu compared to the initial inoculum. (C) Bacterial nuclease activity in supernatants of A. hydrophila cultures grown in RPMI (as shown in B): Nuclease activity was analysed by measuring degradation of calf thymus DNA using Picogreen and compared to a standard curve based on micrococcal nuclease activity. Data in (C) are shown as mean ± SEM of n=6 independent experiments.
3.1 A. hydrophila uses nucleases to degrade NETs

Until now, NET formation in fish has been proven for fathead minnow (Pimephales promelas) [2] and zebrafish (Danio rerio)-derived kidney neutrophils [7]. Here in this study we investigated the NET formation in head kidney-derived neutrophils from carp by immunofluorescence microscopy using an antibody against histone-DNA-complexes. Unexpectedly, untreated control cells showed high spontaneous release of NETs, indicated by Alexa 488-labelled extracellular fibrous structures shown in Fig. 1A (Control). Immunofluorescence-microscopic quantification of NETs revealed approximately 50% of the area to be covered with NETs (Fig. 1C, Control). Interestingly, the addition of A. hydrophila resulted in significantly reduced area covered with NETs (Fig. 1A and C, Control versus Bacteria). A. hydrophila is widely considered as a major fish and amphibian pathogen and its pathogenicity is mediated by a number of extracellular proteins [11]. Among those extracellular proteins, the bacterium is able to produce a nuclease [12]. The role of this nuclease in A. hydrophila virulence is still not entirely clear. Here we hypothesise that A. hydrophila is able to degrade NETs, resulting in a significantly reduced area covered with NETs in infected cells compared to non-infected cells (Fig 1A and C, Control versus Bacteria). Control experiments were performed to exclude that the bacteria do not block NET formation: Since NET formation has been shown to be the result of a novel cell death mechanism called NETosis [3], control experiments analysed relative death of the cells using Sytox Green. As shown in Fig. 1D cells incubated in the presence or absence of A. hydrophila showed no significant differences in cell death, indicating that the bacteria do not block NETosis. Higher magnification of immunofluorescence micrographs revealed that cells co-incubated with A. hydrophila showed characteristic features of NETosis, including histone decondensation, loss of lobulated nuclei and subsequent extracellular release of nuclear DNA-histone-complexes (Fig. 1B). However, as described above, a significantly lower percentage area of NET coverage was detectable after co-incubation of cells with bacteria compared to non-infected cells, indicating that NETs are degraded by bacterial nucleases (Fig. 1A and C, Control versus Bacteria).
3.2 Effect of β-glucan on degradation of NETs by A. hydrophila

Interestingly, the co-treatment of neutrophils with β-glucan significantly protected the NETs against degradation by A. hydrophila nuclease: As seen in Fig. 1A and 1C (Bacteria versus Bacteria + BG), cells co-incubated with bacteria and treated with β-glucan showed significantly more NETs compared to infected cells not treated with β-glucan. Importantly, control experiments revealed no significant difference in the area of NET coverage comparing uninfected cells treated with or without β-glucan (Fig. 1A and C, Control versus BG). The cell death assay using Sytox Green (Fig. 1D) gave similar results as shown by immunofluorescence microscopy for the same uninfected groups (Fig. 1 C, Control versus BG), indicating that the addition of β-glucan did not induce a significant difference in the level of NETosis. These data differ from previously published data in zebrafish [7]. These authors showed that MacroGard® is able to induce NETosis in zebrafish kidney-derived cells [7]. Whether β-glucan induces NET-formation in carp neutrophils at other treatment time points and/or conditions, e.g. when less spontaneous NET formation can be found in unstimulated cells, remains to be determined and is under current investigation in our lab. However, since in our control experiments β-glucan shows no effect on NET formation, we can conclude that β-glucan is protecting the NETs against degradation by A. hydrophila nuclease.

3.3 Effect of β-glucan on bacterial growth and nuclease activity

To exclude that the NET-degradation in the presence and absence of β-glucan is not just a secondary effect based on the phenomenon that β-glucan affects the growth of A. hydrophila, the bacterial growth was monitored in the presence and absence of β-glucan over time. As seen in Fig. 2A, β-glucan showed no effect on the exponential growth rate of bacteria in LB medium over a period of 7 hours until reaching the stationary phase. However, when counting colony forming units/bacterial growth of A. hydrophila under similar
experimental conditions as used for the NET-degradation assay, β-glucan showed a slight but significant reduction of bacterial colony forming units compared to the initial inoculum used for NET-degradation assay (Fig. 2B). Interestingly, during the same 1 hour time period the nuclease activity in the respective bacterial supernatant was induced (Fig. 2C). Thus, it seems that β-glucan is stressing bacteria by slightly retarding its growth in RPMI and by activating its nuclease activity. Importantly, despite the β-glucan-mediated increase of \textit{A. hydrophila} nuclease activity (Fig. 2C), the degradation of NETs has been shown to be impaired in the presence of β-glucan (Fig. 1A and C, Bacteria versus Bacteria + BG). Thus, it might be assumed that β-glucan might effect the composition and stability of NETs and thereby making the NETs less susceptible to degradation by \textit{A. hydrophila} nuclease.

4. Conclusions

In this study we have shown for the first time that a β-glucan-feed material, namely MacroGard®, is able to protect NETs derived from carp neutrophils against degradation by \textit{A. hydrophila}. Interestingly, this phenomenon was not observed in fish that were fed a diet without any β-glucan addition (Supplemental Fig. 1). This supports the assumption, that β-glucan feed supplementation is modulating the fish immune system and thereby increasing protection against bacterial infections. Various immunomodulatory functions including increasing superoxide production or chemokine production as well as protective effects against bacterial infections of β-glucans have already been shown for instance for Nile tilapia [13], \textit{Anabas testudineu} [14], zebrafish [15] and Asian catfish [16], [17]. Interestingly, Selvaraj et al. [18] showed that the injection of β-glucan led to an increase in the relative percent survival of carp infected with \textit{A. hydrophila}. However, the detailed mechanisms behind this phenomenon are still not entirely clear. Based on our data and the study published by Palic et al. 2007 [7], it might be hypothesised that β-glucan is able to boost the host innate immune defence by inducing NET formation and by stabilising NETs against bacterial nuclease degradation, and thereby reduce severity of an infection with \textit{A. hydrophila}. Future work is
focussing on the role of β-glucan-associated NET-formation against \textit{A. hydrophila} infections \textit{in vivo} and on the mechanism of β-glucan-mediated stabilisation of NETs against bacterial nucleases.

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We wish to thank Marc Monestier, Temple University School of Medicine, Philadelphia, PA, for kindly providing us the H2A-H2B-DNA complex antibody. We would also like to thank Rolf Nordmo (Biorigin, Norway) for providing the MacroGard\textsuperscript{®}. The research leading to these results has received funding from the European Community's Seventh Framework Program (FP7/2007-2013) under grant agreement number PITN-GA-2008-21405.
Chapter 3 Aeromonas hydrophila degrades carp NETs

References

**Supplemental Fig. 1.** Effect of β-glucan on degradation of NETs by *A. hydrophila* using carp that were fed the same experimental diet without Macrogard®. Total cells were isolated from head kidney of the carp and incubated in the presence or absence of *A. hydrophila* (bacteria; MOI 0.01) and/or β-glucan (BG; 200 µg/ml MacroGard®). Presence of NETs was visualised by immunofluorescence microscopy using an antibody against H2A-H2B-DNA complexes (green) in combination with Dapi to stain all cell nuclei (blue): Control cells show spontaneous NET release (Control). Co-incubation of the cells with *A. hydrophila* led to the degradation of NETs (Bacteria). Treatment of the cells with β-glucan (200 µg/ml) did not protect the NETs against degradation by *A. hydrophila* (Bacteria + BG). Data are shown as mean ± SEM of *n*=6 individual fish.
Chapter 4

Cholesterol-depletion in human blood-derived neutrophils by methyl-β-cyclodextrin leads to formation of neutrophil extracellular traps.

Ariane Neumann*, Graham Brogden*, Natalja Jerjomicева, Susanne Brodesser, Hassan Y. Naim†, Maren von Köckritz-Blickwede†
Chapter 4 Role of cholesterol in NET formation

Foreword: In chapter 2 and 3 we have shown that β-glucan induces and stabilises NETs during *A. hydrophila* infections. Thus, it might be hypothesised that β-glucan affects elementary structures and/or signalling platforms in the neutrophil e.g. the cell membrane or lipid rafts. As the formation of NETs by neutrophils is a relatively new field of research, there are still many open questions surrounding the underlying mechanisms of NET formation. We have also showed in chapter 2 and 3 that carp NETs share morphological and functional similarities with human NETs. Since human neutrophils can be isolated very easily with a high yield of cells, they may serve as an excellent model to study the mechanisms in NET-formation. Therefore, we investigated the role of major membrane lipids, specifically cholesterol and sphingomyelin in NET formation by using human neutrophils isolated from peripheral blood as a model.

The extent of Graham Brogden’s contribution to the article is evaluated according to the following scale:

A. has contributed to collaboration (0-33%).
B. has contributed significantly (34-66%).
C. has essentially performed this study independently (67-100%).

1. Design of the project including design of individual experiments: B
2. Performing of the experimental part of the study: B
3. Analysis of the experiments: B
4. Presentation and discussion of the study in article form: B
Cholesterol-depletion in human blood-derived neutrophils by methyl-β-cyclodextrin leads to formation of neutrophil extracellular traps

Short title: Effect of cholesterol depletion on neutrophil extracellular traps

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SYNOPSIS

The formation of neutrophil extracellular traps (NETs) as a host innate immune defence mechanism has been shown to be the result of a novel cell death process called NETosis. Here we demonstrate that cholesterol depletion by methyl-β-cyclodextrin (MβCD) mediates the formation of NETs in a process that is independent of NADPH-oxidases.

Keywords: neutrophils, neutrophil extracellular traps, methyl-beta-cyclodextrin, NETosis, N-butyl-deoxynojirimycin, NADPH-oxidases
INTRODUCTION

Neutrophil extracellular traps (NETs) have recently been described as a novel phagocytosis-independent host innate immune defence mechanism against microbes [1]. Interestingly, NET-releasing neutrophils have been shown to enter a cell death program called NETosis [2] which clearly differs from classical cell death pathways such as apoptosis or necrosis. During NETosis the nuclear and cellular membrane dissolve, and the components, such as chromatin decorated with granular proteins are released into the extracellular space [3]. Those extracellular NETs can then be used by the dying cell to entrap and kill various microbial pathogens [4].

Mechanisms leading to the release of NETs are still not completely understood. Importantly, Chow et al. found that statin treatment of primary blood-derived neutrophils induced the formation of NETs [5]. Statins block the rate-limiting enzymes in the cholesterol biosynthesis through the inhibition of the HMG-CoA reductase. However, it remained unclear if this statin-mediated NET-formation is a result of direct cholesterol depletion in the cell or eventually only a secondary effect caused by statin-mediated transcriptional changes as reported for simvastatin-mediated changes in transcript expression of various cytokines in monocytes [6]. The objective of the study was to investigate the effect of cholesterol depletion on the formation of NETs.

MATERIALS AND METHODS

Isolation of neutrophils

The blood of healthy human donors was used for the isolation of polymorphnuclear cells (PMNs). Therefore blood was layered onto Polymorphprep™ (Progen) without mixing. A centrifugation step with 30 min at 370 x g without brake was performed. The plasma and mononuclear cells were removed and the PMNs including neutrophils were washed with 1X PBS (PAA); contaminating erythrocytes were lysed using water (Roth, pure water, sterile,
pyrogen free). Finally, the pellet was resuspended in 1 ml RPMI 1640 (PAA, without Phenol red, with L-glutamin).

**Lipid extraction and preparation**

To gain an insight into the effect of MβCD on the lipid composition of neutrophils, high performance thin layer chromatography (HPTLC) was performed. Therefore 1*10^5/ml blood-derived human neutrophils were treated with three concentrations of MβCD (1 mM, 5 mM and 10 mM) for two hours at 37°C with 5% CO₂. Subsequently the cells were lysed using 1% SDS and a 26 G cannula, and prepared for TLC as described by Bligh and Dyer [7]. Briefly, 2 ml methanol and 1 ml chloroform were added to each sample. Subsequently, the samples were rotated at room temperature for 30 min followed by centrifugation at 7°C for 5 min at 1952 x g. The supernatant was transferred to a new glass tube and 1 ml chloroform and 1 ml water was added, mixed briefly and centrifuged again at 7°C for 10 min at 17000 x g. After centrifugation, the upper aqueous layer was removed and the remaining organic phase was vacuum dried and stored at -20°C for further analysis.

**Thin layer chromatography**

Lipids were resuspended in 150 µl chloroform/methanol (1:1). Five µl of the lipid extract was loaded onto 20 x 10 cm HPTLC silica gel 60 plates (Merck). The HPTLC plates were developed using three solvent systems consecutively. Firstly, acetic acid ethyl ester/1-propanol/chloroform/methanol/0.25% potassium chloride (27:27:11:10), followed by n-hexane/diethyl ether/ acetic acid (75:23:2), and finally n-hexane. The plates were then stained with a phosphoric acid/copper sulphate (10:7.5) in water. Finally plates were heated at 170 °C for 10 min. The lipid bands were identified by comparison to authentic standard
substances and analysed using the CP ATLAS software (Lazarsoftware). Lipid identification was confirmed by Electrospray Ionisation-Tandem Mass Spectrometry (ESI-MS/MS) using a TLC-MS Interface (CAMAG) coupled to the pump of a 1200 Series Binary LC System (Agilent) and to the Turbo V ESI source of a 4000 QTrap mass spectrometer (AB SCIEX). Lipid bands were extracted using methanol with 5 mM ammonium acetate at a flow rate of 0.1 ml/min. Fatty acyl subspecies were determined in the positive (SM, PC, PS, PE, DAG, cholesterol, CE) or the negative (PI, fatty acids) ion mode by specific precursor ion or neutral loss scans. Precursor ions of m/z 184 were monitored for the determination SM and PC subspecies, precursors of m/z 369 for cholesterol and CE subspecies, and precursors of m/z 241 for the analysis of PI subspecies. A neutral loss of 185 was selected for the determination of PS subspecies, a loss of 141 for PE subspecies, and a loss of 35 for the analysis of DAG subspecies. Fatty acids were analysed in the full scan mode. The instrument settings for nebuliser gas (Gas 1), turbogas (Gas 2), curtain gas, and collision gas were 50 psi, 55 psi, 20 psi, and medium, respectively. The interface heater was on, the ESI source temperature was 350 °C, and the ionspray voltage was 5.5 kV. For all scans the values for declustering potential, entrance potential, and cell exit potential were 80 V, 10 V, and 10 V, respectively. The collision energies ranged from 35 to 55 V.

NET induction

To examine the implication of membrane lipids in NET formation, cholesterol was depleted by using different concentrations of MβCD (Sigma, final 1 mM, 5 mM, and 10 mM) and sphingolipid synthesis was blocked by N-butyl-deoxynojirimycin (NB-DNJ; Actelion Pharmaceuticals) at a final concentration of 5 µM, 50 µM, 250 µM and 500 µM). As a positive control for NET release, PMA was used at a final concentration of 25 nM (Sigma). $2 \times 10^5$/100 µl neutrophils were seeded into a 48-well-plate (Nunc), on poly-L-lysine (Sigma, 0.01%) coated glass slides. The plate was centrifuged for 5 min at 370 x g at room temperature, then incubated for 2 hours at 37°C with 5% CO₂ and fixed with 4% PFA. For the time kinetic
approach, the cells were treated with 10 mM of MβCD for 10, 30, 60, 90 and 120 min. Untreated neutrophils were used as a negative control. To test the role of NADPH-oxidases in NET-formation, neutrophils were incubated with 10 mM of MβCD in the presence and absence of 10 µg/ml diphenylene iodonium (DPI, Sigma) for 2 h at 37°C with 5% CO₂ to block. Finally the cells were fixed with 4% PFA.

**Immunofluorescence microscopy**

For visualisation of the NETs, glass slides were washed three times with PBS. Then cells were permeabilised and blocked using 2% BSA, 0.2% Triton X-100 in PBS for 45min at room temperature. Next, neutrophils were incubated with a mouse monoclonal antibody against H2A-H2B-DNA complex (PL2-6 [8], 2.65mg/ml, diluted 1:2000 in PBS containing 2% BSA, 0.2% Triton X-100) over night at 4°C. Finally, an Alexa 488-conjugated goat-anti-mouse antibody (Dy Light 488 conjugated highly cross-absorbed, Thermo; diluted 1:1000 in in PBS containing 2% BSA, 0.2% Triton X-100) was used as secondary antibody, and after washing the glass slides were embedded in ProLong® Gold antifade reagent with Dapi (Invitrogen). Samples were recorded using a Leica TCS SP5 confocal inverted-base fluorescence microscope with a HCX PL APO 40× 0.75-1.25 oil immersion objective. Settings were adjusted with control preparations using an isotype control antibody. For each sample, a minimum of 6 randomly selected images per independent experiment were acquired and used for quantification of NET-producing cells. Data were expressed as percentages of NET-forming cells in relation to the total number of cells.

**Quantitative cell death measurement by LDH release**

For the quantification of LDH release, supernatant of the cells from time kinetic experiments were collected prior to fixation. The supernatants were stored at -20°C until usage. For the total cell lysate, 25 µl of 1% Triton X-100 was added to the well. Finally, 100 µl of cells and
100 µl CytoTox-ONE™ reagent (Promega) were incubated for 10 min at 22°C on a 96 well plate. After incubation, 50 µl of stop solution was added and the fluorescence was recorded with an excitation wavelength of 560 nm and an emission wavelength of 590 nm, according to the manufacturer’s instructions.

**Statistical analysis**

Data were analysed by using Excel 2003 (Microsoft) and GraphPad Prism 5.0 (GraphPad Software). The NETs visualisation experiments were performed with 2 to 4 replicates in at least 3 independent experiments. Differences between 2 groups were analysed by using a paired, one-tailed Student’s t-test. The lipid results were obtained from 3 independent experiments each performed on a separate HPTLC plate. Differences between the treatment groups within each lipid class were analysed by using repeated measures 1-way ANOVA. The significance is indicated as * p<0.05; ** p< 0.005 and *** p< 0.001.
RESULTS AND DISCUSSION
**Figure 1.** MβCD depletes cholesterol from human blood-derived neutrophils.

(A) $1\times10^7$/ml human blood-derived neutrophils were prepared for lipid extraction according to Bligh & Dyer. HPTLC was utilised to display the lipid composition pattern of the neutrophils after treatment with 1 mM, 5 mM and 10 mM of MβCD (denoted as CD) for 2 h; lipid bands are referred to abbreviations given in 1B. (B) Table representing abbreviations of lipid classes displayed as bands in 1A. (C) $1\times10^7$/ml cells were treated with 1 mM, 5 mM and 10 mM of MβCD for 2 h. After lipid extraction and HPTLC separation, the lipid bands were analysed using CpAtlas software. Among all analysed lipids only cholesterol was significantly affected by MβCD treatment (5 mM and 10 mM). Other lipid classes like SM were also affected, but the change was not significant. Means of three independent experiments on three independently developed silica plates with **<0.01, ***<0.001 are shown.
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A

% cells releasing NETs

B

C

D

% cells releasing NETs

E

Relative fluorescence units

F

% cells releasing NETs

G

H

I

J

K

L

M

N

O

P

Q

R

S

T

U

V

W

X

Y

Z
Figure 2. Cholesterol depletion by MβCD leads to formation of NETs.

(A) 5*10^5 human blood-derived neutrophils were treated for 2 h with three different concentrations of MβCD (1 mM, 5 mM and 10 mM), fixed with 4% PFA and immunostained with NET-specific antibodies. NET release was visualised and quantified using fluorescence confocal microscopy. All three concentrations of MβCD significantly induced the formation of NETs. (B) Representative fluorescence micrographs displaying the results from (A), with NETs stained in green and nuclei in blue; untreated neutrophils served as control. (C) 5*10^5 cells were treated for 2 h with three different concentrations of NB-DNJ (final 5 µM, 50 µM, 250 µM and 500 µM) and PMA as a positive control (final concentration of 25 nM), fixed with 4% PFA and immunostained to visualize NET-formation. NB-DNJ was not able to induce NET release in three independent experiments. (D) 5*10^5 human blood-derived neutrophils were stimulated with 10 mM of MβCD for 10 min, 30 min, 60 min, 90 min and 120 min, untreated neutrophils served as control; a time-dependent NET release was already observed after 30 min. (E) Supernatants of time kinetic experiment shown in (D) were used to quantify loss of membrane integrity (cell death) by measuring relative fluorescence units of CytoTox-ONE™ reagent (Promega) as a marker for LDH release. (F) 5*10^5 cells were incubated for 2 h with 10 mM of MβCD in the presence and absence of 10 µg/ml DPI to block NADPH-oxidase activity. While the PMA-mediated NET release was significantly affected by the DPI-treatment, MβCD-treated cells showed no change in the NET release when treated either with or without DPI All data are shown as minimum of n=3 independent experiments; *<0.05, **<0.01, ***< 0.001.
Cyclodextrins, such as MβCD have been shown to be reliable and effective tools used to investigate the physiological role of cholesterol [9]. Cyclodextrins are cyclic oligosaccharides which are able to form complexes with hydrophobic molecules. As such MβCD has been widely used to deplete cholesterol from cellular membranes by increasing the water solubility of the sterol [10]. Here we investigated whether cholesterol depletion by MβCD may influence the formation of NETs.

Therefore, primary blood-derived neutrophils were treated with different concentrations of MβCD for 2 h. The effect of MβCD on the lipid composition of the cells was determined using HPTLC analysis. The identities of lipids separated by HPTLC were confirmed by MS/MS. As shown in Figure 1C, treatment of human neutrophils with MβCD revealed several changes in the lipid composition after 2 h of incubation. These changes were only statistically significant for cholesterol at a concentration of 5 mM and 10 mM MβCD (Figure 1C). Sphingomyelins (SM) were also slightly affected by the MβCD treatment, but this effect was not statistically significant (Figure 1C).

After having confirmed the successful depletion of cholesterol from membranes of primary neutrophils, the formation of NETs was studied using immunofluorescence microscopy. Neutrophils incubated for 2 h with 1 mM, 5 mM or 10 mM MβCD showed a dose-dependent significant increase in NET production (Figure 2A and B). These data indicate that cholesterol-depletion by MβCD induce the formation of NETs. To confirm the cholesterol-specificity and to exclude that general membrane damage by MβCD has caused the NET-formation, we tested the effect of NB-DNJ on NET-formation. NB-DNJ has been shown to interfere with the formation of glycosphingolipids by inhibiting the ceramide specific glucosyltransferase enzyme which is responsible for initiating the glycosphogolipid synthesis pathway [11]. NB-DNJ is often used to treat lysosomal storage diseases such as Fabry disease [12] and Niemann-Pick type C disease [13] by reducing the levels of glycosphingolipids and sphingomyelin associated with the diseases. As shown in Figure 2C, we could show that the treatment of the neutrophils with NB-DNJ had no effect on NET
formation compared to the PMA control. Since other lipid classes apart from cholesterol were not affected by the MβCD treatment, our data strongly suggest that NET release is dependent on the cholesterol content in the membranes.

When using only 10 mM of MβCD in a time kinetic approach, we found a time-dependent NET release, which was already significant after 30 min of incubation (Figure 2D). Since NET formation has been shown to be the result of a novel cell death mechanism called NETosis, cell death was quantified using an LDH assay. In good correlation to the above mentioned data, a statistically significant increase in LDH release as an indicator for cell death was already observed at 10 min of incubation with MβCD (Figure 2E). Since ROS-dependent [2] and ROS-independent [14] pathways have been described to be involved in NETosis mediated NET-formation, we finally tested whether NADPH-oxidases play a role in the MβCD-mediated formation of NETs. The cells were treated with DPI to block NADPH-oxidase-dependent ROS-formation at the same time when treating the cells with MβCD. While the control experiment revealed a significant reduction in PMA-induced NET release upon blocking of NADPH-oxidases with DPI, the MβCD-induced NET formation was not affected (Figure 2F). Thus, our data indicate that the MβCD-mediated NET-formation process is independent of NADPH-oxidases.

In conclusion, the present study shows that MβCD treatment of primary blood-derived neutrophils leads to the depletion of cholesterol on the cell and thereby facilitates NADPH-oxidase-independent formation of NETs. Our results correlate with a previous report that demonstrated an induction of NETs in a NADPH-oxidase-independent process by statins [5]. Nevertheless, in these studies it was unclear if cholesterol depletion or transcriptional changes were responsible for that phenotype [5]. Our data extend these findings and unequivocally demonstrate that cholesterol depletion can directly induce NETosis in a short period of time, already after 30 min of treatment with 10 mM MβCD. Thus, these data provide important new information on the role of cholesterol in NET-formation. Given that cholesterol is an essential lipid in the composition of membrane microdomains (lipid rafts) [15] and
cellular signalling events involved in host-pathogen interactions [16], it would be intriguing to assess the implication of these membrane structures in regulatory mechanisms leading to NETosis as a novel host innate immune defence mechanism against invading pathogens.

**AUTHOR CONTRIBUTION**

A.N. and G.B. performed research, analysed and interpreted data and wrote the manuscript; N.J. performed research, analysed and interpreted data; S.B. performed TLC-MS analysis, provided interpretation of data and critical proof-reading of the manuscript; H.Y.N. and M.v.K.-B designed research, interpreted data and wrote the manuscript.

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Chapter 5

Isolation and analysis of membrane lipids and lipid rafts in common carp (*Cyprinus carpio* L.).

Graham Brogden, Marcus Propsting, Mikolaj Adamek, Hassan Y Naim, Dieter Steinhagen
Foreword: The lipid membrane acts as a barrier between the external and internal environments of a cell. The cell membrane comprises of cholesterol rich lipid microdomains, termed lipid rafts, which are interspersed like islands in a sea of phospholipids. A large amount of research has been conducted into lipid rafts in mammals, however relatively little work has been performed on fish lipid rafts. Therefore, we established a protocol allowing for the isolation and characterisation of carp lipid rafts and detergent soluble membrane fractions. This method will be used as a basis for future biochemical studies on fish cell membranes.

The extent of Graham Brogden’s contribution to the article is evaluated according to the following scale:

A. has contributed to collaboration (0-33%).
B. has contributed significantly (34-66%).
C. has essentially performed this study independently (67-100%).

1. Design of the project including design of individual experiments: B
2. Performing of the experimental part of the study: C
3. Analysis of the experiments: C
4. Presentation and discussion of the study in article form: C
Isolation and analysis of membrane lipids and lipid rafts in common carp (Cyprinus carpio L.)

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Abstract

Cell membranes act as an interface between the interior of the cell and the exterior environment and facilitate a range of essential functions including cell signalling, cell structure, nutrient uptake and protection. It is composed of a lipid bilayer with integrated proteins, and the inner leaflet of the lipid bilayer comprises of Liquid ordered (Lo) and Liquid disordered (Ld) domains. Lo microdomains, also named as lipid rafts are enriched in cholesterol, sphingomyelin and certain types of proteins, which facilitate cell signalling and nutrient uptake. Lipid rafts have been extensively researched in mammals and the presence of functional lipid rafts was recently demonstrated in goldfish, but there is currently very little knowledge about their composition and function in fish. Therefore a protocol was established for the analysis of lipid rafts and membranous lipids in Common Carp (Cyprinus carpio L.) tissues. Twelve lipids were identified and analysed in the detergent soluble fractions of the membrane with the most predominant lipids found in all tissues being; triglycerides, cholesterol, PE and PC. Four lipids were identified in lipid rafts in all tissues analysed, triglycerides (33-62%) always found in the highest concentration followed by cholesterol (24-32%), phosphatidylcholine and sphingomyelin. Isolation of lipid rafts was confirmed by identifying the presence of the lipid raft associated protein flotillin, present at higher concentrations in the detergent resistant fraction. The data provided here build a lipid library of important carp tissues as a base line for further studies into virus entry, protein trafficking or environmental stress analysis.

Keywords: Carp, lipid rafts, lipid analysis, DRM, DSM, Flotillin.
Chapter 5 Characterisation of carp lipid rafts

1 Introduction

The cell membrane acts as an interface between the interior machinery of the cell and the exterior environment. Cell membranes are composed of a lipid bilayer with integrated proteins, which facilitate the movements of ions or serve as adhesion and signalling molecules. In the outer leaflet of the lipid bilayer, both a liquid ordered phase, \( L_0 \) and a liquid disordered phase \( L_d \) is present (Rietveld et al. 1998). The \( L_0 \) and \( L_d \) phases consist of proteins and lipids, of which a higher percentage of cholesterol and sphingolipids are found in the \( L_0 \) phase. The \( L_0 \) phase is currently defined as dynamic, nanoscale, sterol–sphingolipid-enriched, ordered assemblies of proteins and lipids and these microdomains are associated with a range of important functions including cell signalling and nutrient transport (Simons et al. 2010). Experimentally, \( L_0 \) and \( L_d \) membrane fractions can be isolated with a non-ionic detergent such as Triton X-100 under ice cold conditions using a protocol described by Alfalah et al. (Alfalah et al. 2005). Therefore, \( L_0 \) membrane fractions were also described as detergent resistant membranes (DRMs) or lipid rafts (Brown 2006). Although these membrane fractions were first discovered over 15 years ago, there is still a lot of contention surrounding these membrane microdomains, including the size and longevity. The \textit{in vitro} methodology used has for instance led to discussions questioning if the DRM fractions are indeed real and not artefacts produced from the methodology (Munro 2003), whereas data produced from \textit{in vivo} studies could not always be reproduced \textit{in vitro} (Munro 2003).

Nevertheless, lipid rafts have been extensively studied in mammals and meanwhile the field has grown rapidly (Lingwood et al. 2010). The lipid raft model is now used to investigate a large range of processes including virus entry (Nguyen et al. 2000; Ono et al. 2001), protein trafficking (Brown et al. 1998), metabolic diseases (Maalouf et al. 2009) and cell signalling (Varma et al. 1998). In fish, lipid rafts were extracted from the brush border membranes (BBM) of enterocytes from Atlantic cod (Gylfason et al. 2010), rainbow trout hepatocyte membranes (Zehmer et al. 2003; Zehmer et al. 2004; Zehmer et al. 2005), and recently functional lipid rafts were demonstrated in the cell membrane of goldfish leukocytes (Garcia-Garcia et al. 2012).

Compared to mammals, however very little is known about lipid rafts in aquatic species. There is currently no published work on the lipid composition of different fish tissues and of the composition of lipid rafts isolated from these tissues in fish.

One of the advantages of using fish as a model species to examine the roles of DRMs and DSMs is that it is possible to investigate the effects of environmental stresses on membranes in an \textit{in vivo} system. Laboratory lipid raft models have been produced to analyse the environmental stresses on the membrane and its fractions, however it can be argued that these membranes are artificial and produce results which are difficult to verify. Zehmer and Hazel (Zehmer et al. 2003; Zehmer et al. 2004; Zehmer et al. 2005) investigated the effects of temperature on rainbow trout hepatocyte membranes, by keeping the fish at 20°C and a lower temperature of 5°C. The membrane fractions were isolated and analysed using a sucrose density gradient. The hepatocyte cells showed significant
changes in the lipid composition of the lipid rafts; however the order of the lipid rafts appeared to remain unchanged.

Analysis of these membrane fractions will help in our understanding of how fish cells function and respond to different challenges and environments.

Therefore, in this work we are presenting the lipid composition of both DRMs and detergent soluble membrane (DSM) fractions of six carp tissues. The ultimate goal of the study was to build a lipid raft library of important tissues so that the results and methodology can be used as a basis for future studies into virus entry, protein trafficking, metabolic diseases, signalling and environmental stress experiments.

2 Materials and Methods

2.1 Fish

Common carp (Cyprinus carpio L.) used in this study were produced from crossing 2 polish carp lines, line K with line R3. The fish were obtained from the Institute of Ichthyobiology & Aquaculture in Gołyś (Poland). The fish were grown in pathogen free conditions in re-circulated tap water, fed 2 % body weight per day and kept at a temperature of 22±1°C. All animal experimentation was performed regarding EU standards for animal experimentation according to the EU Directive 201/63/EU and national German legislation.

2.2 Extraction of detergent resistant membranes fractions

Detergent resistant membrane fractions were isolated from common carp by using a method adapted from Alfalah et al. (Alfalah et al. 2005). Carp were killed by an immersion into a solution of 0.5 g/L tricane methan solphonate (MS 222, Pharmaq, UK) and samples from the kidney, head kidney, spleen, liver, gut and gills, each approximately 25 mg, were collected and placed on ice in 1 ml 1% Triton X-100 PBS solution. Samples were processed firstly by homogenising for 10 seconds with an ultra Turrax (Juergens), followed by a further homogenisation step for 1 min in a glass potter (B. Braun Melsungen). The samples were then further processed through syringing, firstly 20 times with a 0.8 mm canule syringe followed by a 0.45 mm canule syringe 15 times. The homogenates were incubated whilst rotating overnight at 4°C. From the homogenates, the cell debris was pelleted by centrifugation at 4°C at 13 000 x g for 30 min. Then the DRM fraction was separated from DSM fraction by ultracentrifugation of the supernatant at 100 000 x g for 90 min at 4°C. After centrifugation, the DSM supernatant fraction was removed from the tube using a Pasteur pipette and the DRM pellet was resuspended in 300 µl distilled water. The two fractions were stored at -20°C for later analysis.
2.3 Lipid extraction and preparation

From DRM and DSM fractions, lipids were separated from the proteins by chloroform/methanol as described by Bligh and Dyer (1957) with minor modifications. The protocol was performed on ice unless otherwise stated. Briefly, the samples were homogenised in a potter for 1 min, then 2 ml methanol of and 1 ml of chloroform were added and the samples homogenised again for 1 min. The samples were then continuously mixed for 30 min at room temperature and centrifuged for 5 min at 600 x g and 7°C. The supernatant was transferred into a clean glass tube and the protein pellet was dried and stored at -20°C for future analysis. One ml of both chloroform and distilled water was added to the supernatant. The sample was then centrifuged again for 10 min at 600 x g and 7°C. The second phase was removed and the remaining fraction was vacuum dried and stored at -20°C.

2.4 Lipid analysis

Lipid samples were re-suspended in 250 µl chloroform/methanol solution. The samples were analysed by High Performance Thin Layer Chromatography (HPTLC) on 20 x 10 cm TLC Silica gel 60 glass plates (Merck, Germany). Ten µl of the lipid solution was loaded drop-wise onto the plate in triplicates. The plates were placed in three running solutions, the first running solution comprised of ethyl acetate, 1-propanol, chloroform, methanol and potassium chloride. The second solution consisted of n-hexane, diethyl ether and citric acid. The third running solution was 100 % n-hexane. The distance the samples migrated in the running solutions were optimised depending on if the sample contained more polar (DRM fraction) or non-polar (DSM fraction) lipids. To visualise lipid bands, the plates were stained in copper sulphate solution for approximately 7 seconds.

2.5 Lipid identification and qualification

The lipid bands were analysed using CP Atlas (Lazersoftware), which assigned each lipid an ‘rf’ value indicating the distance that each band migrated. The rf value of each lipid was compared to a known standard comprising of 14 commonly found lipids in mammalian cell membranes (see table 1). The results are expressed as a total of 100% from the lipids detected at significant concentrations. Lipids were donated as ‘trace’ when a lipid produced a clearly visible band, consistent in every sample on the HPTLC plate, however the band was below the reliable detection limit for that particular lipid.
2.6 Western blotting

Kidney and spleen samples were collected and placed into 1 ml triton X-100 and stored on ice. Lipid rafts were isolated using the method described in section 2.2, with the only amendment being the addition of protease inhibitors prior to homogenising. The pellet was resuspended in distilled water and 5% of the total volume was taken from both the pellet and the supernatant fraction. Briefly, the samples were loaded onto a 12 % polyacrylamide gel and ran for the first 30 min at 80 V and thereafter at 120 V. The proteins in the gel were then transferred onto a PVDF membrane (Roth) at 250 Amps for 120 min. The membrane was blocked overnight in 5 % milk-PBS at 4 °C and then incubated with anti-flotillin 2 (B-6) antibody (Santa Cruz) at a concentration of 1:5000 in 2 % milk-PBS. The membrane was then washed three times with 1 % Tween-PBS and incubated with the secondary polyclonal anti mouse HRP conjugated antibody (Amersham Biosciences) for 1 hour at room temperature at a concentration of 1:5000 dissolved in 2 % milk-PBS. The bands were then visualised by using SuperSignal® ELISA Femto Maximum Sensitivity Substrate from Pierce and detected using a ChemiDoc XRS Molecular Imager (Bio-Rad) according to the manufacturer’s guidelines.

2.7 Statistical analysis

Statistical analysis was performed using SigmaPlot 12. All data presented as percentages was first transformed using arcsin. Statistical analysis investigating the differing concentrations of lipids between tissues was performed using a 1 way ANOVA with Tukey on arcsin transformed data. A two way ANOVA coupled with the Holm-Sidak test was used to compare the differences between lipids within the same tissue.
3 Results

**Figure 1**: Analysis of Flotillin in detergent soluble (DSM) and detergent resistant (DRM) membrane fraction of carp kidney and spleen by western blot. Higher concentrations of flotillin, a protein associated with lipid rafts in mammalian cells were found in the DRM fraction compared to the DSM fraction in both spleen and kidney samples. The marker (arrow) represents 55 kDa and the expected size of mammalian flotillin is 48-49 kDa.
Figure 2: Lipid composition of detergent soluble membrane fractions (DSM) of six carp tissues. In total 12 lipids were identified and analysed in all samples collected from three individual carp and analysed by HTPLC. The lipids were identified by comparing the band to a known standard and expressed as a percentage (+SD). Key to the abbreviations see table 1.
Figure 3: Lipid composition of detergent resistant membrane (DRM) fractions of six carp tissues. 9 lipids were identified and analysed in samples collected from three individual carp and analysed by HTPLC. The lipids were identified by comparing the band to a known standard expressed as a percentage (+SD). Lipid shown to have trace amounts were determined as having a band intensity that was below the identified detection limit for this particular lipid. Key to the abbreviations see table 1.
Chapter 5 Characterisation of carp lipid rafts

**Figure 4**: Comparison of polar and non-polar lipids in detergent resistant (DRM) and detergent soluble (DSM) membrane fraction of carp tissues. Samples were collected from three individual carp and analysed by HTPLC.

**Figure 5**: Relative amount of cholesterol, PC and SM in detergent resistant membrane fractions of 6 different tissues in common carp. The average ratio of cholesterol, PC and SM was 72:20:8. N=3
Chapter 5 Characterisation of carp lipid rafts

3.1 Isolation of lipid rafts

Using the method described in sections 2.2, 2.3 and 2.4, DRM and DSM fractions were consistently and successfully separated from all six tissues analysed. When equal amounts of each fraction were analysed for the presence of flotillin, a lipid raft associated membrane protein, a much stronger band was found in the DRM fraction isolated from both the spleen and kidney. This indicates that using this methodology, the isolated DRM fractions share the same lipid and flotillin distribution characteristics as observed in mammalian tissues (Eckert et al. 2003) and are therefore enriched in lipid rafts.

3.2 Composition of DRMs (lipid rafts)

A total of nine different lipids were identified in lipid raft samples across the six different tissues. Sphingomyelin (SM), phosphatidylcholine (PC), cholesterol (Chol) and free fatty acids (FFA) were present in all tissues samples and in addition phosphoserine (PS), triglycerides (TG), plus trace amounts of phosphoinositol (PI), cardiolipin (CA) and phosphethanolamine (PE) were also detected.

All lipid rafts analysed showed similar traits. Lipid rafts contained a much higher concentration of non-polar lipids compared to polar lipids (Fig. 4). The difference between the two groups of lipids was statistically significant ($p \geq 0.001$), however no difference was found between tissues. High concentration of the non-polar lipids, cholesterol and free fatty acids were present in all tissues (Fig. 3 and 4) and lower concentrations of SM, PC and, when present, PS. Cholesterol was found at significantly higher concentrations, compared to PC and SM in every tissue tested ($p \geq 0.05$). Likewise, PC was also found at significantly higher concentrations, when compared to SM in every tissue analysed ($p \geq 0.05$). There is an uneven distribution of the lipids analysed in each tissue, with cholesterol, FFA and in the head kidney and gills triglycerides, at much higher concentrations compared to PC, PS and SM. There were no significant differences in the lipid concentrations between tissues. Higher concentrations of FFA relative to cholesterol were also present in the lipid rafts of every tissue analysed (Fig. 3).

Trace measurements of PS were found in the spleen, and traces of PI and CA were found in the spleen, gills and gut isolated lipid rafts. The gut-isolated lipid rafts also had an additional band present at a height corresponding to PE. Five out of the six tissues showed the absence of PE, with the exception being the lipid rafts isolated from the gut. FFA was present in all analysed tissues. PC and SM were also present in every tissue, with PC always present in a higher concentration relative to SM.

3.3 Lipid composition of DSMs
DSM fractions were isolated from six different carp tissues and their lipid composition ascertained.

All tissues contained high concentrations of PC, PE, FFA, cholesterol and, where analysed, TG. PE was found in high concentrations in all tissues analysed except for the gills, and was usually the 3rd or 4th most significant lipid. However, the level of PE in the gills was found to be significantly lower than in the spleen, gut, kidney and head kidney ($p \geq 0.05$). TG was found in higher concentrations than cholesterol/FFA in four out of the six tissues analysed, with the exceptions being head kidney and spleen, however TG was not analysed in the spleen.

An unidentified lipid was identified as a persistent band which had a migration pattern between that of PC and PS, however it was only present in significant concentrations in the gills.

Comparing the polar versus non–polar ratio, illustrated in Fig. 4, the results showed that five out of the six analysed tissues contain a higher concentration of polar lipids, with the exception being the gills. The gills have a significantly higher concentration of non-polar lipids compared to polar lipids. The percentage of polar lipids ranges from 69 to 50 % and non-polar from 31 to 50 %. All DSM fractions (Figs. 2 and 4), when compared to their corresponding lipid rafts were also statistically different regarding the polar/non-polar lipid compositions ($p \geq 0.001$).

### 3.4 Comparison of lipid raft with DSM fractions

The lipid compositions of lipid rafts (Fig. 3) and DSM fractions (Fig. 2) were significantly different. Every DSM fraction contained relatively high levels of PE, however in every corresponding tissue, except for the gut where a trace of PE was present, no PE was detected in the lipid raft fraction. Likewise, there was a greater range of phospholipids present in the DSM compared to the lipid raft fraction. In all analysed samples, the lipid raft fraction contained a higher percentage of cholesterol and FFA, plus a lower concentration of PC, SM and PS, relative to the corresponding DSM fraction.

Assigning lipids into polar and non-polar groups showed clear, statistically significant differences between lipid rafts and DSM fractions. The lipid raft fraction contains a high percentage of non-polar lipids, ranging from 81 to 92%, and a much lower percentage of polar lipids ranging from 8 to 19% depending on the tissue.
4. Discussion

This article describes the isolation and characterisation of DRM (lipid rafts) and DSM fractions from different carp tissues. The methodology also describes the analysis of a lipid raft marker protein used to validate this method.

There has been a large volume of published articles using a similar method described to analyse the lipid composition of mammalian tissues and cells (Harder et al. 1998), and more recently a few papers investigating the lipid composition of piscine derived (Zehmer et al. 2003; Zehmer et al. 2004; Zehmer et al. 2005) cells, however these investigation have not focused on the lipid composition and have therefore not used the method described here to define the presence and abundance of various lipids. Therefore in the present investigation the validity of the lipid raft isolation methodology was assessed by comparing the concentrations of a lipid raft marker protein in both the lipid raft and DSM isolated fractions in two different tissues. Flotillin is a ubiquitous predominately lipid raft-associated protein which is involved in the building of lipid rafts and is also commonly used as a lipid raft marker (Garin et al. 2001). Importantly, higher concentrations of flotillin were detected in lipid raft fractions compared to DSM fractions. Furthermore, the protein distribution was also ascertained, showing that approximately 25% of the membrane protein was located in Triton X-100 lipid rafts (data not shown). This result also corresponds with data published in mammalian cells (Fabelo et al. 2011).

Analysis of lipid raft membrane fractions from the six tissues, head kidney, kidney, liver, spleen, gut and gills, revealed that the Triton X-100 microdomains consist of a minimum of four basic lipids at significant, measurable concentrations. Cholesterol, FFA, PC and SM were consistently present. However other lipids were detected in the majority of tissues and in several samples, but some of these lipids could not be reliably and consistently measured with this methodology, such as cholesteryl ester, monoacylglycerol and triglycerides.

The presence of the four common lipids fits with previously published data on the composition of lipid rafts and shows the importance of these lipids in both mammalian and fish cell membranes (Brown et al. 1998). Lipid rafts all have a higher concentration of cholesterol compared to the detergent soluble fraction, whereas the lipid rafts are comprised of approximately 30% cholesterol, with the DSM fractions ranging from 25% (cholesterol and Free fatty acid combined total) in the gills to 8% (cholesterol and free fatty acid combined total) in the spleen. These results are in accordance with the role of cholesterol, which has been shown to be associated with membrane rigidity, and are required for lipid rafts to function (Silvius 2003).

In lipid rafts from mammalian cells, the levels of cholesterol, PC and SM have been shown to be all at similar levels, where in vitro studies use cholesterol/PC/SM at a ratio of 1:1:1 (de Almeida et al. 2003). Interestingly, the results presented here show a much greater concentration of cholesterol, with the ratio closer to 72:20:8. This difference may be attributed to the different environment that
the fish inhabits, specifically the lower temperature ranges. Cholesterol is critical for the stability and function of lipid rafts, as studies have shown the importance of cholesterol in the formation of lipid rafts (Silvius 2003) and, when cholesterol is depleted from the cells by using methyl-beta-cyclodextrin, the function of lipid rafts is adversely affected (Zidovetzki et al. 2007). Therefore significant concentrations of cholesterol were expected to be present in carp cell membranes.

All lipid raft samples analysed comprised of a high percentage of cholesterol and triglycerides (polar lipids) and a lower concentration of SM ranging from approximately 3.5 to 9.5% of the total measured lipid composition. SM was also an ever present lipid in every lipid raft sample analysed at significant concentrations, underlying the importance of this lipid in the cell membrane of fish. However the low levels at which SM was detected are surprising given that in mammalian cells it is found at much higher concentrations. SM has been described as an important lipid component of lipid rafts in mammals (Brown et al. 1998), and the data presented here suggests also in carp, has been shown to be important in the sorting of GPI-anchored membrane proteins (Brown et al. 1992).

Zehmer et al. (Zehmer et al. 2003; Zehmer et al. 2004; Zehmer et al. 2005) investigated the effects of temperature on Atlantic salmon hepatocyte membranes, by keeping the fish at 20°C and a lower temperature of 5°C. The membrane fractions were isolated and analysed through a sucrose density gradient followed by TLC and mass spectrometry. Following acclimatisation, the hepatocyte cells showed a decrease in PC from 56 to 30% and an increase in both PI and PS from 8 to 20% in fish kept at 5°C and 20°C respectively (Zehmer et al. 2005). Cholesterol was also shown to decrease by 69% from cold acclimatised trout but the order of the raft appeared unchanged (Zehmer et al. 2004). Using a comparable method the same authors showed that in trout the lipid raft fraction contained a significantly higher concentration of phospholipids compared to cholesterol, which is contradictory to our results, showing a much higher concentration of cholesterol to phospholipids in the lipid raft fraction. The effects of temperature and lipid composition has also been described by Avery and colleagues (Avery et al. 1995), where they showed that chilling amoeba A. castellanii induced a short-term inhibition of phagocytosis activity, however the activity increased again when the degree of fatty acid saturation increased.

Analysis of DSM fractions revealed a minimum of seven lipids present at significantly high concentrations. Generally all tissues were found to contain significant concentrations of TG, FFA, cholesterol, PE, PC, PS and CA. Every sample from every tissue analysed always contained the higher concentrations of PE, which is predominately present in the detergent soluble fractions and not the detergent resistant fractions, further supporting this methodology for the isolation of lipid rafts with the non-ionic detergent Triton-X 100 (Fabelo et al. 2011).

The DSM fractions showed a completely different lipid profile when compared to DRM fractions, with considerable variations between tissues. PE is an ever-present lipid found in all DSM samples, however large differences were found between spleen and gill DSM fractions, with 16% and 5%
concentrations analysed in each tissue respectively and an average of 13.5% for the other five tissues excluding the gills. The gills are also the only tissue analysed which contained similar levels of triglycerides to free fatty acids and cholesterol. The concentration of PE in the gills was found to be significantly lower than in the gut, spleen, head kidney and kidney. A similar trait was also shown in the liver, however the difference was not significant.

Interestingly, when compared to mammalian tissues, where PE is commonly the second or third most prevalent lipid (Wubbolts et al. 2003; Subra et al. 2007), the six analysed carp tissues all had relatively low levels of PE indicating that low levels of PE may be a fish specific cell membrane phenotype. This is surprising because PE is associated in having an important role in cell homeostasis. These differences may be down to the unique structure and function of the gills as an organ specialised in the exchange of gases. It could be argued that the higher than average concentrations of cholesterol in both the lipid raft and DSM fractions could therefore compensate for the lower levels of PE and provide added stability to the membranes of gill cells.

It is assumed that the function of each tissue will be reflected in the lipid composition of the tissue and the role of each particular lipid. For example, the results show that the liver contains very high levels of TG in the DSM fraction. TG are important energy storage lipids, so it is interesting that the liver contains relatively high levels of this metabolically important lipid.

The results obtained from the liver lipid composition were in accordance with the function of the tissue as a site for metabolism, with high concentrations of free fatty acids in the DSM and triglycerides in the lipid raft fraction. The gut sample contained a more diversified range of cell types, mucus and also the micro flora residing within the gut, which might explain the lipid composition of this tissue containing a broader range of lipids and a higher concentration of PC, which is important for bacterial growth (Garsin 2010), compared to other tissues analysed.

The head kidney can be used as a further example to show the relationship between the lipid composition of each tissue and the known role each lipid can play. The head kidney is regarded as an important immune competent organ in fish responsible for the production of pluripotent cells and is functioning similarly to bone marrow in mammals. High levels of FFA and cholesterol, and also very low levels of TG were found in this tissue, which relates to the importance of theses lipids in immune functions. Analysis of common carp head kidney cells showed that the tissue comprised of approximately 39% lymphocytes, 36% neutrophils 20% monocytes and 4% basophils (Scharsack et al. 2003). Previous work has shown the importance of cholesterol in the activity of immune cells, for example, statin-induced cholesterol depletion showed the importance of cholesterol synthesis on the function of neutrophils (Chow et al. 2010). However this relationship will be more complex in fish due to the seasonal temperature changes in the fishes’ habitat and would therefore require further investigation.
Chapter 5 Characterisation of carp lipid rafts

It is expected that the cell membranes of mammalians and fish perform similar basic functions, therefore the structure is also expected to be similar. The presence of lipid rafts in carp, goldfish (Garcia-Garcia et al. 2012) and Atlantic cod (Gylfason et al. 2008) shows that this membrane structure has been conserved between fish species and mammals. However, differences in the environment such as temperature and osmotic variations which can fluctuate seasonally depending on the species will require a greater flexibility in the membrane. Migratory species, such as trout, eels and salmon, and species living in areas where seasonal flooding occurs, for example in the Amazon basin will need to adapt to different conditions and probably employ a system to combat changes in temperature or water parameters.

Future investigations can also be conducted investigating the importance and roles the cell membrane, and in particular the lipid rafts play in host-parasite interactions and how these functions compare between aquatic species.

5. Conclusion

Lipid rafts and DSM fractions were successfully isolated from six different carp tissues. Lipid raft fractions consisted of 4 main ever-present lipids and higher concentrations of flotillin. The lipid rafts were found to have a similar lipid composition in each analysed tissue, however, interestingly there were large differences evident between the lipid composition of carp tissues-derived lipid rafts and mammalian lipid rafts. Lipid rafts have to perform the same basic functions, trafficking and signalling, which would explain why the lipid composition between tissues is conserved. However, differences between mammals and fish are likely to be because of the different environments that fish inhabit compared to mammals, especially regarding temperature.

The DSM fractions contained a broader range of lipids, with at least seven different lipids consistently present in all tissues. Interestingly, large differences were observed between DSM fractions between different tissues. As differences are also observed in mammals between differing cell origins, the differences can be attributed to the different functions and environments surrounding the cells in each tissue within the fish.

The establishment of this method in fish allows for further studies to be performed, such as investigating the effects of feeding-enriched feeds on the lipid raft composition. Further investigations are needed on fish specific situations, such as certain environmental factors including climate change associated temperature and salinity alterations.
Acknowledgements

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References


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Table 1. Overview of lipids included in the standard for TLC analysis

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Abbreviation</th>
<th>Polarity</th>
<th>rf value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysophosphatidylcholine</td>
<td>LPC</td>
<td>Polar</td>
<td>0.95</td>
</tr>
<tr>
<td>Sphingomyelin</td>
<td>SM</td>
<td>Polar</td>
<td>0.92</td>
</tr>
<tr>
<td>Phosphatidyl choline</td>
<td>PC</td>
<td>Polar</td>
<td>0.89</td>
</tr>
<tr>
<td>Unidentified</td>
<td>Un</td>
<td>Polar</td>
<td>0.85</td>
</tr>
<tr>
<td>Phosphatidyl serine</td>
<td>PS</td>
<td>Polar</td>
<td>0.80</td>
</tr>
<tr>
<td>Phosphatidyl inositol</td>
<td>PI</td>
<td>Polar</td>
<td>0.77</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>CA</td>
<td>Polar</td>
<td>0.71</td>
</tr>
<tr>
<td>Phosphoethanolamine</td>
<td>PE</td>
<td>Polar</td>
<td>0.66</td>
</tr>
<tr>
<td>Monoacylglycerol</td>
<td>MG</td>
<td>Non-polar</td>
<td>0.29</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>Chol</td>
<td>Non-polar</td>
<td>0.21</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>FFA</td>
<td>0.14</td>
<td></td>
</tr>
<tr>
<td>Triglyceride</td>
<td>TG</td>
<td>Non-polar</td>
<td>0.11</td>
</tr>
</tbody>
</table>
Supplementary 1 Typical HPTLC image showing a detergent resistant membrane (DRM) and lipid raft sample compared to the prepared standard. Band identities are as follows: 1) Triglycerides, 2) free fatty acids, 3) cholesterol, 4) monoglycerides, 5) phosphoethanolamine, 6) cardiolipin, 7) phosphatidylinositol, 8) phosphatidylserine, 9) phosphatidylcholine, 10) sphingomyelin.
Chapter 6

The Cyprinid herpesvirus-3 (CyHV-3) uses lipid rafts as a mode of entry into carp cells.

Graham Brogden*, Mikolaj Adamek*, Marcus J. Proepsting, Hassam Y. Naim#, Dieter Steinhagen#
Foreword: CyHV-3 is one of the most economically and socially important fish pathogens in the world. Research has shown that members of the Herpesvirales order require lipid rafts as a mode of entry into a host cell. As we have characterised carp lipid rafts and detergent soluble membrane fractions, we wanted to investigate the interaction between CyHV-3 and the cell membrane, specifically lipid rafts, and the importance of lipid rafts in the replication cycle of the virus.

The extent of Graham Brogden’s contribution to the article is evaluated according to the following scale:

A. has contributed to collaboration (0-33%).
B. has contributed significantly (34-66%).
C. has essentially performed this study independently (67-100%).

1. Design of the project including design of individual experiments: B
2. Performing of the experimental part of the study: B
3. Analysis of the experiments: B
4. Presentation and discussion of the study in article form: C
The Cyprinid herpesvirus-3 (CyHV-3) uses lipid rafts as a mode of entry into carp cells

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Summary

The Cyprinus herpesvirus-3 (CyHV-3) is a member of the new *Alloherpesviridae* virus family in the *Herpesvirales* order. CyHV-3 has been implicated in a large number of disease outbreaks in carp populations causing up to 100% mortality. The aim of this study was to investigate the requirement of cholesterol-rich lipid rafts in CyHV-3 entry into carp cells. Plasma membrane cholesterol was depleted from carp CCB cells with methyl-β-cyclodextrin (MβCD). Treated and non-treated cells were infected with CyHV-3 and infection parameters were assessed using RT-qPCR and immunocytochemistry. The effect of cholesterol reduction severely stunted virus replication rates *in vitro*, however after cholesterol replenishment virus replication rates were similar to the control infection. Lipid analysis of both the CyHV-3 and CCB lipid rafts revealed strong similarities in the lipid composition. The results were confirmed by comparative analysis of the lipid compositions of CyHV-3 and CCB membrane fractions. The results show that lipid raft mediated virus entry/exit is conserved amongst herpes viruses.
Chapter 6 CyHV-3 uses lipid rafts as mode of entry

1. Introduction

The Alloherpesviridae is a new virus family in the Herpesvirales order. It is comprised of both piscine and amphibian herpesviruses (McGeoch et al., 2006) and is evolutionary distinct from the other families of the order Herpesvirales (Davison et al., 2009; Hanson et al., 2011; Waltzek et al., 2009). Members of the Alloherpesviridae family are increasingly recognised as pathogens in aquaculture. One important pathogen is the Cyprinid herpesvirus-3 (CyHV-3), a novel herpesvirus from the Cyprinivirus genus which infects common carp, Cyprinus carpio and its coloured variety, the koi (Waltzek et al., 2009). CyHV-3 infections may cause severe outbreaks of the so called koi herpesvirus disease (KHVD) leading to up to 100% mortalities in infected populations, consequently causing a severe negative impact on carp aquaculture and koi ornamental trade. In surviving carp, the virus has been shown to have a latency phase, where it has been publicised to reside in leukocytes at low copy numbers (Eide et al., 2011). From the latency phase, the virus can be reactivated under stress situations such as netting or transportation (Bergmann & Kempter, 2011), released into the holding water and thus transferred to previously uninfected populations. Virus distribution through the trade of healthy looking latent infected carp is considered to contribute considerably to the fast spread of the infection over many countries in Europe, America and Asia within a short period of time after the first detection of the infection in Europe, Israel and North America in the late 1990s ((Haenen et al., 2004; Hedrick et al., 2000; Neukirch et al., 1999; Perelberg et al., 2003). Currently, CyHV-3 infection is considered the most serious disease affecting carp aquaculture worldwide (Cheng et al.).

Characteristic features of the virus include a large 295 kbp long linear genome with 156 potential ORFs (Aoki et al., 2007) encoding for at least 40 proteins building the mature viron (Michel et al., 2010). The viral envelope is required for the critical steps of fusion and fission to the host’s cell membrane. The CyHV-3 virion has an icosahedral capsid, an amorphic protein tegument and a lipid envelope containing virus glycoproteins (Dishon et al., 2005; Hutoran et al., 2005) which it acquires during the budding step from infected cells. In studies on the assembly of CyHV-3 in infected cells,
nucleocapsids appear to bud from the inner nuclear membrane into the perinuclear space. When the
nucleocapsids cross the outer nuclear membrane into the cytoplasm the primary envelope is lost and
a second envelope is acquired through budding into cytoplasmic vesicles (Hanson et al., 2011; Miwa
et al., 2007). This process is similar to the mechanism observed for mammalian herpesviruses
(Mettenleiter, 2002).

Because of the morphogenesis process, the lipid composition of the virus is therefore likely to
be representative/comparative to the place where budding occurred (Vangenderen et al., 1994). In a
variety of viruses, the lipid composition is distinct from that of the host cell plasma membrane from
which they are derived. This lead to the assumption that viruses might bud from specific
microdomains in the plasma membrane (Scheiffele et al., 1999). This view is supported by the
observation that annexin A2, a cellular lipid rafts-associated protein not found in viruses (Aoki et al.,
2007), was identified in the CyHV-3 virion (Michel et al., 2010) The plasma membrane of cells
consists of a variety of proteins and lipids, which the virus can utilise throughout its replication cycle,
in a non-uniform plasma membrane, comprising of both liquid ordered and liquid disordered

Liquid ordered microdomains, also assigned as lipid rafts, are defined as dynamic, nanoscale,
(chole)sterol– sphingolipid-enriched, ordered assemblies of proteins and lipids in the cell membrane
(Simons & Toomre, 2000) and these microdomains are associated with a range of important cellular
and physiological functions including cell signalling, membrane and protein trafficking and sorting
and nutrient transport. Lipid rafts have been extensively studied in mammals, and in the last two
decades the field has grown rapidly. In fish, lipid rafts have been isolated from rainbow trout
(Zehmer & Hazel, 2003) and Atlantic cod (Gylfason & Asgeirsson, 2008), and recently the presence of
functional lipid rafts in goldfish macrophages was shown (Garcia-Garcia et al., 2012). The lipid raft
model is used to investigate a large range of processes including; protein trafficking (Ikonen, 2001),
metabolic diseases (Maalouf et al.) and cell signalling (Magee et al., 2002; Varma & Mayor, 1998). In
addition, lipid rafts and cell membrane cholesterol were found to be involved in various stages of the
viral life cycle, such as virus entry (Nguyen & Hildreth, 2000; Ono & Freed, 2001), assembly and budding (Chazal & Gerlier, 2003).

The entry of enveloped viruses into host cells depends on binding to specific cellular receptors and fusion of the viral membrane with the cellular membrane. Several enveloped viruses enter host cells in a cholesterol-dependent manner, including coronavirus (Choi et al., 2005), poxvirus (Chung et al., 2005), paramyxovirus (Martin et al., 2012) and herpes virus (Bender et al., 2003). Fish infecting herpesviruses from the alloherpesviridae family are evolutionary distinct from mammalian herpesviruses (Davison et al., 2009) and at present little knowledge, if at all, is available regarding the host cell interactions of members from this herpesvirus family, and in particular regarding the role of cell membrane cholesterol in cell entry and trafficking. In the present communication we therefore examine the role of cholesterol in CyHV-3 entry into CCB cells, a carp derived fibroblastic cell line. We demonstrate that depletion of cellular cholesterol by methyl-βcyclodextrin (MβCD) reduces CyHV-3 replication in the cell and replenishment of cholesterol levels restores replication. Furthermore, the analysis of CyHV-3 lipid profiles showed a greater similarity to the cholesterol rich, lipid ordered membrane microdomains suggesting a role for lipid rafts in virus trafficking in the cell. To our knowledge, this is the first communication which addresses the role of lipid membrane components during the infection of fish cells with an aquatic herpesvirus. An understanding of the involvement of membrane lipids in virus entry should be useful for the development of vaccines or antiviral agents.
Chapter 6 CyHV-3 uses lipid rafts as mode of entry

2 Results

Table 1. Sequences of primers used in this work. Primers marked with ‘Q’ were used in RT-qPCR expression analyses, primers marked with ‘P’ were used for the amplification of gene fragments for plasmid based quantification of gene expression, primers marked with ‘E’ were used for the detection with end point PCR of viral and carp DNA in samples during purification of the virus.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Sequence 5’-3’</th>
<th>GenBank ID</th>
<th>Use</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>40S ribosomal protein S11</td>
<td>q40S.FW1</td>
<td>CCGTGAGGCTGGACATCGTTACA</td>
<td>AB012087</td>
<td>P, Q</td>
<td>(Gonzalez et al., 2007)</td>
</tr>
<tr>
<td></td>
<td>q40S.RV1</td>
<td>TCAGGACATTGAACCTACCTGCT</td>
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<td>Interferon α1S</td>
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<td>GATGGCTCGGAGATATGGGGA</td>
<td>EC393381</td>
<td>E</td>
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</tr>
<tr>
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<td>IFN_R</td>
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<td>M-KHV1-F</td>
<td>TACCAGAACAGAACACTTGTC</td>
<td>AJ535112</td>
<td>E</td>
<td>(Rakus et al., 2012)</td>
</tr>
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<td></td>
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<td>(Adamek et al., 2012)</td>
</tr>
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<td></td>
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<tr>
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<td>CyHV3_OFR92_F1</td>
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<td>DQ177346</td>
<td>P</td>
<td>(Adamek et al., 2012)</td>
</tr>
<tr>
<td></td>
<td>CyHV3_OFR92_R1</td>
<td>GACCCAGTCCCCCTGACGTTG</td>
<td></td>
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<tr>
<td></td>
<td>CyHV3_O92_qF1</td>
<td>AGCCACCTTGGTGCCTG</td>
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<tr>
<td></td>
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<td>CyHV3_TK_qF1</td>
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<td>P, Q</td>
<td>(Adamek et al., 2012)</td>
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Figure 1. Effect of Methyl-beta-cyclodextrin treatment on CCB cells on cellular levels of cholesterol, free fatty acids and phosphocholine. Cells were incubated for 1 hour with MβCD and the lipid compositions determined by TLC analysis.

With the addition of MβCD, the quantities of FFA and cholesterol in the cells decreased relative to an increase in MβCD concentration. However the concentration of Phosphocholine (PC) remained constant except when CCB cells were incubated with 10 mM of MβCD, where a much lower value of PC was detected. This lower PC value correlates to a decrease in viable cells due to the toxic effects of high concentrations of MβCD. Shown are mean values and standard deviation of n=3 experiments.
Figure 2. Effect of MβCD treatment on cholesterol levels in CCB cells. The cells were treated with 10 mM of MβCD for 1 hour and samples were taken for analysis 1 hour after incubation. The levels of cholesterol were measured by HPTLC and analysed using the CpAtlas software. Results showed a decrease of over 70% after incubation with MβCD up to 2 hours post treatment. However, the cholesterol levels were completely replenished 48 hours after treatment. Shown are mean values and standard deviation of n=3 experiments.
Figure 3. Expression profiles of Cyprinid herpesvirus 3 genes in CCB cells from cell cultures during CyHV-3 infection. CCB monolayers were treated with MβCD, MβCD and cholesterol or left untreated prior to infection with the virus. Expression levels are shown as the mean copy number (±SD) of the gene normalised for 100 000 copies of 40S ribosomal protein S11 (n=3). The mRNA expression of the CyHV-3 genes were used as a marker for CyHV-3 replication, thymidine kinase TK (A), major capsid protein MCP (B), and DNA polymerase DP (C) all genes showed significantly lower transcription rates in the MβCD treated cells. Viral genes were measured 2 days and 5 days post infection.
Figure 4. Immunocytochemistry of CyHV-3 in infected CCB cells. Cells were treated in four groups; with or without MβCD, MβCD and cholesterol co-treatment and after cholesterol reduction and replenishment. Depicted is the percentage of CCB cells positive for CyHV-3 glycoprotein on 4 and 7 days post infection. No virus associated protein was detected in the MβCD treated cells after 4 days post infection. After 7 days post infection, a significantly lower percentage of cells showed positive for the presence of CyHV-3 protein compared to untreated and cells treated with both MβCD and cholesterol (p≤0.01). There was no significant difference in the percentage of infected cells between cholesterol depleted cells and cells naturally replenished with cholesterol. Virus protein was not detected in non-infected control cells. Results are displayed as a percentage (±SD)
Figure 5. PCR analysis of isolated and purified CyHV-3. The virus was purified on a sucrose density gradient and each fraction run on an SDS-Page gel stained with comassi blue. A clear band appeared at approximately 35-40 KDa (image not shown). The same band containing fraction was then amplified using CyHV-3 specific thymidine kinase primers and carp IFN type 1 primers. In both the virus containing pellet and the supernatant, a band was identified at 500 bp. A band of 500 bp was also detected in the positive control, with no visible band detected in the negative control. A PCR was also performed using a carp IFN type 1 primer set, where no visible bands were detected in either the virus containing pellet, the supernatant or the negative control, however a 2000 bp band was detected in the positive control.
Chapter 6 CyHV-3 uses lipid rafts as mode of entry

**Graphs:**
- DSM
- LR
- CYHV-3
Chapter 6 CyHV-3 uses lipid rafts as mode of entry

**Figure 6.** Lipid analysis by TLC showing the lipid composition of CyHV-3 and the DSM and lipid rafts fractions (LR) from CCB cells. The results are displayed as a total percentage of all the lipids analysed using the TLC method described. * In the DSM fraction the Cholesterol and FFA accounted for a combined total of 14.9% and have been displayed in the above graph as 7.5% shared equally. SM Sphingomyelin, PC: phosphocholine, PS: phosphoserine, PI: phosphoinositol, CA: cardiolipin, PE: phosphoethanolamine, GC: galactoceramide, MG, Chol: cholesterol, FFA: free fatty acids, TG: triglycerides, CE: cholesterol-ester. N=3

![Comparison of polar vs non-polar lipids](image)

**Figure 7.** Graph displaying the percentage of polar lipids against non-polar lipids in the DSM and lipid raft fractions of CCB cells and the CyHV-3.
2.1 Treatment of CCB cells with MβCD induces a concentration dependent depletion of cholesterol

To investigate the importance of cholesterol and lipid rafts during virus entry, a cholesterol depletion and replenishment assay was first established *in vitro* using the common carp derived CCB cell line. The aim of the cholesterol depletion assay was to remove a significantly high concentration of cholesterol whilst keeping a viable cell monolayer. For this, CCB cells were incubated with 0, 5, 7.5, 10 and 15 mM of MβCD. The results illustrated in figure 1 showed that there was an MβCD concentration dependent reduction in cellular levels of cholesterol after one hour of incubation. Interestingly free fatty acids were also depleted by similar levels, however the levels of phosphocholine remained relatively stable, excluding the cells incubated with 15 mM MβCD (fig. 1). At this concentration a higher level of cell death was observed as described in 2.2 (data not shown). Incubation with 7.5 and 10 mM of MβCD led to a significant reduction in cholesterol levels whilst not disrupting the cell monolayer. Subsequently the kinetics of MβCD induced cholesterol depletion was examined including the rate of cholesterol replenishment. Therefore for further experimentation, 10 mM MβCD and an incubation period of one hour were selected.

Incubation of CCB cells with 10 mM MβCD for one hour led to an almost 70% reduction in the cholesterol content of the cells at one hour and two hours post incubation (Fig. 2). At 48 hours post incubation, the cellular cholesterol was replenished to the pre-treatment level. The monolayer remained intact and the cells remained viable. Both the untreated cells and the cells incubated with both 10 mM MβCD and 2.5 mM cholesterol remained viable and the cholesterol levels were maintained at the pre-treatment level (fig. 2).
2.2 The effect of MβCD on virus entry

The mRNA expression of CyHV-3 genes which were used as a marker for CyHV-3 replication, thymidine kinase (TK), major capsid protein (MCP), and DNA polymerase (DP), all showed significantly lower expression levels in the MβCD treated cells. Viral genes were measured 2 and 5 days post infection. In infected cell cultures, the expression levels of the CyHV-3 TK gene increased during the time of infection, and therefore the expression level of this gene corresponds to the progress of infection in an in vitro setting (Adamek et al., 2012).

The viral gene expression levels were significantly lower in the MβCD treated cells when compared to the cells co-treated with MβCD and cholesterol as well as in non-treated cells in both time points (P<0.001). At 2 days p.i., the mRNA expression in MβCD treated cells was 56x lower for CyHV-3 TK, 65x for CyHV-3 DP and 85x lower for CyHV-3 MCP compared to non-treated cells (fig. 2). The differences were even higher at 5 days p.i. (from 73x for CyHV-3 DP to 110x for CyHV-3 MCP) (fig. 3). Interestingly, this assay also showed (similarly to FICC) that the MβCD and cholesterol co-treated cells showed slightly higher expression of viral genes (form 1.2x to 1.6x) than the non-treated cells (fig. 3).

Importantly, the FICC results also showed a similar trait to the viral gene expression analysis. Infected cell monolayers treated with MβCD also contained a significantly lower percentage of infected cells at both 4 and 7 days p.i., when compared to the two control treatments, MβCD plus cholesterol and untreated medium. The cells incubated with either MβCD and cholesterol, or left untreated had similar levels of infected cells of 85.5% and 90% respectively whereas the cells treated from MβCD showed a significantly lower (P<0.001) average of 25.5% of cells presenting virus proteins (Fig. 4). Critically, cells depleted of their cholesterol and then naturally replenished over a 3 day period, showed comparable levels of infection to the untreated samples. These data show that both the replication rate and spread
of the virus in the culture was lower in MβCD treated cells when compared to the two control groups.

2.3 CyHV-3 virions have a high concentration of cholesterol and have a similar lipid composition to lipid rafts

In order to examine a possible association of the virus with the cell membrane and in particular with specific plasma membrane microdomains, the lipids profiles of detergent soluble membrane fractions (DSM), lipid raft or detergent resistant membrane fractions (DRM) and CyHV-3 were determined.

For this, CyHV-3 was isolated and purified from infected tissue by sucrose density gradient centrifugation. The resulting pellet fraction was tested for the absence of a contamination with cell debris by the amplification of the gene encoding for carp IFN and subsequently used to determine the lipid composition of the virus. The cyca-IFN primers produced a band of the expected size only in the positive control, however no band was present in either the supernatant or importantly the virus containing pellet. This result indicated that there was no tissue contamination present in the pellet (Fig. 5). The PCR designed to test for the presence of the virus revealed strong bands in both the purified pellet fraction and the supernatant, showing that the CyHV-3 was present in both fractions (Fig. 5).

HPTLC analysis revealed that the CyHV-3 contained a wide variety of lipids. Eleven individual lipid species were identified at significantly high concentrations, with especially high concentrations of triglycerides, free fatty acids, cholesterol, monoacylglycerol and phosphatidylcholine, with trace amounts of galactocerebrosides, phophatidylethanolamine, cardiolipin, phosphatidylinositol and sphingomyelin detected (Fig. 6). In CyHV-3 the ratio of polar/non-polar lipids was 17:83 (Fig. 7).
In cell membrane fractions separated by Triton X-100 treatment into the detergent resistant membrane (DRM) or lipid raft fraction and detergent soluble (DSM) fractions, two distinctly different lipid profiles were observed. The DSM contained a broader range of 10 lipids present at measurable concentrations. This fraction was particularly rich in phospholipids with a very high concentration of phosphatidylocholine (Fig. 6). In the lipid raft fraction, seven lipids were detected at measurable concentrations, with high levels of free fatty acids and cholesterol present, however many phospholipids such as phosphatidylethanolamine were below the minimum detection level (fig. 6). In the DSM fraction, a ratio of polar / non-polar lipids of 59:41 was found while in the lipid raft fraction the ratio of polar / non-polar lipids was 8:92, which was similar to the ratio 17:83 as it was found in CyHV-3 (Fig. 7).

3 Discussion

Cholesterol, a major component of lipid rafts, has been shown to be critically important in the life cycle of several enveloped viruses from different families, (Bavari et al., 2002; Huang et al., 2011; Ren et al., 2008) including herpesviruses (Bender et al., 2003; Ren et al., 2011) The present study provides evidence that cholesterol is also important in the life cycle of CyHV-3, a herpesvirus from a fish host. Based on the criteria of different gene contents (McGeoch et al., 2006), herpesviruses (HV) from amphibian and fish were recently separated from mammalian HVs and assigned to the family alloherpesviridae within the order Herpesvirales (www.ictvonline.org; (Davison et al., 2009; Waltzek et al., 2009)). Further phylogenetic analysis provided strong evidence of a monophyly of fish and amphibian herpesviruses (HV) within a larger clade containing all HVs (Waltzek et al., 2009). The cohesion of the family alloherpesviridae is also supported by a detectable conservation of at least 13 genes (Aoki et al., 2007). The sequence analysis however revealed that these viruses are but tenuously related to mammalian, avian and reptile HVs, which share approximately 43 homologous genes (McGeoch et al., 2006). The best evidence supporting a
common ancestry for all HVs is provided by sequence conservation of 5 regions from a single gene that encodes the putative ATPase subunit of terminase, an enzyme complex involved in packing DNA into capsids (McGeoch et al., 2006; Waltzek et al., 2009).

In the search for additional lines of evidence for an evolutionary relationship among the different groups of HVs, further common properties of viruses from the 3 HV families could be considered. In respect of biological characteristics, the development of latency is a hallmark of herpesviruses (Howley Roizman, 2001). Evidence for a long term carrier state, which may involve latency, has been described in several fish infection viruses, in particular in CyHV-3 (Bercovier et al., 2005; Eide et al., 2011; St-Hilaire et al., 2005). These reports indicate that long-term infection (perhaps latency) is caused by various viruses from the family alloherpesviridae.

Another biological characteristic that could support the evolutionary relationship amongst HVs is the mode of virus entry. Mammalian HVs use different pathways for cell entry. In addition to endocytosis, HVs can enter cells via cholesterol dependent fusion at the plasma membrane (Rahn et al., 2011). The importance of cholesterol in the cellular membrane in establishing an infection with alloherpesvirus CyHV-3 in carp cells was shown in the present work by several lines of evidence. Firstly, MβCD-treatment of cells reduced the virus multiplication rate and a reduction of virus protein synthesis in infected cells. Secondly, the effect of cholesterol removal was reversible: when cholesterol was replenished in the cell or when additional cholesterol was added to MβCD-treated cells, the virus multiplication was restored. Thirdly, lipids isolated from the virus envelope are similar in composition to that of the lipid raft fraction of CCB cells and dissimilar to the detergent soluble non-raft fraction.

Lipid rafts have been shown to be involved in several important cellular functions, such as signalling where lipid rafts function as a site for high concentrations of membrane bound receptors. Cholesterol is thought to keep the raft assembly together (Simons & Toomre, 2000) and a sequestration of cholesterol by pore forming agents such as saponin or by MβCD allows for the
Chapter 6 CyHV-3 uses lipid rafts as mode of entry

manipulation of the constituents of lipid rafts (Simons & Toomre, 2000). In particular MβCD is widely used to study the importance of cholesterol in infection process of several viruses (Bender et al., 2003; Chung et al., 2005; Martin et al., 2012; Ren et al., 2011), since it captures cholesterol and removes it from cell membrane. As a result, lipid microdomains are disrupted with concomitant blocking of biological processes that depend on them are blocked (Zidovetzki & Levitan, 2007). Here we here could show that incubation with increasing amounts of MβCD resulted in a substantial decrease of cholesterol levels in carp derived CCB cells whilst still retaining an intact monolayer of viable cells. The incubation of goldfish kidney derived macrophages with MβCD caused a shifting of flotillin-1, a protein commonly associated with lipid rafts in mammalian cells, from the lipid raft fraction to the non-lipid raft associated fraction of the membrane (Garcia-Garcia et al., 2012). This indicates that like in mammalian cells, the incubation of fish derived cells with MβCD leads to a disruption of lipid rafts in a fashion similar to mammalian cells. At the same time, unlike other cholesterol binding agents that become incorporated into membranes, MβCD is strictly surface-acting and can be rapidly removed when cells are incubated with fresh medium. In CCB cells, the cholesterol level remained low until at least 2 hours post MβCD treatment, and entry of CyHV-3 to CCB cells during this period of time resulted in reduced virus multiplication and virus protein expression. However, by 48 hr post MβCD incubation, the cholesterol levels of CCB cells had been completely replenished. Once the cholesterol had been replenished, the virus replication and spread followed a similar trend to that of the control/untreated infected cells.

During morphogenesis, CyHV-3 acquires its envelope through budding into cytoplasmic vesicles (Hanson et al., 2011; Miwa et al., 2007). The lipid profile of virus appears similar to the lipid raft fraction from the CCB cells and differs from that of the DSM fraction. In particular when the lipid content was displayed as polar and non-polar lipids, the composition of the lipid raft fraction is significantly different when compared to the DSM fraction. Interestingly, there is no significant difference between the polar: non-polar ratio of the virus and the lipid rafts. Viruses acquire their lipid envelope directly from their immediate surrounding area, therefore the similarity between the
lipid raft and virus lipid composition suggests that lipid rafts are involved in entry, trafficking and/or budding of CyHV-3. In addition, Michel et al. (Michel et al., 2010) identified Annexin 2a, a lipid raft associated protein in the CyHV-3 virion but not in the CyHV-3 genome, suggesting that this protein was derived from the cell and not directly produced by the virus. This would support the hypothesis that the CyHV-3 uses lipid rafts as a portal of entry/exit where this protein could be incorporated into the mature virion.

In summary, this is the first study that demonstrates an implication of cholesterol in the life cycle of a piscine herpesvirus. As CyHV-3 is a member of the family alloherpesviridae, which is an ancient phylogenetic diversification of herpesviruses, our data suggests that this method is probably conserved amongst all members of the larger clade of the herpesvirales and might represent an ancient pathway. Further studies are needed to understand the mechanisms involved in the cholesterol dependence of CyHV-3 attachment to and penetration into cells. An understanding of entry pathways would be useful for designing antiviral agents or vaccines.

4. Materials and methods

4.1. Cell and virus cultivation

Common carp brain (CCB) cells (Neukirch et al., 1999) were cultured in minimum essential medium (MEM) with Earle’s salts supplemented with 1x non-essential amino acids (NEAA), 10% fetal bovine serum, 0.35% glucose, 100 IU ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin and incubated at 25°C in a humidified atmosphere containing 2% CO₂.

CyHV-3 (strain KHV I, (Hedrick et al., 2000)) was re-isolated from the skin of intraperitonealy infected carp using CCB cells according to a standard protocol. Next, the virus was propagated in CCB cells in 2 following passages. The virus stock suspension was stored at -80°C until further use.

4.2 Establishment of a cholesterol reduction assay using Methyl-beta-cyclodextrin.
In order to determine the effects of Methyl-beta-cyclodextrin (MβCD) (Sigma, Germany) CCB cells were plated in 24-well plates and treated with a range of MβCD concentrations (5 mM, 7.5 mM, 10 mM and 15 mM) for a time period ranging from two to six hours and the cell viability was assessed using crystal violet. The results showed that a significant number of cells died in wells treated with 15 mM MβCD, however the cell monolayer remained intact for concentrations of 10 mM and lower, therefore 10 mM MβCD was used for further studies (data not included). CCB cells were seeded in 24 well plates and cultured until confluent. The cells were then treated in triplicates for one hour with 10 mM MβCD (dissolved in RPMI 1640, Sigma), 2.5 mM MβCD plus cholesterol (cholesterol dissolved in Ethanol at a concentration of 80 mM) or RPMI culture medium. The cells were then washed twice with PBS to remove all traces of MβCD and excess cholesterol and untreated RPMI medium was added. After the cells have been incubated for the desired time in RPMI medium (1, 2 or 48 hours), the cells were lysed with 1% Triton X-100 and stored at -20°C for future processing.

### 4.3 Influence of cholesterol reduction on virus entry

To assess the importance of cholesterol for the entrance of the CyHV-3, the cells were treated with MβCD to remove a significant concentration of cholesterol. Next, 150 TCID\textsubscript{50} virus was added to the cells and incubated for a further 1 hour. After infection, the virus containing medium was removed and the cells washed twice before fresh untreated medium was added to the cells. Infected cell cultures on 24-well plates were incubated in 25 °C and on 48h and 120h post stimulation/infection cells were harvested from 6 wells of each treatment type by replacing the medium with TriFast reagent (PeqLab) and frozen in – 80 °C until further use.

### 4.4 Fish and infection.
Eighteen month old, specific pathogen free common carp (*Cyprinus carpio* L.) were used for a CyHV-3 bath infection. The fish from an R3xR8 cross were obtained as fertilised eggs from Wageningen University (The Netherlands) and kept in a recirculation system with bio-filters. The water temperature was $24 \pm 1^\circ$C. Before challenge, fish ($n=3$; mean weight $\pm$ SD = 57g $\pm$ 16g) were transferred to a small plastic tank and infected with 300 TCID$_{50}$/ml for 30 min. After infection the fish were transferred in to 400 L plastic tanks and kept until death 7 days post infection. The whole fish were stored at -80°C until further use. The infection experiment was performed in accordance with the legal requirements approved by LAVES Niedersachsen under agreement 08/1446.

5.5 Lipid isolation and analysis

4.5.1 Virus isolation

Samples from CyHV-3 infected frozen carp bodies were cut into smaller pieces and then further lysed by Qiagen tissue lyser II (Qiagen, US) twice for 2 min at 20 Hz. The tissue debris was pelleted by centrifugation for 10 min at 10 000 x g, 4°C (Heraeus Megafuge) and the virus containing supernatant removed. The virus was then pelleted by centrifugation in a Beckman 70 Ti rotor for 90 min at 45 000 x g (Beckman L8-70M). Pellets were re-suspended in PBS and loaded on a 10 to 60% (w/v) sucrose gradient prepared in PBS and centrifuged for 60 min at 26 000 x g in a Beckman SW41 rotor. Bands were aspirated from the corresponding gradient fraction, diluted 10 fold in PBS and re-pelleted. The pellets were re-suspended in PBS and frozen at -80°C for further investigation.

The purified product was tested for contamination with cell debris using primers designed to bind to the cyca-IFN gene, and for the presence of the virus using primers designed to bind to the CyHV-3 thymidine kinase gene.

4.5.2 Isolation and extraction of detergent resistant and detergent-soluble membrane fractions.
Tissue samples were collected and placed on ice in 1 ml 1% Triton X-100 PBS solution. Samples were processed firstly by homogenising for 10 seconds (B. Braun Melsungen AG, Germany), followed by a further homogenisation step for 1 min in a glass potter (B. Braun Melsungen AG, Germany). The samples were then further processed through syringing, firstly 20 times with a 0.8 mm canule syringe followed by a 0.45 mm canule syringe 15 times. Samples were incubated whilst rotating overnight at 4°C. The cell debris was pelleted by centrifugation at 4°C at 13 000 rpm for 30 min. The detergent resistant membrane (DRM), or lipid raft fraction, was separated from the detergent soluble fraction by ultracentrifugation at 100 000 x g for 90 min at 4°C. The two fractions were stored at -20°C for later analysis.

4.5.3 Lipid extraction and preparation

Lipids were extracted from cell membrane samples as well as from virus samples. Lipids were separated from the proteins by chloroform/methanol as described by Bligh and Dyer (Bligh & Dyer, 1959) with minor modifications. Briefly, the lipid sample was loaded into a potter and the total volume equated to 800 µl with the addition of distilled water. The samples were homogenised for 1 min. Two ml Methanol and 1 ml Chloroform was added to each sample and the mixture was homogenised again for 1 min. The samples were then transferred to glass tubes and rotated at room temperature for 30 min followed by centrifugation at 7°C for 5 min at 1952 x g (Beckmann centrifuge). The protein pellet was removed, dried via vacuum centrifugation and stored at -20°C for later analysis. The supernatant was transferred to a new glass tube and 1 ml chloroform and 1 ml water was added, mixed briefly and centrifuged again at 7°C for 10 min at 17 000 x g. After centrifugation the upper layer was removed and the remaining liquid and lipid sample was vacuum dried and stored at -20°C for future analysis.

4.5.4 Lipid analysis
Lipid samples were re-suspended in 250µl chloroform/methanol solution. The samples were analysed by high performance thin layer chromatography (HPTLC) on silica gel 60 plates from Merck (Germany). 10µl of the lipid solution was loaded drop-wise onto the plates in triplicates. The TLC plates were placed in three separate running solutions. Firstly a solution comprising of acetic acid, 1-propanol, chloroform, methanol and potassium chloride, followed by a second solution containing n-hexane, diethyl ether and acetic acid, and finally ran in n-hexane. The plates were then stained with a copper sulphate solution. The bands found in the samples were compared to a lipid standard solution and analysed using the CP ATLAS software (Lazarsoftware).

4.6 Virus quantification

4.6.1 PCR confirmation of the viral and fish DNA.

End point PCR amplification of a fragment of CyHV-3 specific DNA in between ORF 55 and ORF 56 by the use of M-KHV1-F and M-KHV1-R primers (as described by Rakus et al. (Rakus et al., 2012)) were used for confirmation of CyHV-3 DNA in the purified virus sample. Possible contamination of the purified virus samples by carp DNA was detected by the amplification of carp specific genomic DNA using the primers Cyca IFN F and Cyca IFN R amplifying a fragment of IFN type I gene of common carp. Briefly: PCR amplification of the target PCR product was carried out in 20 µl of reaction mix using 100x diluted pellet and sucrose solution used for virus purification, 200 nM of each primer, 200 µM of each dNTP, 1 x Advantage 2 Buffer, and 0.2 units of Advantage 2 polymerase (Clontech, Japan). The PCR profile included an initial denaturation step at 94°C for 3 min, followed by 30 cycles of 95°C for 30 s, 62°C for 30 s, 72°C for 45 s, and a final extension step at 72°C for 7 min. PCRs were performed using an Eppendorf Mastercycler Gradient thermocycler (Eppendorf, Germany). PCR products were detected in a 1% agarose gel with GelRED staining (Biotium, USA) and visualised using a UV gel capture system (INTAS, Germany).
4.6.2 Expression of viral genes

For checking the expression dynamics of the CyHV-3 genes encoding for thymidine kinase (TK), major capsid protein (MCP) and DNA polymerase (DP).

Total RNA was extracted from collected samples using TriFast reagent (PeqLab, Germany) in accordance to the manufacturer’s instructions. Any remaining genomic DNA was digested with 1U of DNase I (Fermentas, Germany) as directed by the manufacturer. Synthesis of cDNA from 300 ng total RNA was performed using the 100U Maxima RT (Fermentas, Germany), a mix of 25 pM of random hexamer primers (Fermentas, Germany), oligo dT(18) (Fermentas, Germany) and 0.5 mM of a dNTP mix (Fermentas, Germany). A non-reverse transcriptase control was included for each sample. cDNA samples were diluted 20x prior to real time quantitative PCR analysis. Please refer to table 2 with the primer description.

Real time quantitative PCR (qPCR) was used for expression analysis. Reactions were performed using the Maxima SYBR Green 2x mastermix (Fermentas, Germany) in a Stratagene Mx300P cycler (Agilent, USA). The RT-qPCR master-mix was prepared as follows: 1x Maxima SYBR Green, 2x mastermix (with 10nM of ROX), 200nM of each primer, 5.0μl of 20x diluted cDNA and nuclease free water to a final volume of 25μl. The amplification program included an initial denaturation at 95°C for 10 min, followed by 40 cycles with denaturation at 95°C for 30s, annealing at 60°C for 30s and elongation at 72°C for 30s. For each gene and each sample qPCR was done in duplicate. Furthermore non template control and non reverse transcriptase control were performed for each reaction mix and sample. At the end of each run the dissociation curve of the amplicon was recorded. Fluorescence data from RT-qPCR experiments were analysed using the Stratagene MxPro Software and exported to Microsoft Excel (Microsoft, USA). The quantification of expression was performed as described by Adamek and co-workers (Adamek et al., 2012). Briefly: A standard curve based quantification was performed using serially diluted recombinant plasmids (from $10^7$ to $10^1$...
copies). For normalisation of the results the expression of carp 40S ribosomal protein S11 served as a reference gene. The level of gene expression was shown as copy number of the gene normalised for 1 x 10^4 copies of the 40S ribosomal protein S11 according to the following formula:

$$\text{Normalised copy number} = \frac{\text{mRNA copies per PCR for target gene}}{\text{(mRNA copies per PCR for reference gene/10^4)}}$$

4.6.3 Fluorescent immunocytochemistry (FICC)

Cells were collected at 4 and 7 days post infection from n=3 cell cultures per time point per treatment. Furthermore the cells which were infected 3 days post MβCD treatments and cultivated for 7 days post infection were also collected. To characterise the infection dynamics of the CyHV-3, CCB cells infected with the virus were collected after 3d, 4d, 6d, 7d, post infection.

The presence of CyHV-3 in infected cell cultures was confirmed by CPE formation, and by means of fluorescent immunocytochemical (FICC) detection of virus proteins. For the FICC analysis of virus protein expression infected CCB cells were labelled with the mouse monoclonal P14 KHV antibody (Aquatic, UK) and subsequently with fluorescence isothiocyanate conjugated rabbit anti mouse antiserum (RAM-FITC, Dako Cytomation, Denmark). Briefly: the cells were scraped from tissue culture vessels, transferred on superfrost microscopy slides and dried overnight at 4°C. Next, cells were fixed in acetone for 10 minutes and air dried in RT. On top of the cells 1% bovine albumin fraction V (in RPMI 1640 medium) was placed and incubated for 30 min at room temperature. Subsequently the cells were washed with RPMI medium, incubated with the monoclonal antibody KHV P14 (diluted 1: 10 with the same medium) for 1h. After the first antibody was washed away three times using PBS, the cells were incubated with RAM-FITC diluted 1:200 in the same medium for 1 h at room temperature. The cells were then washed again three times using PBS, then a drop of dabco buffer (2.5% 1,4-Diazobicyclo[2.2.2]octane in 90% glycerol solution containing 2 µg/ml of propidium iodide) was added on top of the cells with the covering glass and assessed by blue laser
(488nm) UV microscopy (Zeiss, Germany). Three types of controls were performed (i) non infected cells were stained in the same way as samples from the infection experiment, (ii) the primary antibody was replaced by the blocking solution, (iii) the primary antibody was replaced by control mice IgG.

4.7 Statistical analysis

SigmaPlot 12 software was used for statistical analysis. The differences between the treatments were considered as statistically significant at $P \leq 0.05$. The percentages of virus positive cells (assessed with FICC) were subjected to arcsin transformation before statistical analyses. The normalised expression levels of viral genes expression were transformed with Log(10). The differences in between the treatments in the percentages of FICC virus positive cells and viral gene expression were assessed using two-way ANOVA with a pairwise multiple comparison using the Holm-Sidak's procedure.
Chapter 6 CyHV-3 uses lipid rafts as mode of entry

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Chapter 6 CyHV-3 uses lipid rafts as mode of entry


Chapter 7

Discussion
Chapter 7 Discussion

Aquaculture is the fastest growing sector of food production, however there are limiting factors such as bacterial and viral diseases that cause huge financial losses to the industry (FAO 2010). Bacterial infections can be treated using antibiotics, however due to the likelihood of antibiotic resistance occurring, the use of antibiotics is not sustainable for the medium to long term future of aquaculture. Additional knowledge about the host-pathogen interactions in fish are needed to identify new therapeutic targets to combat microbial infections in aquaculture. Therefore, research is currently being focused on different ways of boosting the immune response against pathogens to reduce disease outbreaks and severity without the use of antibiotics. One promising avenue of research is the use of feed additives that are able to modulate the immune response, such as β-glucan. β-glucan has been shown to modulate neutrophil functions in several animal species. Human derived peripheral blood neutrophils treated with β 1,3 glucan and β 1,6 glucan showed increased levels of engulfment, ROS production and expression of heat shock proteins, with β 1,6 glucan inducing a higher level of neutrophil activity compared to β 1,6 glucan (RUBIN-BEJERANO et al. 2007).

Furthermore, β-glucan has been shown to function as a PAMP on the yeast Candida albicans, which was responsible for the induction of NETs in human derived peripheral blood via the complement receptor 3 (BYRD et al. 2013). Interestingly, β-glucan has also been shown to induce NET production in zebrafish (Danio rerio) (PALIC et al. 2007).

Recent research has been focusing on the mechanisms behind NET formation, with evidence presented for both ROS dependent and independent pathways. A role of lipid modulations is also suggested by (CHOW et al. 2010) showing that cholesterol depletion may be involved in NET formation.

Interestingly, cholesterol has been shown to play a crucial role in host-pathogen interactions. Cholesterol is an important constituent of plasma membrane lipid rafts which host a range of receptors and are responsible for commencing a signalling cascade once a foreign antigen is detected. Furthermore, bacteria (SHIN et al. 2000) and viruses (ONO u. FREED 2001) have been shown to use lipid rafts as a mode of entry into a cell.
Therefore, host-pathogen interactions at a cellular level were investigated in this thesis in common carp, with a focus on the effects of β-glucan on carp neutrophil-bacteria interactions (chapter 2 and 3) and on the host cell membrane (chapter 4, 5 and 6). A methodology which facilitated the isolated and characterisation of carp lipid rafts and DSM fractions from the cell membrane was established (chapter 4). Additionally, the role of cholesterol in NET formation and virus entry was investigated (chapter 4 and 6). In the following paragraph, the data presented in chapter 2-6 will be discussed more in detail.

In chapter 2 we characterised the production and function of carp kidney and head kidney derived neutrophils. Since Brinkmann and colleagues (BRINKMANN et al. 2004) first published the existence of NETs in humans, they have also been discovered in a wide range of species including; cats (WARDINI et al. 2010), mice (ERMERT et al. 2009), bovine (LIPPOLIS et al. 2006), pigs (BREA et al. 2012) and fish (PALIC et al. 2007). Although the majority of work investigating the underlying mechanisms of NET formation has been performed with mice and human derived neutrophils, the presence of NETs and the effects of stress on NET-formation has been characterised in zebrafish (PALIC et al. 2007). Additionally, the effects of titanium oxide pollution in fat head minnow (Pimephales promelas) have been investigated (JOVANOVIC et al. 2011). However, the role of NETs in response to bacterial infections e.g. A. hydrophila in fish and the functionality of carp kidney and head kidney derived neutrophils remain to be investigated. Therefore, we wanted to characterise carp kidney and head kidney derived NETs and investigate the role of these NETs in the carp immune system against a bacterial challenge.

Firstly, the effects of β-glucan on the time kinetics of NET formation of carp derived kidney and head kidney neutrophils was investigated. The results showed that NET production is time and β-glucan concentration dependent and that after 30 min incubation, a higher percentage of kidney derived neutrophils produced NETs relative to head kidney derived neutrophils. Secondly, as NETs have been shown to be capable of entrapping bacteria (FUCHS et al. 2007), we investigated this phenomenon in carp. The results showed that carp derived neutrophils are able to entrap A. hydrophila, again with the kidney neutrophils being more efficient compared to head kidney derived neutrophils. In literature NETs have also been associated with the extracellular killing of some Gram-positive bacterial species. Interestingly, carp NETs were unable to kill A. hydrophila (chapter 2). These results show
that carp NETs function in a similar trait to mammalian NETs as they are produced rapidly, can react to stimuli and entrap bacteria.

Mammalian NETs have been shown to be able to entrap a wide range of bacteria (FUCHS et al. 2007) however extracellular killing of bacteria is species specific, (MENEGAZZI et al. 2012). Therefore, it appears that at least one of the main functions of NETs has been evolutionary conserved, with external killing ability still remains to be shown. However, like in mammals, NETs are produced rapidly from stimulated neutrophils and can be visualised 45 min post plating, comprising of 30 min seeding and 15 min incubation with either β-glucan or RPMI medium. Interestingly, as seen in both chapter 2 and 3, carp neutrophils produce relatively high levels of NETs when cultured in RPMI medium alone. Conversely, Palic and colleagues (PALIC et al. 2007) incubated zebrafish kidney derived neutrophils in HBSS and detected no NETs in the un-stimulated samples. This could be due to the extensive range of nutrients and ions such as Ca$^{2+}$ and Mg$^{2+}$, which have been shown to stimulate neutrophils and may be required to induce NET formation. Further work will need to be performed to assess the role of ions in NET formation.

Interestingly we found that there were significant differences between neutrophil function depending on the tissues of origin, which has not yet been shown in mammals. It has been suggested that the head kidney in fish is the site of pluripotent stem cell production (ZAPATA et al. 2006; URIBE et al. 2011), thus functioning as the direct replacement for the bone marrow in mammals. However the kidney performs a similar role in mammals as it does in fish (ZAPATA et al. 2006). Interestingly, the peripheral blood granulocyte population is very low compared to mammals, however the kidney and head kidney contain relatively high concentrations of granulocytes, with approximately 45% of the cells isolated from each tissue staining positive for granulocytes (Chapter 3). It could be hypothesised that neutrophils are produced in the head kidney and migrate and mature in the kidney where they are exposed to antigens as described by (ZAPATA et al. 2006).

In addition to showing that β-glucan can induce NET production and enhance bacterial entrapment, we also investigated the interaction of carp NETs with A. hydrophila and if β-glucan influences this host-pathogen interaction (chapter 3). To investigate this interaction, we fed carp a diet enriched in 1 % β-glucan, isolated the head kidney and infected the cells with A. hydrophila, in the presence of medium supplemented with or without 200 µg/ml
MacroGard® (approximately 60% β-glucan). Immunofluorescence analysis of NET formation revealed that NETs were produced by the head kidney neutrophils, however the DNA-protein based NETs were degraded by nucleases produced by *A. hydrophila*. Interestingly, the addition of MacroGard® was able to stabilise and thus protect the NETs against this host-evasion strategy in the form of nucleases employed by the bacteria.

As shown in chapter 2, β-glucan in the form of MacroGard® can induce NET production in carp head kidney and kidney derived neutrophils and Palic *et al.* (PALIC et al. 2007) also showed that MacroGard® can induce NET formation in zebrafish derived neutrophils. We additionally showed that MacroGard® can stabilise the NETs against bacteria produced nucleases, however the underlying mechanisms behind this phenotype remain to be explained. The protein composition of NETs has recently been described (URBAN et al. 2009) and Neumann *et al.* (NEUMANN et al. 2012) showed proteins play an important role in NET functions by demonstrating that LL-37, an antimicrobial peptide, is responsible for stabilising NETs and can protect the DNA-protein based strands against nucleases. Interestingly, MacroGard® has been shown to modulate the expression of certain antimicrobial peptides in feeding experiments (FALCO et al. 2012; SYAKURI et al. 2013). However, these peptides have not yet been shown to play a role in NET formation or stabilisation, although this is due to this being a relatively new field of research (VON KOCKRITZ-BLICKWEDE u. NIZET 2009).

Interestingly, cathelicidin, which is in the same family of proteins as LL-37, has been identified in a range of salmonids and gadiforms (MAIER et al. 2008) so it can be hypothesised that MacroGard® could affect the protein composition of some of the described NET associated proteins.

Alternatively, due to the rapid production of NETs in response to β-glucan treatment that we detected, the relatively low levels of RNA and the short life spans of neutrophils, it could also be suggested that β-glucan may not be able to modulate protein expression, but β-glucan may be able to influence the charge of certain proteins *in vitro*. Neumann *et al.* (NEUMANN et al. 2012) also demonstrated that the charge of the NET associated LL-37 is important to its function of NET-induction and stabilisation. Therefore, it is possible to hypothesise that pre-stimulation of immune cells such as neutrophils by β-glucan feeding, may modulate the protein composition and thus produce neutrophils that are tailor made to a specific pathogen and are able to resist NET degrading nucleases produced by *A. hydrophila*,...
whereas neutrophils from fish fed a diet without β-glucan may contain lower levels of NET stabilising proteins such as cathelicidins. Further research would need to be conducted in this area to determine the mechanisms of how β-glucan can lead to the stabilisation of NETs and increased entrapment of bacterial pathogens. Future experiments could include the protein analysis of possible β-glucan-induced protein modulations by 2-D gel electrophoresis coupled to MALDI-TOF using a method described by Urban et al. (URBAN et al. 2009), or protein changes in the charge of the protein using a similar method described by Neumann et al. (NEUMANN et al. 2012).

Additionally, the results showed that this stabilising role of MacroGard® against A. hydrophila nuclease was only evident in fish already fed a diet enriched in 1 % MacroGard® (chapter 3, supplementary 1). The addition of MacroGard® in vitro exhibited no significant protective effect against A. hydrophila nuclease. At the time of writing, β-glucan has not been shown to pass through the intestinal barrier of fish, although recently M-like cells, which could facilitate the uptake of β-glucan through the gut, have been discovered (FUGLEM et al. 2010). If neutrophils do not come into contact with β-glucan directly, it would suggest that smaller fragments of β-glucan or antigens may be transported through the intestinal membrane and detected by immune cells such as T cells and neutrophils or activated platelets (CLARK et al. 2007). β-glucan has been shown to function as a PAMP, therefore this result may indicate that pre-stimulation with β-glucan via feeding may prime the neutrophils to this particular PAMP and once the neutrophil encounters this particular PAMP again, a stronger more effective immune response is observed. However to investigate this fully, comparisons must be made between NET producing neutrophils harvested from fish fed a diet with and without β-glucan.

Interestingly, these results could suggest that either neutrophils, or another immune cell which can stimulate neutrophils, may have a memory to recognise certain PAMPs, or more likely that neutrophil precursors can be influenced by a certain PAMP and be sensitised to a certain PAMP, which would result in a stronger or more efficient immune response. Alternatively, as the cell populations that we were working with were not pure, memory T cells may have come into contact with the PAMP and then they can produce cytokines, such as IL 6 (FUJIKI et al. 2003) which would in-turn modulate and induce NET production (VON KOCKRITZ-BLICKWEDE u. NIZET 2009).
Taken together these results show that carp derived neutrophils can rapidly produce NETs in a time and β-glucan dependant manner and are able to entrap but not kill *A. hydrophila*. As these results are in line with results published in mammalian species, they show that NETs have been evolutionary conserved in fish.

![Diagram showing β-glucan induced NET formation](image)

**Figure 1.** As depicted in the above figure, fish NETs play a role in the innate immune response of carp. Previous studies have shown that β-glucan has a positive influence on the outcome of bacterial infections in fish (DALMO u. BOGWALD 2008). Furthermore, our results show an additional role that neutrophils can perform during an infection with *A. hydrophila*, namely the production of extracellular traps which are capable of entrapping bacteria and hindering the spread of the infection. Therefore, these findings support previously published work that supports the use of β-glucan as a feed additive in aquaculture.

As well as possible protein modulations pre- and post- neutrophil maturation, lipid modulations may also play a role in NETs. Chow and colleagues (CHOW et al. 2010) showed that cholesterol is involved in human NET formation. Lipids are known to play a role in immune reactions and zymosan has also been described to cause glycerophospholipid
remodelling during murine macrophage phagocytosis (ROUZER et al. 2007). As the formation and role of NETs is a relatively new phenomenon, there are still large gaps in our knowledge about the mechanisms involved and new techniques are being developed to investigate NETs (YIPP et al. 2012) in a range of differing species. The formation of NETs has been shown to be ROS dependent (FUCHS et al. 2007), however one instance of ROS independent (REMIJSEN et al. 2011) NETosis has also been found in human embryos. Furthermore, NET formation by inhibiting cholesterol synthesis with statins has also been shown (CHOW et al. 2010). Recently more research is being conducted into the underlying mechanisms of NET formation, where research has shown that once the neutrophil is stimulated by a cytokine or a PAMP, which results in the production of NADPH oxidase and the formation of ROS. This in turn leads to PAD4-mediated histone H3 citrullination which induces chromatin decondensation (WANG et al. 2009). Finally, nuclear and granular proteins mix and protrude from the cell, forming an extracellular trap (VON KOCKRITZ-BLICKWEDE u. NIZET 2009; REMIJSEN et al. 2011). Interestingly, the role of lipids and in particular cholesterol has been suggested (CHOW et al. 2010), but more research needs to be performed to show the role of cholesterol in NET formation.

Unfortunately, at the time of writing there are several technical obstacles which prevented us from analysing the role of cholesterol in fish neutrophil functions. Firstly, although a highly purified neutrophil fraction can be isolated from fish tissues by magnetic antibody cell sorting, if a neutrophil antibody is available, or by chemotaxis through a filter membrane (BENARD et al. 1999), both of these methods would involve stimulating the neutrophils and thus distorting the results. Highly purified neutrophils fractions can be isolated from mammalian blood with minimal stimulation by Polymorphprep™ gradient as described in chapter 5. Secondly, little is known about carp NETs, except for the data presented in chapters 2 and 3, and a large amount of mechanistic data is absent from fish NETs, which would make any meaningful results more difficult to interpret. Thirdly, as cholesterol depletion by MβCD has not yet been performed on neutrophils and due to their short life spans, using purified human derived neutrophils as a model system was much more practical.

To investigate the role of cholesterol in NET induction, human derived neutrophils were isolated and a cholesterol reduction assay was optimised. The MβCD induced lipid
modulations were quantified using the HPTLC method described in chapter 4. The results showed that MβCD was able to induce NETosis both in a concentration and time dependent manner. Co-incubation with MβCD and DPI, a ROS inhibiting compound, showed that MβCD induced NETosis is ROS independent. Importantly cholesterol specificity was shown by incubating the neutrophils with NB-DNJ, which blocks sphingolipid synthesis, and resulted in no increase in NET production. The results showed that cholesterol depletion triggers NET production in human neutrophils. Furthermore, these results will help in understanding the mechanisms involved in NET formation. Interestingly the importance of cholesterol in NET formation may point towards a role of lipid rafts in NET formation or that membrane degradation is achieved by the cell during NETosis by depleting the membrane-stabilising cholesterol from the plasma membrane.

The role of some lipids such as cholesterol on innate immune functions has been investigated by others, showing that cholesterol can regulate the function of antimicrobial peptides (BRENDER et al. 2012). Possible explanations for this could be that cholesterol prevents the disruption of lipid bilayers due to the enhanced stability that cholesterol adds to the membrane, which inhibits the AMPs from binding (EVANS u. WAUGH 1977; HENRIKSEN et al. 2006). Therefore a decrease in cholesterol would aid AMP binding to the membrane and enable increased functionality. Further research needs to be conducted into the role of certain lipids in NET induction and if lipid modulations affect the function of NETs. Preliminary studies by me and my supervisors (data not shown) have shown that β-glucan feeding induces cell membrane lipid modulations in carp. Therefore, as it has been shown that β-glucan can influence AMP expression, which in turn may play a role in NET potency against bacteria, it would also be interesting to investigate if β-glucan induced lipid modifications could modulate the lipid composition of neutrophils and lead to a change in NET formation and entrapment efficiency.

In summary, the results described in chapter 4 show that the cell membrane plays an important role in the immune system. However, as this research was performed in human derived neutrophils, a similar study would need to be conducted to see if cholesterol also plays a role in fish NETs. Interestingly, as temperature has been shown to alter the cholesterol content of rainbow trout lipid rafts (ZEHMER u. HAZEL 2003, 2004, 2005), the role of temperature should also be investigated in NET formation.
Very little work has also been conducted on the cell membrane of carp and especially the lipid composition of carp cell membranes. This basic information is required as a basis for future experiments which will focus on the role of carp and fish cell membranes during bacterial and viral infections. The cell membrane acts as an interface between the cell and the surrounding environment, functioning as a port where nutrients and molecules can be transported and their exchanges regulated. Furthermore, this interface represents the junction where the pathogens come into contact with host cells, which must be recognised and an appropriate immune response initiated.

The cell membrane was described by Singer (SINGER u. NICOLSON 1972) as existing as a lipid and protein bilayer surrounding the cell, which complied with the proposed fluid mosaic model of lipid organisation. Subsequently, special microdomains were identified within the lipid bilayer (SIMONS u. IKONEN 1997), which showed that the lipid bilayer is not homogeneous in nature. These lipid rafts are associated with several cellular functions, such as signalling, transport and trafficking (BROWN u. LONDON 1998). Since the identification of these microdomains, a large amount of research has been conducted into the roles of lipid rafts under various circumstances, however research into lipid rafts in fish has been largely neglected. Furthermore, it has been shown that a wide range of environmental factors are able to influence the lipid and/or protein composition of these lipid rafts. Therefore as described in chapter 6, a protocol was established which facilitated the isolation and characterisation of common carp lipid rafts and DSM fractions.

Firstly the lipid raft and DSM isolation protocol was verified by comparing the flotillin and lipid distribution between the two membrane fractions with published data from mammalian tissues and cell lines (SALZER u. PROHASKA 2001). The establishment of this methodology allowed for the characterisation of carp lipid rafts and DSM fractions from different tissues. Furthermore, it allowed for comparisons to be made between the lipid rafts and DSM fractions within a tissue, between tissues and between carp and mammalian species. The results showed that carp and mammals contain similar lipid species albeit in differing concentrations. Furthermore, results showed that there are large differences between the DSM fractions of each tissue, whereas there were no significant differences between lipid raft fractions from different tissues. Interestingly, there were also large
differences between carp-derived lipid rafts and DSM fractions and mammalian derived lipid rafts and DSM fractions.

As it can be assumed that lipid rafts play similar functions in fish and mammals, the differences are likely to be attributed to the different environments that fish inhabit compared to mammals. An important environmental factor is the differing temperatures, where carp have a temperature range from 4°C to 33°C (www.fishbase.org/summary/1450), most mammals range between 36-39°C. The effect of temperature on lipid membranes has been studied both in vitro and in vivo, where the cholesterol content of Rainbow trout (Oncorhynchus mykiss) isolated lipid rafts was shown to decrease with a decrease in temperature (ZEHMER u. HAZEL 2003), however these results were limited as they did not present a wide range of lipids and no comparisons were made between the lipid raft and the DSM fractions from the gut enterocytes. The methodology presented in this thesis allows for the detection of a broader range of lipids and the cross comparison between lipid raft and DSM fractions and between six different tissues.

Importantly, the establishment and optimisation of this methodology allowed for further experiments such as investigating the role that lipid rafts play in infections, described in chapter 6. One of the great advantages of using fish as a model organism is that they are poikilothermic in nature and the fish lipid raft model detailed in this thesis would allow for important in vivo analysis investigating the effects of temperature changes on lipid rafts. Munro et al. (MUNRO 2003) described the lack of an in vivo model for studying the effects of certain environmental parameters such as the effects of low temperatures on the plasma membrane and its associated microdomains as a major challenge. Some of the points raised in this review can in part be answered using the method established in chapter 5 of this thesis, which would therefore further our knowledge of fish and mammalian lipid rafts. An example of the difficulties of using mammalian derived cell lines to investigate temperature changes would be investigating trafficking mechanisms and the role lipid rafts play in these. This is due to the blocking of endocytosis at temperatures below 20°C, whereas fish derived cells can still function normally, which would allow these studies to be performed in fish cells. Using the method described in chapter 5, which was applied under experimental conditions in chapters 4 and 6, would allow for lipid analysis, protein distribution and functional studies of lipid rafts to be conducted in fish derived tissues or cell lines. Future
research could also be conducted in the role of climate change, nutrient intake, fluctuations in water parameters, including quality and salinity, and the effect of hypoxia on lipid rafts (BOTTO et al. 2008).

Garcia-Garcia et al. (GARCIA-GARCIA et al. 2012) showed that goldfish lipid rafts are functional in fish by depleting the cholesterol-rich lipid raft microdomains with MβCD, which is able to deplete high concentrations of cholesterol from the plasma membrane (Levitin 2007). In mammals they have been shown to be required for protein trafficking (BROWN u. LONDON 1998), signalling (VARMA u. MAYOR 1998), entry of some viruses (ONO u. FREED 2001) and we have shown that they are implicated in metabolic diseases such as Fabry’s disease (MAALOUF et al. 2009). Therefore, as it is known that lipid rafts are important for fish homeostasis, we investigated the role of lipid rafts in the infection process of a piscine virus. The interactions between fish membrane microdomains and piscine viruses have not been addressed in fish.

To assess the role of cholesterol and lipid rafts in virus entry in a carp cell line, a cholesterol depletion assay had to be established. I chose the CyHV-3 virus and the CCB cell line as my infection model as the both the fish and virus are economically important (FAO 2010) and the virus infection system was reliable (ADAMEK et al. 2012). Cyclodextrins, such as MβCD have been widely used in vitro to deplete membrane bound cholesterol, which allows for investigations into the role of lipid rafts in virus entry (ONO u. FREED 2001), apoptosis in tumour cells (ONODERA et al. 2013), glycoprotein absorption in the intestine (ZHANG et al. 2013) plus many more disciplines.

Firstly, the lipid composition of CCB cells and the purified CyHV-3 was ascertained by using the method described in chapter 6. The results showed that the lipid raft fraction contained a similar lipid composition and polar:non polar lipid ratio to that of the virus. Secondly, a cholesterol depletion assay was established for CCB cells and approximately 70% of the total cholesterol in the plasma membrane was removed. Thirdly treated and untreated cells were infected with CyHV-3, with the results showing that cholesterol depletion significantly reduced virus entry and replication, however once cells had replenished their cholesterol levels, the number of virus copies measured by real time PCR and Immunocytochemistry were consistent with untreated cells.
These findings emphasised the role that cholesterol plays in pathogen interactions and illustrates that pathogens are able to hijack and utilise lipid rafts to facilitate binding, entry and replication of the virus in carp cells. Furthermore, these results coupled with those from Garcia-Garcia et al. (GARCIA-GARCIA et al. 2012) show that the role that lipid rafts play in internalising and trafficking proteins is likely to be conserved with mammalian cells. The results presented in chapter 5 will help us better understand the CyHV-3 replication cycle and valuable information for future vaccine development. Furthermore these findings open up many potential avenues for research to be directed such as vaccine development, studies on nutrient uptake, protein synthesis and trafficking to the cellular membrane and basic cell-pathogen interactions at a biochemical level.

Final conclusion

To summarise the results presented here in this thesis, we have shown that certain lipids such as cholesterol are important in the immune system e.g. in the formation of NETs. Furthermore, we isolated and characterised carp lipid rafts and showed that they are involved in CyHV-3 infection. Lastly we characterised carp kidney and head kidney derived NETs, showing that they are able to entrap, but not kill A. hydrophila. β-glucan was also shown to have a stimulating effect on NET production and β-glucan can also protect against NET degrading nucleases produced by A. hydrophila, illustrating that β-glucan induced NET production results in better quality NETs possibly due to either protein or lipid modulations.

All together the results of this study characterised host-pathogen interaction during microbial carp infections. This knowledge can help to identify novel targets against infections for new treatment or prophylactic strategies in fish aquaculture. For example, a better understanding of how carp neutrophils interact with A. hydrophila and the role of the feed additive β-glucan in this interaction will help to reduce the severity and frequency of disease outbreaks in carp aquaculture. Furthermore, increased knowledge of the carp cell membrane and the mechanisms used by CyHV-3 to replicate within a host cell will help in the development of a vaccine against this economically important virus.
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Chapter 8
Summary
Chapter 8 Summary

Graham Brogden:  Cell-pathogen interactions in common carp (Cyprinus carpio L.):
Studies on cell membranes and neutrophil responses

The aim of this study was to investigate host-pathogen interactions at the cell level in common carp. We wanted to investigate and describe how the feed additive β-glucan can improve the outcome of an infection by modulating the formation, stabilisation and bactericidal ability of NETs when exposed to Aeromonas hydrophila. Additionally, we wanted to explore the mechanisms behind NET formation and the role of cholesterol in this immune reaction. Furthermore, we wanted to establish a method to investigate the lipid composition of a carp cell membrane and to ascertain the role of cholesterol-rich lipid rafts in CyHV-3 entry into a cell.

In chapter 2, we described the effects of β-glucan on NET formation and the role of NETs against Aeromonas hydrophila. The results show that NET formation is both time and β-glucan concentration dependent. Furthermore, we showed that carp NETs are capable of entrapping but not killing A. hydrophila and that the percentage of entrapped bacteria was increased in the presence of β-glucan. Interestingly, a higher percentage of neutrophils isolated from the kidney produced a NET compared to head kidney derived neutrophils. Similarly, kidney derived neutrophils were also able to entrap a higher percentage of bacteria relative to head kidney derived neutrophils.

Additionally, in chapter 3 we investigated the interaction between carp NETs and a pathogenic strain of A. hydrophila. As we showed previously that A. hydrophila can be trapped in the protein-DNA NET complexes released from carp neutrophils, we demonstrated that A. hydrophila is able to produce DNA degrading nucleases which were shown to destroy carp NETs. Importantly, the addition of β-glucan in vitro stabilised the NETs against the host-evasion strategy employed by the bacteria.

In chapter 4 we described the role of cholesterol in the formation of NETs. This work was performed using human peripheral blood derived neutrophils due to the limitations of using carp neutrophils. The results show that MβCD-induced cholesterol depletion led to an
increase in NET production. Importantly, treatment with the sphingolipid synthesis inhibitor NB-DNJ had no effect on NET production. Interestingly, MβCD-induced NET production was also shown to be ROS independent. Therefore, these results show that cholesterol plays a significant role in mechanism leading to NET production.

As we have shown the importance of lipids and particularly cholesterol in immune functions, we wanted to characterise the lipid composition of the cell membrane in carp (chapter 5). Therefore, a lipid raft isolation and analysis protocol was established, whereby lipid rafts were isolated with Triton X-100 and the lipid composition visualised by using high performance thin layer chromatography. The method was validated by western blotting which showed high concentrations of the lipid raft associated flotillin protein in the lipid raft fraction. Higher concentrations of cholesterol, free fatty acids, triglycerides and sphingomyelin were detected in lipid rafts fractions, whereas higher concentrations of phospholipids were detected in the DSM fraction.

Lastly, using the cholesterol reduction and lipid analysis methods described in this thesis, we wanted to investigate the role lipid rafts play during a CyHV-3 infection (chapter 6). The results showed that after cholesterol-depleting MβCD treatment of CCB cells, virus entry was hindered, which was determined by lower levels of virus replication and virus copies evaluated by qRT-PCR and immunocytochemistry respectively.

Taken together, the results presented in this thesis have characterised host-pathogen interactions during bacterial and viral carp infections. Furthermore, methodologies presented here can be used for more detailed studies of host-pathogen interactions and the knowledge gained can help to identify novel targets against infections for new treatment or prophylactic strategies in fish aquaculture.
Chapter 9

Zusammenfassung
Zusammenfassung

Graham Brogden: Zell-Pathogen Interaktionen in Karpfen (Cyprinus carpio L.): Studien an Zellmembranen und Reaktionen neutrophiler Granulozyten


In Kapitel 3 untersuchten wir die Wechselbeziehung zwischen Karpfen NETs und einem pathogenen A. hydrophila Stamm. Wie wir im Vorfeld gezeigt haben, kann A. hydrophila in den Protein-DNA-Komplexen (NETs) von neutrophilen Granulozyten eingeschlossen werden. Weiterhin konnten wir zeigen, dass A. hydrophila in der Lage ist, DNA abbauende Nukleasen zu produzieren, um NETs von Karpfen zu zerstören. Interessanterweise zeigte sich so, dass der Zusatz von β-Glukanen die NETs gegenüber den Bakterien in vitro stabilisiert.

In Kapitel 4 wurde die Rolle des Cholesterols in der NET Bildung beschrieben. Diese Untersuchung wurde aufgrund der begrenzten Möglichkeiten neutrophile Granulozyten von


Zusammenfassend zeigten die Ergebnisse, die in dieser Studie vorgestellt wurden, die Wirt-Pathogen Interaktionen während einer bakteriellen und einer viralen Infektion. Weiterhin können die hier vorgestellten Methoden für detailgenauere Studien der Wirt-Pathogen Interaktion genutzt werden und die erhaltenen Erkenntnisse können bei der Identifizierung neuer Ziele der Infektionsabwehr für neue Behandlungsmöglichkeiten oder prophylaktische Strategien in der Aquakultur genutzt werden.
Affidavit

I herewith declare that I autonomously carried out the PhD-thesis entitled “Cell pathogen interactions in common carp (Cyprinus carpio L.): Studies on cell membranes and neutrophil responses”.

No third party assistance has been used. I did not receive any assistance in return for payment by consulting agencies or any other person. No one received any kind of payment for direct or indirect assistance in correlation to the content of the submitted thesis.

I conducted the project at the following institutions:

1. Fish Disease Research Unit, University of Veterinary Medicine Hannover, Germany
2. Department of Physiological chemistry, University of Veterinary Medicine Hannover, Germany

The thesis has not been submitted elsewhere for an exam, as thesis or for evaluation in a similar context.

I hereby affirm the above statements to be complete and true to the best of my knowledge.

_______________________________
[Date], signature
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