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Changes of IgM expression levels in the head kidney of *Cyprinus carpio* following an infection with *Sphareospora molnari*

Bachelor Thesis in Biological Chemistry

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Annotation

Sphaerospora molnar is a myxozoan inducing sphaerosporosis in common carp and causing serious damage to secondary lamellae and epithelia of the gills. The understanding of the host immune response against *S. molnari* as well as other myxozoans is very limited in part due to the unresolved life cycle. The focus of this thesis was the quantification of membrane bound and secretory IgM expression levels in the head kidney over a period of 9 weeks following an intraperitoneal injection of *S. molnari* blood stages. Significant increases in the expression levels of both IgMs were observed reaching its maximum at 42 days after infection. Two weeks following the infection the first significant increases of secretory IgM were detected.

Declaration

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Viktor Sieranski

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1 Introduction

1.1 Myxozoa

Myxozoa are a very diverse class of endoparasitic cnidarians which comprises approximately 2,200 species (Okamura et al. 2015; Lom and Dykova 2006). The Myxozoa can be split into two clades, Malacosporea, which utilize bryozoans as definitive hosts, and Myxosporea, having annelids as their final hosts. One of the major characteristics of Myxozoa is a relatively complex life cycle, incorporating both, invertebrate and vertabrate hosts (Holzer et al. 2018; Okamura et al. 2015). The initial hosts for myxozoans were most likely annelids and bryozoans. Fish are the most widespread intermediate hosts for myxozoans, but infections have been reported in amphibians (Jirků et al. 2007; Ohlmacher 1893; McAllister and Trauth 1995), reptiles (Eiras et al. 2005), small mammals (Szekely et al. 2015) and waterfowl (Bartholomew et al. 2008). Although the effect on fish infected by myxozoa is often relatively modest, infections with some species can have a drastic effect on fish, causing diseases like proliferative kidney disease (Longshaw 2002) or proliferative gill disease (Pote et al. 2000) as well as various types of sphaerosporoses (Lom et al. 1983) . As these diseased fish can contribute to economic losses, especially for fish farms, studies of these parasites are of major commercial interest.

Transmission of Myxozoa between the hosts occurs through multicellular spores, which have valve cells harboring the infectious sporoplasms on the inside. Attachment of the spores to the host is facilitated by polar capsules. These are intracellular organelles homologous to nematocysts (Canning and Okamura 2004). The amount of actionospores necessary to successfully infect a fish is remarkably low, with a single spore being enough in some cases to cause serious disease (Bjork and Bartholomew 2009; Longshaw et al. 2002). In water, the longevity of the spores is affected by the temperature. Actinospores might remain infectious for as little as a few hours (Kinkelin et al. 2002) and need a vertebrate host to survive and continue the lifecycle. Some myxospores can continue being vital and infectious for months or even years as they are protected by thicker walls (Okamura et al. 2015).

Historically, Myxozoa used to be characterized solely based on their morphology, tissue tropism and specificity in vertebrate host. This approach is often insufficient as the

appearance can vary significantly through the myxozoan lifecycle and there are only few morphological characteristics (Atkinson et al. 2015). More recently DNA sequencing techniques are used as well, but they also come with certain limitations; the DNA of the host can be amplified, PCR amplification bias and difficulties of analyzing tissues infected with multiple parasitic species. The most comprehensive database for analysing myxozoan phylogeny is based on the SSU rDNA sequences (Holzer et al. 2018).

The first myxozoan lifecycle described was the one of *Myxobolus cerebralis* which showed that in fact two hosts are incorporated. It was also found that there are two morphologically different types of spores, namely Actinosporea occurring in invertebrates (e.g. Annelids) and Myxosporea in vertebrate hosts (predominately in fish) (Wolf and Markiw 1984). Other representatives of the myxozoan subphylum confirmed that both vertebrate and invertebrate hosts are necessary for completion of the myxozoan lifecycle but they do not necessarily have to be fish and annelids. Fish appeared a long time after the emergence of the Myxozoa during evolution indicating invertebrates like bryozoans and annelids as original hosts (Holzer et al. 2018).

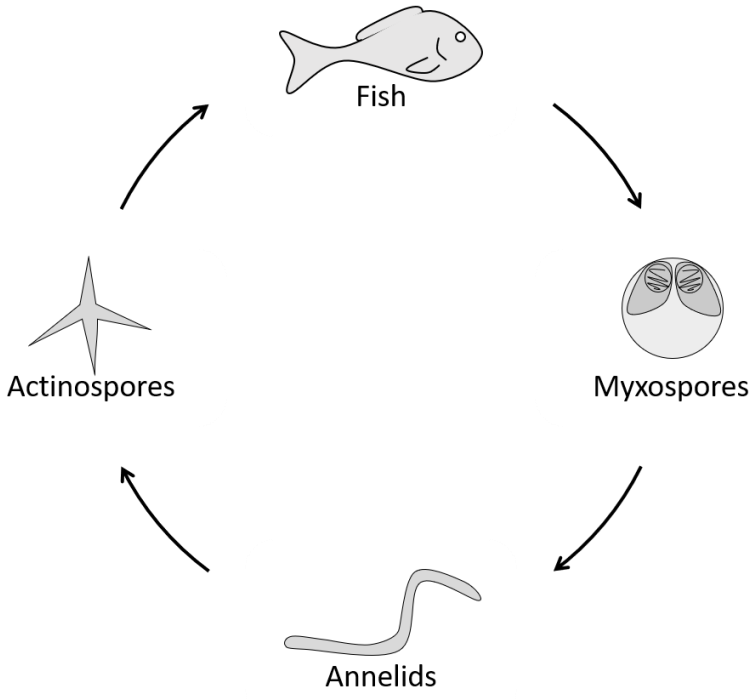

Figure 1: General lifecyle of Myxozoans based on Myxobolus cerabralis

1.1.1 *Sphaerospora molnari*

Species belonging to the genus *Sphaerospora* (Thelohan 1892) typically have more or less spherical myxospores, which can be smooth or feature ridges on the outside. Apart from the spore shape, the most characteristic features are spherical polar capsules and that the suture line lies in a plane perpendicular to the polar capsules. Fresh mature *Sphaerospora molnari* myxospores are subspherical, 10.5 µm wide and 10.3 µm long. The suture line is more noticeable on the anterior end of the spore. The polar capsules are 4-5 µm in length and 4-4.5 µm wide. (Lom et al. 1983)

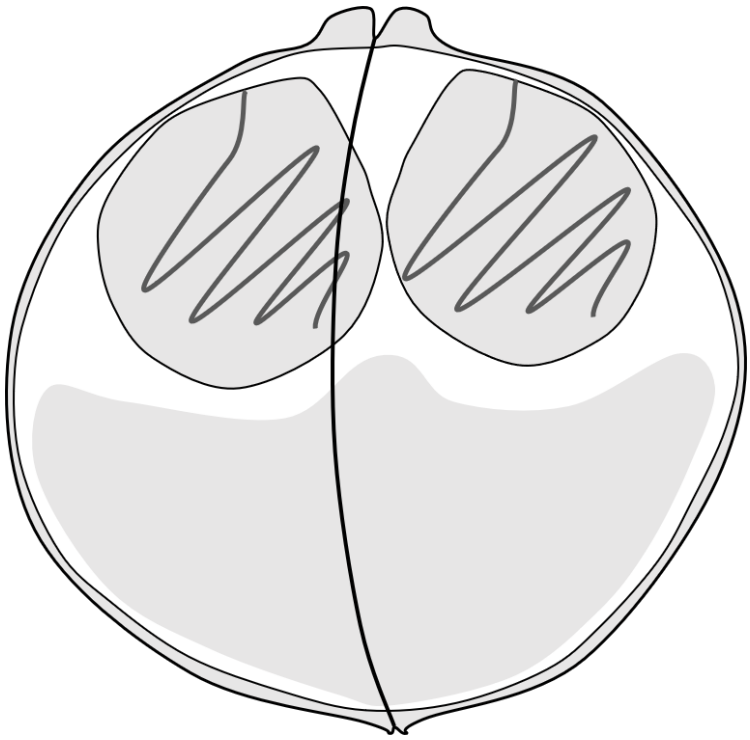

Figure 2: Schematic visualization of a Sphaerospora molnari myxospore according to (Lom et al. 1983)

In contrast to most *Sphaerospora* spp., which inhibit cavities of amphibians and fish (coelozoic) and usually have an impact on their excretory system, *S. molnari* is living in the tissues of *Cyprinus carpio* (Eszterbauer et al. 2013; Jirků et al. 2007) and is likely to be

oioxenic (Dyková and Lom 1988). *S. molnari* is linked directly to the development of gill sphaerosporosis (Eszterbauer et al. 2013). Infected fish can suffer from serious damage to secondary lamellae and epithelia of the gills which lead to reduced gas exchange in the gills and osmoregulatory problems. Spores are released into the surrounding water when the cell membranes in the necrotic tissue rupture (Lom et al. 1983). The skin epithelium can be affected as well or nasal pits (Lom et al. 1983). Sphaerosporosis can lead to deaths in 50-80% and in some cases up to 98-100% of fry (Waluga D. 1983). Although it is likely an understatement, 20% of carp fingerling stocks analyzed in Czechoslovakia were infected with *S. molnari*, which shows how common this parasite really is. In further investigations some of the seemingly healthy fish were kept in laboratory conditions and developed signs of an infection after some time (Dyková and Lom 1988). The complete lifecycle of *S. molnari* has not been resolved to this day, with the only reported one in the *Sphaerospora* clade being *Sphaerospora dykovae* (formerly *Sphaerospora renicola*) (Molnár et al. 1999).

Prior to the production of spores in the gills or skin, *Sphaerospora molnari* proliferates as a multicellular blood stage in the bloodstream of the host. In this stage *S. molnari* shows a non-directional twitching motility. The rotational motion is achieved by quick formation of folds on the exterior surface of the parasite which disappear rapidly. This movement can be referred to as Membrane Fold Induced Tumbling (MFIT). It has been shown that this type of movement is used as an evasion strategy against host immune cell attachment. (Hartigan et al. 2016)

1.2 Immune response

In fish there are two different types of immune responses present, the innate immune response and the adaptive immune response, mediated by T and B lymphocytes and specific antibodies (=humoral). In contrast to higher vertebrates, innate mechanisms are of major importance in fish. The basic principles behind the immune response in teleosts are remarkably similar to mammals. However, there are still significant differences, the most apparent being the deficiency of bone marrow and lymph nodes (Sunyer 2013) while in fish,the head kidney and spleen are crucial for immune defence (Press and Evensen 1999; Tort et al. 2003).

The first barrier for Myxozoa is the mucus of the fish which can contain molecules rejecting the parasite. For fish, mucosal immunity is essential as the skin and gills represent the portals of entry of any aquatic pathogen. Once the epithelial surface is passed, the first systemic defence mechanism which can directly affect Myxozoa is innate (Sitjà-Bobadilla et al. 2015). Most Myxozoa, in particular the coelozoic species do not cause a major host immune response (Lom and Dykova 1992) and in some cases, the immune reaction can be evaded if located in immunologically privileged sites (Sitjà-Bobadilla et al. 2015). Encapsulation of the parasite in layers of epithelioid and connective tissue, forming so-called granulomata is the most common defence mechanism in infections with Myxozoa. This prevents spreading of the parasite to other areas of the organism, while nutrients and oxygen are not blocked off sufficiently (Koehler et al. 2004). Lymphocytes and macrophages are the main types of leucocytes associated with granuloma formation. Typically, the formation of granulomata coincides with an increased production of melano-macrophage centers. Melano-macrophage centers appear to be analogous to lymph node centers in mammals (Sitjà-Bobadilla et al. 2015). Melano-macrophage centers process and retain antigens and macrophage activation which leads to maintenance of immunological memory (Agius and Roberts 2003).

Some fish do not get diseased after infection with certain genotypes of the myxozoan parasite, as reported with *C. shasta*, but can still show serious inflammatory reactions after exposure to other genotypes of the same parasite (Hurst et al. 2012). There are also significant differences in disease severity between different strains of a fish species following an infection with myxozoans (Grabner and El-Matbouli 2009). During mucosal infections granulocytes and phagocytes are the most common immune cells (Lovy et al. 2011; Sitjà-Bobadilla et al. 2015). Infections caused by myxozoans can modulate the activity of phagocytes which are

crucial for the defence of the host against the parasite. Not only have in vitro studies shown that the creation of reactive oxygen species is stimulated by certain myxozoans (Muñoz et al. 2000) but they also can involve lysozomes or proteases (Lovy et al. 2011; Muñoz et al. 2007). Natural killer (NK) cells are leukocytes which are utilized in innate immunity and can be divided into two groups. The NK-like cells and nonspecific cytotoxic cells (NCC) (Sitjà-Bobadilla et al. 2015). In *Sparus aurata* infected with *Enteromyxum leei* a down-regulation of the Tumor necrosis factor-α, interleukin-1β in the intestine was described (Sitjà-Bobadilla et al. 2008). Rainbow trout with *Myxobolus* cerebralis-infections typically exhibit temporary up-regulation of interleukin-1β 1&2, cyclooxigenase-2 and other pro-inflammatory genes (Severin and El-Matbouli 2007).

1.2.1 **Adaptive Immunity**

As the knowledge about the lifecycles of most myxozoa is very limited there are very few possibilities to study the host responses against those pathogens in a laboratory environment. Nevertheless, specific immunity after preceding exposure to myxozoa has previously been described. In *Salmo solar* a decrease of infection severity over time could be observed. A reintroduction of the parasite at a later point of time resulted in a weaker infection by utilization of MHII β^+/CD83$^+$ Antigen presenting cells as well as induction of CD8$^+$ and IgM$^+$ cells (Braden et al. 2017). The type II interferon IFN-γ might be inducing this upregulation of MHII (major histocompatibility complex class II) genes as type I IFNs only promote the expression of MHI molecules (Davey et al. 2011).

The adapative immune response involves specific antibodies against myxozoans proven in numerous by myxozoans infected fish, for instance in Atlantic salmon against *Kudoa thyrsites* (Braden et al. 2017), or in turbot against *E. scophthalmi* (Sitjà-Bobadilla et al. 2007). The rate of production of antibodies is relatively slow with at least 6 weeks after the fish has been exposed to the parasite (Sitjà-Bobadilla 2008). Location and quantity of plasma and B-cells are altered in fish infected with Myxozoa, but the exact change is dependent on the host species. In gilthead seabream IgM expression levels and IgM positive B cells in the head kidney are more numerous following an infection with *Enteromyxum leei* (Estensoro et al. 2014). In contrast to *E. scophthalmi*-infected turbot, where the number is decreased in the head

kidney and spleen (Bermúdez et al. 2006). In both species, a high number of plasma cells and IgM^+ B cells can be observed in the intestine. As they were observed surrounding the parasites in the submucosa and epithelia it is suggested that T cells might not be the only intraepithelial lymphocytes in teleosts (Estensoro et al. 2014; Sitjà-Bobadilla et al. 2015).

In *Oncorhynchus mykiss* infected with *Tetracapsuloides bryosalmonae* showing symptoms of proliferative kidney disease an up-regulation of the immunoglobulins M and T could be observed. The secretory forms of those immunoglobulins and, in particular, IgT showed a strong positive correlation with distribution and populations of the myxozoan parasite and the amount of kidney swelling. The membrane type IgT only showed weak and the membrane form IgM did not show any correlations with the parasite prevalence. (Gorgoglione et al. 2013)

1.2.2 Immune Evasion Strategies of Myxozoa

As a result of coevolution, hosts have developed methods to fight invading parasites, and Myxozoa have acquired mechanisms to escape the immune response. (Sitjà-Bobadilla 2008). One of the strategies used by Myxozoa is the occupation of host immunologically privileged sites, which have either reduced or highly specific surveillance options. Common immunologically privileged sites are, for example, eyes, testis, and the central nervous system. These routes are probably used sot that the immune response cannot be induced which allows increased proliferation and the targeted tissues can be reached more easily (Sitjà-Bobadilla et al. 2015). For example, there is (almost) no inflammation in some neurotropic myxobolids even though the parasite replaces a large part of the brain (Khoo et al. 2010). And since eyes are already identified as foreign to the body by the immune system due to their isolation during embryonic development they can be considered as immunologically privileged sites (Koevary 2000). In addition to a blood barrier testis are also immunoprivileged so that no autoimmune reaction agains germ cells is raised (Fijak and Meinhardt 2006).

The second evasion strategy is intracellular disguise, which allows concealing from the hosts' immune system, for example in oocyte developed myxozoans escape the immune response. Another benefit for the parasites is that these oocysts are nutrient-rich allowing them to proliferate easily (Swearer and Robertson 1999).

Another possible strategy is antigen-based which allows masking or mimicry of antigens. Even though this is well documented for parasites infecting mammals, there is only low documentation for myxozoans which leaves space for a greater number of studies (Sitjà-Bobadilla et al. 2015).

A better documented strategy is induced immunosuppression by myxozoans inhibiting host reactions (Densmore et al. 2004). Food depletion caused by the parasite is one of many factors that can cause immunosuppression and result in undesired weight loss and atrophy of muscle and brain (Wang et al. 2005). Further possible factors among many others are host leucocytes apoptosis, decreased proliferation of lymphocytes or the modulation of genes that encode for example immunosuppressive molecules (Gorgoglione et al. 2013; Densmore et al. 2004; Sitjà-Bobadilla et al. 2015). B lymphocytes have a crucial role in the production of specific antibodies and in adaptive immunity. Some parasites, e.g. *T. bryosalmonae (Abos et al. 2018)* and protozoan species (Zuniga et al. 2005), have developed mechanisms which limit their functionality to avoid the immune response by inducing polyclonal activation. In this process the production of antibodies which are not specific towards the parasite is increased and therefore reducing the relative amount of parasite-specific antibodies (Abos et al. 2018). It has been shown that hypergammaglobulinaemia, which occurs as a result of polyclonal B cell activation, also correlates with PKD (Olesen and Jørgensen 1985).

2 Aims

- Isolation of total RNA from the head kidney (HK) of common carp infected with *Sphaerospora molnari*

- cDNA synthesis using RT-PCR

- Taqman qPCR to measure the expression levels of membrane bound and secretory IgM in the head kidney of infected *C. carpio* sampled over the course of 9 weeks

3 Materials and Methods

3.1 Infection and Sampling of *Cyprinus carpio*

Fertilized *Cyprinus carpio* eggs originating from cultured carp in production ponds in Vodňany (Czech Republic), were provided by the Faculty of Fisheries and Protection of Waters of the University of South Bohemia. An experimental recirculation system (UV filtration, ozone) was used to accommodate the eggs and further rear the fish. Additionally, a constant water temperature of 21 ± 1 °C was maintained in the fish tanks. Nourishment was supplied daily in form fo a commercial carp diet (Skretting, Stavanger, Norway) at 2% of biomass.

Infection of 57 fish weighing 15 ± 4 g was performed by intraperitoneal injection of motile *S. molnari* blood stages. The parasites were previously isolated from the blood of 18 infected carp aged 0+ by centrifugation of whole blood in heparinized haematocrit capillaries at 4000×g for 5 minutes. The capillaries were broken and a mixture of *S. molnari* blood stages and white blood cells was obtained. PBS was used to wash the cells and subsequently, after centrifugation at 800×g for 5 minutes, the blood stages were resuspended, quantified and diluted to a concentration of 370 parasites/µl. 14 800 parasites were injected by using the corresponding volume of the suspension into the peritoneum of every specific pathogen-free (SPF) fish. Similarly, 40 µl of PBS were injected into 36 control fish. For appropriate anaesthesia all fish were treated with clove oil prior to injections and bleeding. All carp used in this experiment were kept 3 separate 80 litre fish tanks including filtration, aeration, and water changes at a weekly rate. Two aquaria accommodated experimental fish and one the control carp. The fish were sampled at 1, 2, 4, 7 days following the infection and then on a weekly basis until week 9. On every sampling day head kidneys of 5 experimental and of 3 control fish were taken, stabilized in RNAlater (Ambion, Europe Ltd, Huntingdon, UK) and frozen for storage.

3.2 Total RNA purification

For the isolation of the total RNA from head kidney and spleen tissue the NucleoSpin RNA kit (Macherey-Nagel, Düren, Germany) was used according to the manufacturer's instructions. Briefly, mechanical disruption of the sample tissue was performed using a plastic pestle rod fitting tightly into the microtubes. Subsequently, for lysis of the tissue and denaturation of ribonucleases 350 µL Buffer RA1 and 3.5 µL β-mercaptoethanol were added and the mixture was vortexed vigorously. Then, the viscosity of the solution was reduced by passing it through a NucleoSpin® Filter, by centrifuging for 1 minute at 11,000 g. By the addition of 350 µL of 70% ethanol and thorough mixing the RNA binding conditions were adjusted, the lysate was transferred to a Nucleospin® RNA II Column, RNA was left to bind to the silica membrane, followed by centrifugation for 30 seconds at 11,000 g. After applying 350 µL membrane desalting buffer to the column and 1 minute of centrifugation so the membrane can dry, the contaminating DNA needed to be degraded. For this, 95 µL DNase reaction mixture, prepared by mixing 10 µL reconstituted rDNase with 90 µL reaction buffer for rDNase, was added straight onto the membrane and left at room temperature for 15 minutes. To inactivate the rDNase 200 µL Buffer RA2 was added, and the column was centrifuged for 30 seconds. Following the transfer of the column into a new collection tube, 600 µL Buffer RA3 were applied to wash the column and another centrifugation step with a duration of 30 seconds was performed. The flow through was discarded and another washing step with 250 µL Buffer RA3 and 2 minutes of centrifugation was done. The column was placed in a microtube and the purified RNA was eluted by centrifugation with 20-60 µL RNase-free H_2O.

3.3 cDNA synthesis using RT-PCR

cDNA synthesis was performed using the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics): All reagents were thawed and centrifuged before use. To a nuclease-free thin walled PCR tube, which was cooled on ice, 500 ng purified total RNA were added together with 2 µL random hexamer primer (600pmol/µL) and an adequate amount of water. The total volume was added up to 11.4 µL, therefore a maximum of 9.4 µL of the RNA solution can be added, even in the case of a lower concentration. Afterwards, denaturation of the template primer mixture is achieved by heating the tubes to 65 °C for 10 minutes in a block cycler with a heated lid and cooling on ice before further steps. For reverse transcription, the following reagents were added to the mixture so a total volume of 20 µL was reached.

Table 1: Reagents added to the reaction

Reagent	**Volume [µL]**
Protector RNase Inhibitor (40 U/µL)	0.5
DTT	1
Transcriptor High Fidelity Reverse Transcriptase	1.1
Deoxynucleotide mixture (10mM)	2
Transcriptor High Fidelity Reverse Transcriptase Reaction Buffer (5x conc.)	4

Subsequently, the content of the microtubes was mixed and centrifuged to ensure that the contents are at the bottom of the tube. Afterwards, the PCR tubes were placed in a preheated block cycler and the reaction was kept at 29 °C for 10 minutes. Next, everything was brought to 48°C and incubated for 60 minutes. Finally, inactivation of the transcriptase was achieved via incubation of the reaction for 5 minutes at 85°C and the reaction was stopped by cooling it on ice.

3.4 qPCR

A mixture of *S. molnari* blood stages and white blood cells was used to acquire transcriptomic data, employed to mine for IgM heavy chain sequences of *C. carpio*. One of the found isoforms had a transmembrane domain (on B cell/plasma cell surface; GenBank: MH352353) and the other a secretory tail (secreted form; GenBank: MH352354). Using Primer3 (v.0.4.0.; http://bioinfo.ut.ee/primer3-0.4.0/) TaqMan qPCR primers and probes were designed to specifically differentiate between the expression of the secretory tetramer (IgMsec) and the membrane-bound monomer (IgMmem). IgMsec and IgMmem concentrations were estimated relative to host β-actin.

The Lightcycler 480 kit by Roche was used for the analysis of the previously synthesised cDNA samples. As the reagents were stored at a temperature between -15 °C and -25 °C, they need to be defrosted at room temperature and mixed before use. For the qPCR the cDNA was diluted to approximately 100 ng/µL. The primers were diluted to a concentration of 10 µM and the probes to 5 µM. As the probes are sensitive to light they were covered with aluminium foil. Per well a total reaction volume of 25 µL is reached by addition of all necessary reagents, which are listed in the following table. The LightCycler® 480 Probes Master is a mixture of 6.4 mM $MgCl_2$, FastStart Taq DNA Polymerase, a reaction buffer and a deoxynucleoside triphosphate (dNTP) mixture with dUTP replacing dTTP.

Table 2: Reagents and their volumes per qPCR reaction

Reagent	**Volume [µL]**
LightCycler® 480 Probes Master	12.5
Forward Primer	1
Reverse Primer	1
5′ FAM and 3'BHQ1-labelled Taqman probe	1
H_2O	4.5
cDNA	5
total	**25**

The qPCRs were performed in white 96-well plates which were covert with a sealing foil after addition of all chemicals. The plate was then centrifuged for 10 minutes, followed by insertion into the Lightcycler Instrument. Then the reaction was started. First, the denaturation was performed by incubating at 95 °C for 15 minutes. Afterwards, Amplification is achieved by heating to 95 °C for 15 seconds followed by incubation at 60 °C for 1 minute and repeating for a total of 50 cycles.

Table 3: Primers used in the qPCR

Primer	**Sequence 3' to 5'**	**Amplicon length**
IgMsec Forward	TCGTATTAGCACCCCCAGAG	106bp
IgMsec Reverse	TCATCAGCAAGCCAAGACACA	
IgMmem Forwad	GCTAGAGCATCCCTGTACG	94bp
IgMmem Revere	CAAGGTGATGAGGAACAGGAA	
β-actin Forward	AGGTATGGAGTCTTGCGGTA	80bp
β-actin Reverse	ACAGGTCCTTACGGATGTCG	

Table 4: Labelled probes used in the qPCR

Probe	**Sequence**
IgMsec	BHQ1-ACAAAAAGGGTGAACCGATG-FAM
IgMmem	BHQ1-TTGCAAACACTGCCATAACC-FAM
β-actin	BHQ1-TGAGACCACCTTCAACTCCAT-FAM

3.5 Analysis of acquired data

On the basis of the difference of the take-off points of the infected compared to control fish, the calculation of the relative expression ratio of all the samples was performed in accordance with Pfaffl's mathematical model for real-time RT-PCR (Pfaffl 2001) and normalized to the reference gene β-actin. Incorporating log-transformed response variables, differences in IgM expression were evaluated. Subsequent multiple pairwise comparisons for evaluation of the influence of fixed effect were achieved application of Tukey's all-pair comparisons, using the Bonferroni correction adjusting the P-values (package multcomp, v.1.3-3). The program R (R Core Team, v.3.4.2.) was used for analysis and visualization of the acquired data.

4 Results

Expression of the membrane bound, and secretory IgM types were analysed in the head kidney after intraperitoneal injection of *S. molnari* blood stages, performing differential TaqMan assays.

In the infected carp the membrane type immunoglobulin showed a higher expression starting from 21 days post infection (dpi). Before reaching a maximum at 42 dpi the expression of IgMmem kept increasing, significantly more in contrast to days 1 to 7 after infection (LM: $F_{(11,43)} = 4.9$, $P < 0.01$). After this peak the IgMmem expression showed a distinct decrease, returning to relatively low levels levels.

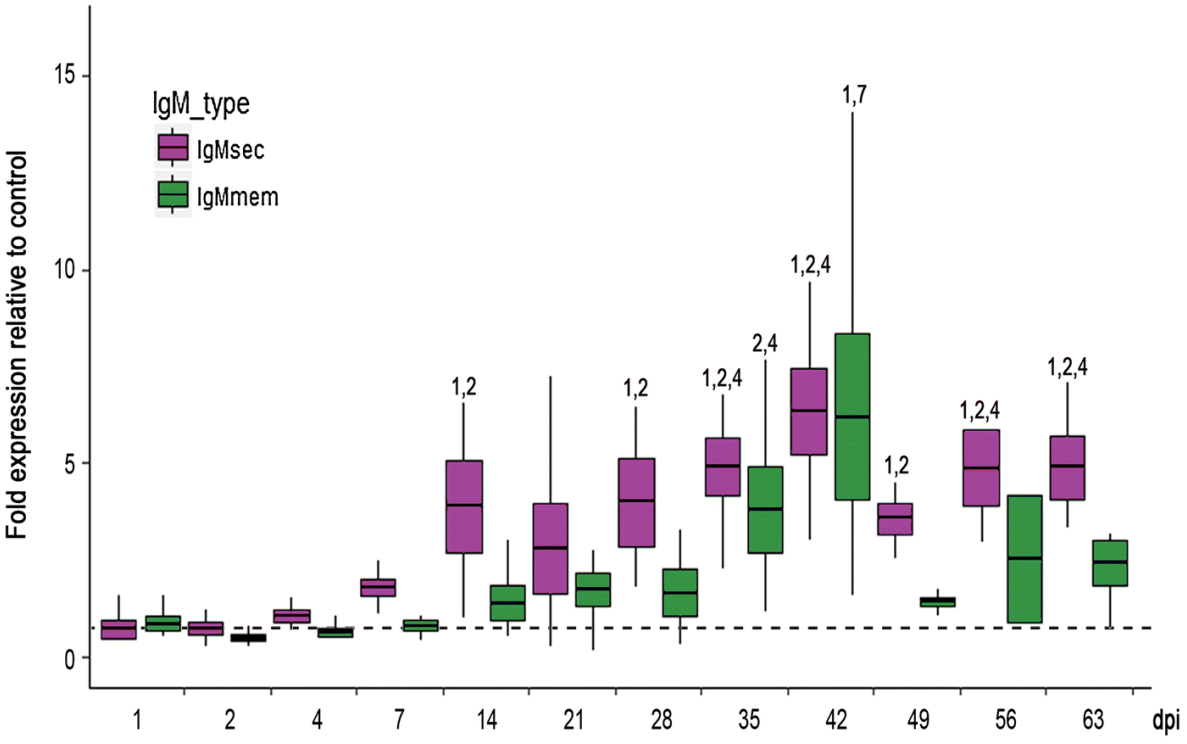

Figure 3: IgM expression of C. carpio over time as a response to S. molnari infection

The standard deviation is visualized with box plots including the mean. The observed range of expressions is indicated by vertical lines. Significance relative to other sampling days is displayed by numbers above the box plots.

The tetrameric secretory IgM (IgMsec) showed significantly elevated expression levels from 14 dpi onwards. Similar to IgMmem, the maximum of IgMsec expression appeared at 42 dpi, showing a up to 10-fold increase (LM: $F_{(11,44)} = 8.2$, $P < 0.001$ to 0.049). After this peak the expression levels dropped but IgMsec remained elevated for the duration of the experiment.

5 Discussion

As can be seen in the results section, Figure 3 shows that membrane bound IgM is already expressed significantly higher than in the control group at 21 dpi. The secretory IgM is expressed even earlier at 14 dpi. The maximum expression of both immunoglobulins could be observed at 42 dpi, followed by a distinct decrease. For this experiment the IgM expression was observed in the head kidney as it plays a fundamental role in the immune response of teleosts. It is the production site of IgM and is also responsible for the immune memory by producing melano-macrophage centers.

Humoral responses in fish towards myxozoa have been studied in the past, showing parasite-specific responses and acquired immunity to *K. thyrsites* in Atlantic salmon (Braden et al. 2017) and against *E. scophthalmi* in turbot (Sitjà-Bobadilla et al. 2007). The distinct increase of IgM expression could be either caused by an increased production of parasite-specific antibodies or due to polyclonal IgM hypersecretion. In polyclonal activation, the production of non-specific antibodies is increased significantly while the expression of antibodies binding to the parasite remains at relatively low levels. Several reports show polyclonal activation in fish as a form of immune evasion induced by parasites, e.g. *Trypanoplasma borreli* in carp (Saeij et al. 2003), also including some myxozoan species, namely *T. bryosalmonae* in rainbow trout (Abos et al. 2018) and *E. leei* in gilthead sea bream

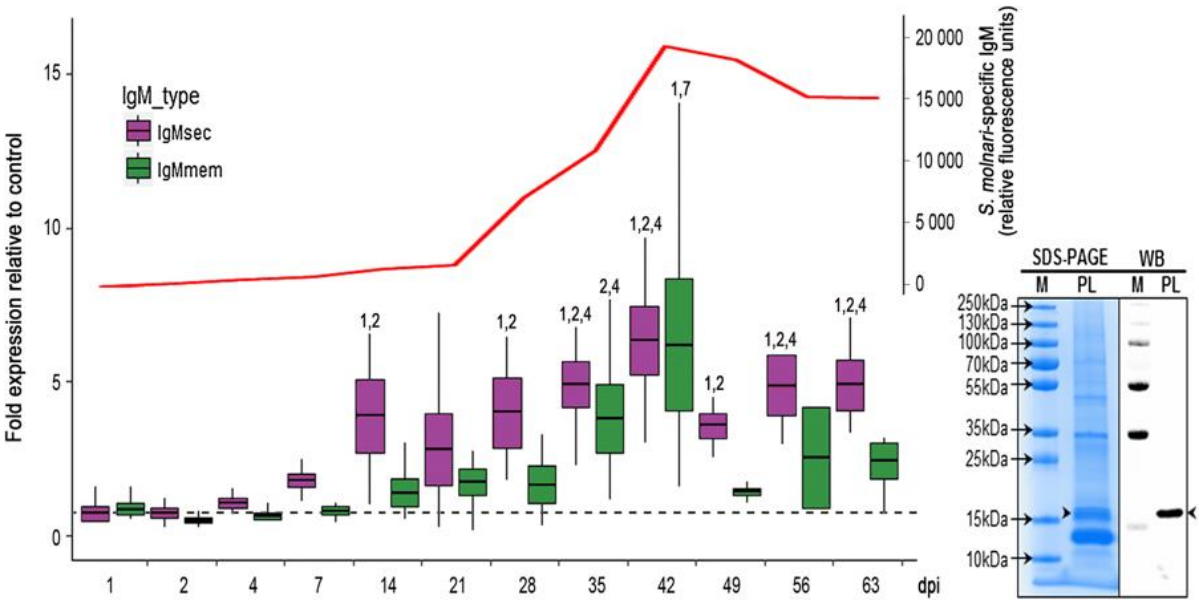

Figure 4: Comparison of IgM expression over time as a response to S. molnari infection to parasite specific antibodies in the sera of infected C. carpio (Korytář et al. 2019)

(Estensoro et al. 2012). The IgM expression levels measured during this experiment do not give any information about the specificity of these antibodies. Detection of specific antibodies in the blood of infected fish was determined in a different experiment by western blotting. Parasite lysates were run through an SDS-PAGE, transferred to a PVDF membrane, followed by blocking and subsequent exposure to the sera of infected fish. Antibodies bound to parasite proteins, were detected using mouse anti-carp IgM and HRP-labelled goat anti-mouse IgG. This confirmed the presence of parasite-specific antibodies following the infection with *S. molnari* (see Figure 4) (Korytář et al. 2019). In combination with data showing a strong increase in IgM^+ B lymphocyte numbers in the blood (Korytář et al. 2019), hypergammaglobulnemia and further the utilization of polyclonal activation by *S. molnari* similar to *T. bryosalmonae* in rainbow trout (Abos et al. 2018) is indicated.

Previous studies concerning humoral responses in fish against myxozoans reported finding the earliest specific antibodies six weeks post infection (Sitjà-Bobadilla 2008). During the experiment this delayed response could not be confirmed, with the first IgM expression increase being detected at 14 dpi. These findings are in accordance with previous studies of the immune response of common carp towards other antigens, namely, trinitrophenyl–lipopolysaccharide (TNP–LPS) and dinitrophenyl–keyhole limpet hemocyanin (DNP_{494}–KLH) (Engelsma et al. 2003). Engelsma showed an anti-TNP–LPS antibody response in common carp kept at 25 °C already after 10 days and first antibodies against DNP after 19 days. The water temperature in the aquaria for the fish in this experiment was kept at 21 °C. As it was also demonstrated that temperature stress is also greatly affecting the immune response of teleosts.

More studies concerning the host-parasite interactions would be beneficial as the knowledge is only limited and a better understanding of the exact mechanisms is crucial for developing treatments and vaccines against *S. molnari*. However, the invertebrate host is yet to be found and there are no methods to produce enough actinospores for comprehensive research. Once a laboratory model for actinospore production of *S. molnari* is established, a more natural infection route could be applied to include immune responses in the mucous of the fish. Future studies should also focus on the identification of myxozoan proteins targeted by specific antibodies produced in infected fish.

6 Conclusion

The aim of the project described within this thesis was to determine the gene expression of the membrane bound IgM and the secretory IgM in the head kidney following an infection with *Sphaerospora molnari*. This was achieved by first extracting the total RNA of this organ. Subsequently, cDNA was synthesized by use of a random hexamer primer and then a Taqman qPCR was performed using β-actin as the reference gene. The secretory IgM expression increased significantly after 14 dpi with the maximum levels being reached at 42 dpi. However, IgMsec expression remained at elevated levels until the end of the experiment. The expression of the membrane bound IgM showed elevated levels after 21 dpi, reaching its peak at 42 dpi.

7 List of abbreviations

dNTP	deoxynucleoside triphosphate
dpi	days post infection
DTT	Dithiothreitol
HK	head kidney
Ig	Immunoglobulin
IgMmem	membrane bound IgM
IgMsec	secretory IgM
LM	linear model
MFIT	Membrane Fold Induced Tumbling
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PVDF	polyvinylidine fluoride
SDS PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SPF	specific pathogen-free

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