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Haematological response of common carp *Cyprinus carpio* to experimental infection with blood stages of the myxozoan parasite *Sphaerospora molnari*

Bachelor thesis

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ANNOTATION

Sphaerospora molnari is a histozoic endoparasite causing severe inflammation during spore-formation in the gills of common carp. As for other myxozoans, little is known about the host response to early proliferation and multiplication of this parasite in the fish host. This thesis is focused on a controlled infection experiment using *S. molnari* blood stages and on determining cellular and haematological changes of common carp as a response to infection, over a 9-week period post intraperitoneal injection. The results show a noticeable increase in cellular components of innate immunity 14 dpi and a massive increase in lymphocyte numbers, likely B cells, 21-28 dpi. The data are discussed in relation to parasite infection intensity in various tissues and organs, and they strongly point to the acquisition of specific immunity of common carp to *S. molnari* resulting in a controlled, low level infection from 49 dpi onwards.

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1 INTRODUCTION

1.1 The Myxozoa

The Myxozoa Grassé, 1970 are a group of metazoan organisms belonging to the phylum Cnidaria. They are microscopic endoparasites, who alternately exploit invertebrate and vertebrate (predominantly fish) hosts in different aquatic environments (Holzer et al. 2018, Okamura et al. 2015). In accordance with their invertebrate hosts and life cycles, Myxozoa are divided into two classes, Myxosporea and Malacosporea (Canning et al. 2000). Myxosporeans use annelids as definitive hosts, whereas malacosporeans use bryozoans as definitive hosts (Okamura et al. 2015). Both use fish as intermediate hosts. Nevertheless, some species of Myxozoa also infect amphibians, reptiles (Eiras 2005, Hartigan et al. 2016), birds (Bartholomew et al. 2008) and mammals (Prunescu et al. 2007, Székely et al. 2015). Currently, more than 2,200 species of Myxozoa were described. This means that the clade represents approximately one fifth of all cnidarians. (Zhang et al. 2011, Okamura et al. 2015). During the process of evolution of the Cnidaria parasitism has evolved 7 times independently (Weinstein and Kuris 2016). However, only the Myxozoa are highly diverse, morphologically extremely simplified microscopic endoparasites with a complex parasitic life cycle.

As transmission stages between invertebrate and vertebrate hosts, myxozoans form hard, durable spores, which help them to survive the period outside the host before invading another one. These multicellular spores are an important characteristic of myxozoans (Fiala et al. 2015). They contain polar capsules with a coiled filament which are homologous to the cnidocyst of free-living cnidarians.

The life cycle of *Myxobolus cerebralis*, the agent of salmonid whirling disease was the first one described (Wolf and Markiw 1984) and the authors showed alternation of two morphologically completely different spore stages formed by the fish and the oligochaete host. Bilaterally symmetrical, commonly round to oval myxospores are formed in fish while spores formed in annelids have a triradiary symmetry and often long floating appendices.

The piscine life cycle is initiated on first contact of the actinospore, released form the definitive host, with the intermediate fish host. After touching the skin or the epithelium of gills and on chemical stimulation (Kallert et al. 2011), the polar filaments are released from the polar capsules to attach the spore to the host. Thereafter, the sporoplasm is released from the valves, and it enters the host body through epidermal or epithelial mucous cells (El-Matbouli and

Hoffmann 1998). Sporoplasms initiate cycles of massive proliferation in different sites (Bjork and Bartholomew 2010, Holzer et al. 2003) before they migrate to the site where sporogony takes place, where plasmodia or pseudoplasmodia are formed and spores are produced within them. Typical for myxozoans is a cell-in-cell development, the endogenously arisen secondary cells persist in the primary cell. Plasmodia can be coelozoic – appear in the cavities of organs, or histozoic – appear in tissues. Plasmodia are composed of a large cell with many vegetative nuclei and endogenously produced generative cells. Plasmodia are polysporic. Pseudoplasmodia are smaller and contain one vegetative nucleus with generative cells necessary for forming one or two spores only. Myxospores leave the host body via faeces and urine, are released from epithelia or require death and composition of the host and thereafter, they invade the definitive host. In the invertebrate host the actinospore stage develops and sexual reproduction occurs just after infection (Morris 2010) and before proliferation and actinospore formation (El-Matbouli and Hoffmann 1998, Lom and Dyková 2006).

Malacosporea, like Myxosporea, have two life cycle phases taking place in two different hosts, but a lot less detail is known. However, spores are relatively undifferentiated (Hartikainen et al. 2014). They begin the life cycle similarly to Myxosporea by spores released from bryozoans attaching to fish hosts by polar filament extrusion. Sporoplasms enter the host via mucous cells (Longshaw et al. 2002). They migrate via the bloodstream to the target kidney tissue, where they proliferate and replicate, whereby the production of malacospores occurs in the renal tubules. These spores are excreted in the urine (Morris and Adams 2008). Malacospores from fish hosts enter the coelom of the filter-feeding definitive hosts, representative of the bryozoans. In the bryozoan coelomic cavity sac or worm-like stages are formed in which sporogenesis occurs (Morris et al. 2002, McGurk et al. 2006, Lom and Dyková 2006). Spores which leave the body attack the intermediate host and the whole cycle repeats itself.

1.2 *Sphaerospora molnari* in common carp

Representatives of the genus *Sphaerospora* Thélohan, 1892 have spherical, subspherical or slightly elongated spores with identical valves that are either smooth or ridged with several protuberances and bumps on the posterolateral end (Lom and Dyková 2006, Fiala et al. 2015). Plasmodia or trophozoites are usually disporic, barely polysporic (Jirků et al. 2007). The genus *Sphaerospora* includes pathogenic species, which are mostly coelozoic and produce spores in the excretory system of fish and amphibians (Jirků et al. 2007). Most Sphaerospora spp. including the type species cluster together in the most basal myxosporean phylogenetic clade, *Sphaerospora sensu stricto* (s. str., Jirků et al. 2007, Bartošová et al. 2013) and are characterized by long insertions in the 18S rDNA region (Holzer et al. 2007). The only known life cycle is that of *S. dykovae* (previously *S. renicola*; Molnár et al. 1999), which uses carp and the oligochaete *Branchiura sowerbyi* as alternate hosts. *Sphaerospora molnari* Lom, Dyková, Pavlásková and Grupcheva, 1983 is a parasite of common carp fry *Cyprinus carpio*. It forms spores in the epithelia of the skin and gills. The disease caused by this parasite is called sphaerosporosis (Lom et al. 1983). Gill sphaerosporosis was first studied by Molnár (1979), who determined *Sphaerospora carasii* Kudo, 1919 as the etiological agent. In another study, he stated that sphaerosporosis of the carp fry can spread to the skin (Molnár 1980). Lom and Dyková found fresh spores in carp skin, which differed from *S. carasii* and other *Sphaerospora* species described in carp (1982). This new species was called *S. molnari* according to its original discoverer (Lom et al. 1983).

S. molnari is a histozoic parasite invading the epithelium of the gill filaments. Unlike most members of the *Sphaerospora spp*., who are coelozotic and sporulate in the excretory system of fish, *S. molnari* produces mature spores in the epithelium of the gill tissue (Estzerbauer et al. 2013). Because *S. molnari* is most closely related to *S. dykovae* in the kidney of carp, it probably developed from coelozoic kidney parasites of freshwater fishes. Its epithelial location is likely a consequence of niche specialization to avoid competition in the kidney tubules of carp (Estzerbauer et al. 2013).

For *S. molnari,* almost spherical spores with prominent suture and central fissure on the anterior site are typical. The average spore length is 10.3 μm and the width is 10.5 μm. Equal subspherical polar capsules contain polar filaments which are coiled most often into 4 coils parallel to the longitudinal axis of the spore. There are two mononucleated sporoplasms that occupy a small part of the spore (Fig. 1, Lom et al. 1983).

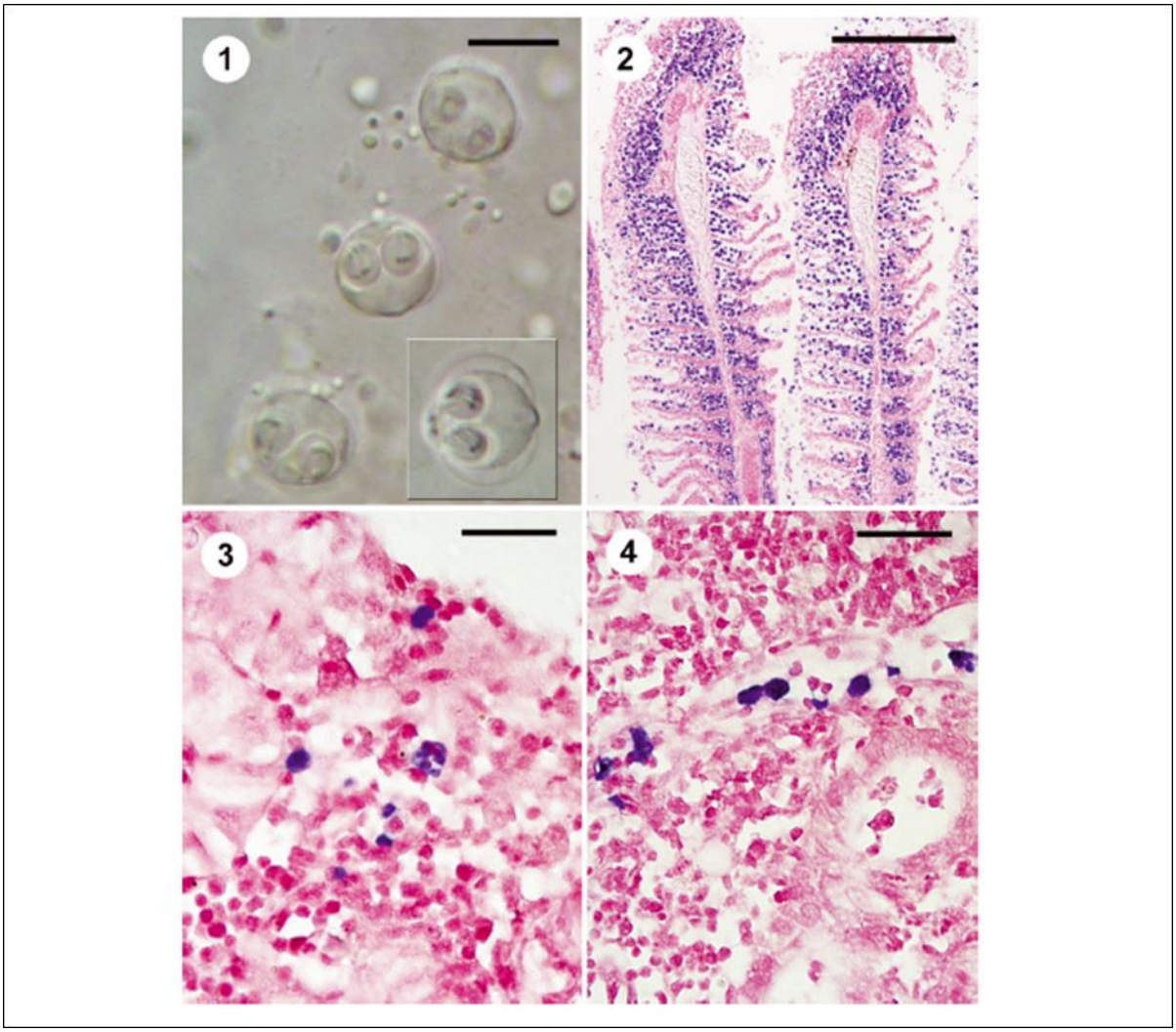

Fig. 1: *Sphaerospora molnari* and *Cyprinus carpio carpio*. (1) *S. molnari* myxospores from gill of common carp fingerling *C. carpio carpio*. Fresh preparation. Bar = 10 µm. Inset: *S. molnari* myxospore with mucous envelope. (2) Gill of common carp fingerlings heavily infected with *S. molnari*. The monosporous pseudoplasmodia develop in high numbers in the epithelium of the gill filaments. Parasites show dark blue coloration. *In situ* hybridization. Bar = 200 µm. (3) Kidney parenchyma of common carp fingerling with *S. molnari* developmental stages. Parasite stages are dark blue. *In situ* hybridization. Bar = 10 µm. (4) *S. molnari* blood stages (stained dark blue) detected in a kidney capillary of common carp fingerling. *In situ* hybridization. Bar = 10 µm (Estzerbauer et al. 2013).

Sphaerospora s. str. species are characterized by presporogonic development and proliferation in the blood of their fish hosts (Lom et al. 1983, Baska et al. 1988, Supamattaya et al. 1991, Holzer et al. 2003). *S. molnari* developed a specific cellular motility characterized by rotation that helps it to evade contact with cells of the immune system (Hartigan et al. 2016). The

earliest stage of the sporogonic phase known is the pseudoplasmodium, where the parental cell with 1-6 sporogonic cells develops to one spore. The pseudoplasmodium can no longer be detected when a sporoblast is formed. A sporoblast contains 2 uninucleated sporoplasms and develops a single mature spore (Lom et al. 1983). Mature spores are released from the carp into the environment by rupture of the external epithelium and invades the definitive host, which is unknown to most species of the *Sphaerospora s. str*. clade.

Sphaerospora molnari is the agent of sphaerosporosis of common carp. This parasite causes severe inflammation in the tissue of gills and skin (Lom et al. 1983, Estzerbauer et al. 2013). Parasites get into the epithelium by diffusion and often form large clusters of developmental stages as well as mature spores of *S. molnari*. If a critical infection is present (Fig. 1) the parasitic cells almost replace the interlamellar cell layer. The afflicted epithelium exhibits signs of dystrophy and necrosis, which causes spores being releasing into the environment (Lom et al. 1983). The pathology related to the massive presporogonic proliferation of *S. molnari* in the blood is unknown to date, but molecular methods confirmed that proliferative stages of *S. molnari* may well contribute to the course of swim bladder inflammation (Holzer et al. 2014) and could potentially be the cause of pathology in other organs, such as the eye (Molnár 1993, Holzer et al. 2014).

1.3 Immune system of fish

The teleost immune system includes the majority of cells and molecules described in mammals. In addition to the evolutionary older innate immunity, teleosts are the first group armed with the BCR/TCR-based adaptive immunity (Flajnik and Kasahara 2010, Sunyer 2013). The cutting line between primary and secondary lymphoid organs is not as obvious as in mammals. The bone marrow is not developed and the lymph nodes are missing. Haematopoiesis takes place in the anterior part of the kidney, which also acts as the secondary lymphoid organ, filtering the blood for antigens. Thymus is the only primary lymphoid organ, specialized in the production of T cells. Spleen acts as the main peripheral lymphoid organ involved in the filtering blood-born antigens and immune complexes as well as in the initiation of the adaptive responses. It homes lymphoid tissue with T and B cells and macrophages in a supporting network of reticular cells (Press and Evensen 1999, Zapata and Amemiya 2000, Zapata et al. 2006, Hadidi et al. 2008, Uribe et al. 2011, Castro and Tafalla 2015). The systemic immune organs are complemented by mucosa-associated lymphoid tissue (MALT) which comprises of four major sites; the gut associated lymphoid tissue (GALT), the skin-associated lymphoid tissue (SALT), the gill associated lymphoid tissue (GIALT) and the nasopharynx-associated lymphoid tissue (NIALT). (Salinas, Zhang and Sunyer 2011, Tacchi et al. 2014, Rombout, Yang, and Kiron 2014). Unlike adaptive immunity, innate immune mechanisms are relatively temperature independent and fast. Acquired immunity is temperature dependent and the formation of antibodies takes several weeks (Elis 2001).

1.3.1 Innate immune response

Innate immune responses are the fundamental defence against pathogens in fish. They are based on cellular and humoral factors and physical barriers of epithelial surfaces separating the fish from the outer environment (Magnadóttir 2006, Castro and Tafalla 2015). If the pathogen crosses the epithelium, it is recognized by the pattern-recognition receptors (PRR), such as TLRs, NLRs, CLRS and PGRPs. These are highly conserved receptors recognizing specific molecules, so called pathogen-associated molecular patterns (PAMPs). PAMPs include a variety of bacterial peptidoglycans and lipopolysaccharides, viral double stranded RNA, bacterial DNA and other molecules. (Elward and Gasque 2003). Blood-borne pathogens can be eliminated by the complement system. Complement is an ancient humoral system comprising about 30 distinct proteins, which can be activated by three pathways. Generally, the activity of complement in teleost is several orders of magnitudes higher than that in

mammals and can be influenced by a number of factors including season, temperature or composition of a diet. (Boshra, Li and Sunyer 2006).

Cellular components of innate immune responses comprise monocytes/macrophages, natural killer (NK)-like cells, non-specific cytotoxic cells (NCC) and granulocytes (Secombes and Wang 2012). Macrophages are responsible for phagocytosis of foreign particles, immune response to those pathogens and maintaining homeostasis (Secombes and Wang 2012). They develop from hemopoietic progenitors, which differentiate via blood monocytes or directly to tissue macrophages (Forlenza et al. 2011). On their surface, macrophages express TLR, scavenger receptors, CLR and complement receptors and produce cytokines and growth factors which stimulate the immune response against pathogens (Secombes and Wang 2012). During infection the number of macrophages increase and morphological changes appear (Ranzani-Paiva et al. 2004). NK-like cells and NCC are mediators of non-specific cell-mediated cytotoxicity (CMC). NCC are organ-derived small lymphocytes which eliminate tumor and virally transformed cells and protozoan parasites. NK cells are blood leukocytes which kill allogeneic cells and other targets by the perforin/granzyme-mediated apoptosis pathway (Praveen et al. 2004, Secombes and Wang 2012). Neutrophils are important cells of inflammatory immune responses. They fight against bacterial, viral, protozoan and fungal pathogens by phagocytosis, toxic reactive oxygen and nitrogen intermediates, degranulation and releasing neutrophil extracellular traps (NETs) (Nathan 2006, Katzenback and Bolosevic 2009). Eosinophilic granulocytes and basophils mediate immune response by degranulation. The presence of these cells is very rare if the organism keeps its homeostasis (Balla et al. 2010, Piačková et al. 2014). Mast cells are components of inflammatory response against pathogens. During degranulation they release a variety of functional proteins (Secombes and Wang 2012). The number of mast cells rise e.g. during parasitic infection (Lauriano et al. 2012). Rodlet cells are present only in epithelia of tissues, not in vascular epithelia (Reite 2005). The number of rodlet cells is usually higher in tissues invaded by parasites (Secombes and Wang 2012). Some research on the immune activity of thrombocytes was conducted and it was confirmed that platelets play an important role in phagocytosis of pathogens (Thuvander et al. 1987, Nagasawa et al. 2014).

1.3.2 Adaptive immune system

The main mediators of adaptive immune response are B and T cells. B cells mediate antibody (humoral) responses and T cells are responsible for cell-mediated immune responses

(Secombes and Wang 2012). Furthermore, the specific immune response includes interaction with receptors, immunoglobulins (Ig) and antigen-presenting cells (APC) (Tort et al. 2003). The humoral adaptive immune response comprises immunoglobulins and B cells. The highest concentration of B cells as well as immunoglobulin occurs in the pronephros (Danilova and Steiner 2002). There are two forms of immunoglobulins, B cell receptors (BCR) and antibodies (immunoglobulins). The BCR is an antigen receptor embedded in the membrane of the B cell. Antibodies are secreted by differentiated B cells known as antibody secreting cells (ASCs) or plasmablasts (plasma cells in mammals) (Kaatari et al. 2009). Three types of immunoglobins, IgM, IgD and IgT/Z were described in teleost (Salinas, Zhang and Sunyer 2011). IgM, the most prevalent serum antibody, is considered to be the functional antibody in mucosal and systemic response (Solem and Stenvik 2006). IgDs are expressed by mature B cells but their function is still unclear (Parra et al. 2016). IgT/Zs are responsible for the gut, skin and gill mucosal immunity (Zhang et al. 2010, Xu et al. 2013, Xu et al. 2016).

Progenitor B cells bind immunoglobulins of a single specificity on their surface. After encountering a pathogen and binding it on its surface, B cells stimulate phagocytotic cells and activate the cascade of complement reactions (Bruce and Brown 2017). Furthermore, a clonal expansion appears, plasma cells and memory B cells are developed. (Schroeder and Cavacini 2010, Secombes and Wang 2012). B cells also have a phagocytic activity (Li et al. 2006). T cells are the mediators of cell immune responses. The peptides of intracellular pathogens are presented by MHC molecules on the cell surface. These molecules are recognized by TCRs. MHC is divided into two classes, MHC I, which is expressed on the surface of almost all cells, activate CD8+ cytotoxic T (T_C) cells. MHC II is only expressed by APC and active CD4+ helper T (T_H) cells (Zhu et al. 2013).

Very important mediators of cellular immune responses are small proteins called cytokines. These proteins act as regulators of immune responses. Cytokines are secreted by several immune cells, nevertheless the main producers are T_H cells and macrophages (Zhang and An 2007).

1.3.3 Host reaction to myxozoan infections

Many myxozoan parasites have little influence on their host and their presence is almost asymptomatic (Schmidt-Posthaus and Wahli 2015). However, some myxozoan species infecting can cause important pathologies resulting in millions of losses in aquaculture production. Currently, no commercial treatments or vaccines are available for myxozoans in

fish destined to human consumption. To be able to mitigate myxozoan diseases it is essential to better understand host-parasite interaction and the reaction of the host immune system to parasite development (Sitjà-Bobadilla et al. 2015).

The common immune response to Myxozoa starts when the sporoplams of actinospores invade the host through gills, skin and gastrointestinal tract covered by mucus, and start to proliferate in the host. In the mucosal layer, the parasite encounters lysozyme, lectins, immunoglobulins or commensal microbiota. Other defence compounds of the mucus are macrophages, granulocytes including mast cells, B cells, T cells and immunoglobulins. If the parasite passes the first barrier it can proliferate and migrate through the blood to the target tissues. As an immune evasion strategy, myxozoans often develop in immunoprivileged sites (eyes, gonads or brain) or try to avoid the immune mechanisms with intercellular disguise, antigen masking or fast proliferation. In blood, the parasitic stages can be eliminated by granulocytes and mast cells, lysozyme, complement, antiproteases and immunoglobulins. When the parasite invades the target tissue, different cellular factors activated by cytokines fight the pathogen. Depending on host-myxozoan model, this interaction causes an acute or chronic inflammatory response. Sporogenesis often leads to necrosis and encapsulation of parasites, and finally granuloma formation. A different type of reaction, often more systemic, may be caused by pre-sporogonic stages (Sitjà-Bobadilla et al. 2015).

Tetracapsuloides bryosalmonae is known as a serious myxozoan pathogen in salmonids and the related disease (Proliferative Kidney Disease) is of emerging economic and environmental concern. *T. bryosalmonae* proliferative stages in the interstitial tissue of the kidney cause an acute host response with pathogenesis shaped by a massive in situ proliferation of lymphocytes and suppression of innate/phagocytotic activity (Chilmonczyk et al. 2002). Immunoglobulins play another major role in myxozoan infections (Zhang et al. 2010) with hyperimmunoglobulaemia (IgM, IgT) being an important characteristic of PKD (Hedrick et al. 1993, Gorgoglione et al. 2013). Furthermore, the pathology of PKD is likely based on a dysregulated T_H cell activity (Gorgolione et al. 2013).

With regard to sphaerosporids, pathological effects have been related to spore-forming stages in the kidney tubules and bowman's capsules (e.g. Dyková and Lom 1982, Sitjà-Bobadilla et al. 1993, Holzer et al. 2013), and pre-sporogonic stages of *S. dykovae* are known as causative agents of swim bladder inflammation of common carp (Kovacs-Gayer 1983, Holzer et al. 2014). None of these infections have been studied in an immunological or cytological

framework or under controlled laboratory conditions, i.e. in single infections and at multiple dates over time. The presporogonic development of *S. molnari* involves massive proliferation in the blood and potentially further parasite multiplication inside certain host organs, especially the liver (Jedličková 2017). The aim of this thesis was to determine the cellular and haematological response of common carp to the massive proliferation of *S. molnari* and to determine if the presence of blood stages and tissue stages can cause a systemic response similar to that observed in *T. bryosalmonae*.

2 OBJECTIVES

The specific aims of this study were:

- To perform a controlled infection experiment by IP injection of blood stages of *Sphaerospora molnari* into specific pathogen-free fish.
- To observe and determine the cellular and haematological response in common carp over a 9-week period post IP injection (12 sampling points).
- To determine the relation between haematological and cellular changes to parasite location and number.

3 MATERIALS AND METHODS

3.1 Experimental exposure of *C. carpio* to *S. molnari*

Specific pathogen-free (SPF) *C. carpio* larvae were obtained from the Faculty of Fisheries and Protection of Waters (FROV) of the University of South Bohemia, in Vodňany, in April 2015. The stock of SPF fish was maintained at 21±1 °C and fed a commercial carp diet. At the age of 1 year SPF fish (15±4.5g) were used for experimental infection with *S. molnari* blood stages. Blood stages originated from one-year-old carp obtained from Malá Outrata pond (Vodňany, Czech Republic), in April 2016. Whole blood was sampled from euthanized fish by caudal vein puncture, using a heparinized syringe (for details see section 3.2.1). Blood of 13 fish was spun in haematocrit tubes, at 5000g for 3 min. Then the leukocyte layer containing a mixture of leukocytes and *S. molnari* blood stages was collected and diluted in plasma. Blood stages were counted in a Bürker chamber (described in section 3.2.3) and further diluted to 370 stages per µl. A volume of 40µl, equalling 14800 blood stages was intraperitoneally injected into 57 SPF carp each. At the same time, 36 control fish were injected with 40µl of PBS. Thereafter, all fish were kept at 21±1 °C, under recirculation conditions and with daily feeding of standard diet. Fish were sampled on day 1, 2, 4, 7, 14, 21, 28, 35, 42, 49, 56 and 63 post injection. On each sampling day, 5 experimental and 3 control fish were sampled. Samplings included full haematology (see section 3.2., aim of this thesis) as well as DNA and RNA samples of several organs for quantification of parasites and immune gene expression (not part of this thesis).

3.2 Analysis of haematological parameters

3.2.1 Blood sampling

The blood volume of fish is relatively small. It comprises only 1-2% of total body weight. There are two possible techniques for taking blood samples from fish, either by cardiac puncture or by caudal venous puncture (Doubek et al. 2003). To prevent the manipulative stress and mechanical harms during taking blood it is necessary to immobilize the fish by anaesthetics. For this purpose, in this experiment all carp were anesthetized separately in buckets with aeration before taking blood samples. Clove oil with a concentration of 0.02-0.04ml/l was used as an anaesthetic (Doubek et al. 2003). Blood was taken with 0.8x25mm needles and 1 ml single use syringes (both Henry Schein, UK). The syringes were rinsed with heparin to prevent blood coagulation. Blood sampling of experimental carp was performed by

puncture of the caudal vessel. When the blood was taken (100-300µl) it was transferred to an Eppendorf tube for the following examinations of haematological parameters.

3.2.2 Haematocrit

Determination of the haematocrit value is a basic research method for studies on fish red blood cells (Doubek et al. 2003). Haematocrit expresses the ratio of the volume of red blood cells to the whole volume of blood. For the determination of the haematocrit value it is necessary to separate the erythrocytes from plasma. This is achieved by centrifugation of the blood based on different density of white and red blood cells (Svobodová et al. 2012). For the experiment, 75mm capillary tubes were used. From whole heparinized blood collected by syringe and transferred into an Eppendorf tube blood was drawn into ¾ of the length of capillary. Then the bottom was closed with sealing compound and the capillary was spun at 5000g for 3 min. After this time interval the blood components were separated into layers (Fig. 2). The size of the erythrocyte layer was divided by the size of the plasma+leukocytes+erythrocytes layer and the result denotes the haematocrit value expressed as % of the blood volume. For each experimental animal two haematocrit values were taken in order to guarantee the accuracy of result.

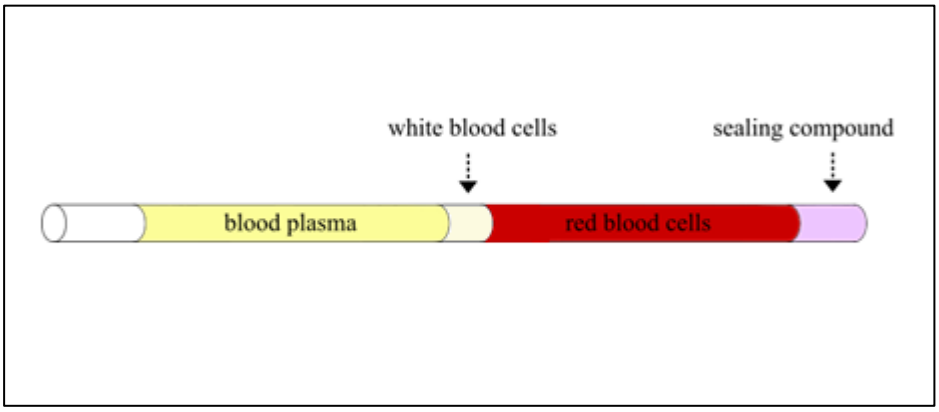

Fig. 2*:* Separated blood components in capillary (adapted from Rohlenová 2011).

3.2.3 Haemoglobin

The haemoglobin content in fish blood was determined using the transformation solution according to Drabkin (0.2g K3[Fe(CN)6], 0.05g KCN, 1.2 g NaHCO3 and distilled water filled up to 1000ml) and the photometric method according to the methodology of Svobodová et al. (2012). Twenty µl of heparinized blood from the Eppendorf tube was added to 4980 µl of transformation solution in 5 ml plastic tubes. The whole content was mixed well and left 15

minutes rest on ice. The sample measurement was performed in a 1 cm^3 cell at 540 nm against the transformation solution (blank). For each fish sample two measurements were accomplished to avoid mistakes.

3.2.4 Erythrocyte, leukocyte and thrombocyte numbers

The number of blood cells was determined in heparinized blood diluted in Hayem's solution (2.5g $HgCl_2$-sublimate, 25g Na_2SO_4, 5g NaCl and 1000ml distilled water) at a ratio of 1:200 (Svobodová et al. 2012). 4975 µl of Hayem's solution were pipetted into a 5ml tube. Then 25 µl of heparinized blood from the Eppendorf tube were added and the whole content of the 5ml tube was mixed well. Thereafter, erythrocytes, leukocytes and thrombocytes were counted in a Bürker chamber under the microscope according to the methodology of Svobodová et al. (2012).

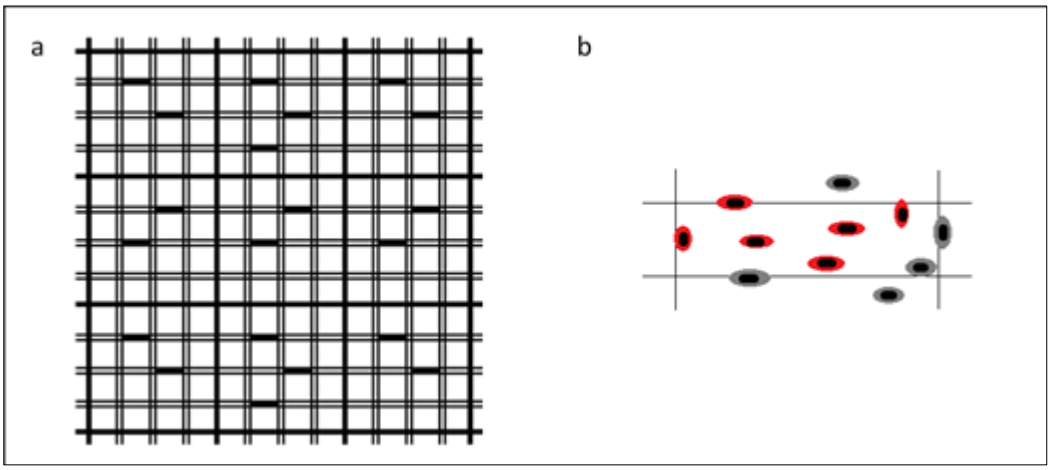

Fig. 3: Counting of red blood cells. (a) 20 rectangles of Bürker chamber where the erythrocytes were counted. (b) Counting cells according to the Bürker's law (adapted from Svobodová et al. 2012).

To determine the number of erythrocytes single cells were counted at 200x magnification in 20 rectangles regularly distributed over the whole lattice of the Bürker counting chamber (Fig. 3a). While counting the erythrocytes the so-called Bürker's rule was followed. According to this rule only the cells lying inside the rectangle and laying on the upper and left rectangle delimitation lines are counted. The cells touching the right and lower delimitation lines were not counted (Fig. 3b). Leukocytes and thrombocytes were counted according to Bürker's rule at 200x magnification in 100 small squares of the Bürker chamber (Fig. 4). The amount of counted red blood cells was divided by 100. The result accounts for the number of erythrocytes

in $x10^6$/µl of blood. The total amount of white blood cells and platelets was divided by 2 and the result denotes the number of leukocytes and thrombocytes in $x10^3$/µl of blood. Thrombocytes and leukocytes can be difficult to differentiate when unstained as they are similar in size. In order to avoid misidentification by different researchers performing the counting (n=2-3 at each sampling event) thrombocytes were included into the total leukocyte count and later deducted after determination of thrombocyte numbers in the differential blood cell count (see 3.2.4).

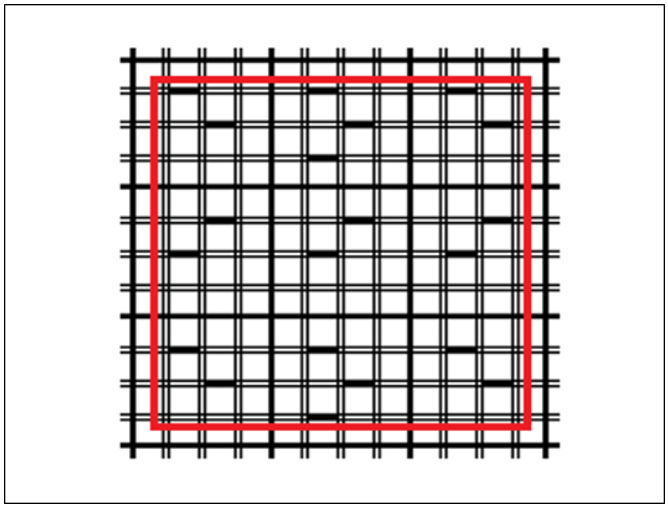

Fig. 4: Counting of white blood cells and thrombocytes in 100 small squares. (adapted from Svobodová et al. 2012).

3.2.5 Mean cell volume (MCV)

MCV of erythrocytes was calculated from haematocrit and erythrocytes numbers for each fish according the following equation.

$$MCV = \frac{haematocrit(\%) \times 10}{erythrocytes\ (\text{x}10^6/\mu\text{l})}$$

The results denote for MCV in fl (Svobodová et al. 2012).

3.2.6 Differential blood cell count

To determine the differential white blood cell count blood smears were created according to the methodology of Svobodová et al. (2012). Blood smears were fixed by methanol and stained by Kwik Diff Stain kit (Thermo Shandon, UK). The kit is a modified version of a compound dye by Paul Ehrlich (1910), which employs an anionic xanthene dye, eosin (orange) and a

cationic thiazine dye (methylene blue). The differential leukocyte count was determined under the light microscope, at 10x100 magnification. Two slides of blood smears were created for each experimental fish and 100 cells (leukocytes+thrombocytes) were counted on every slide. The types of cells were determined according to their morphology and coloration (Fig. 5). Along with leukocytes and thrombocytes, parasites – if present - were counted, too.

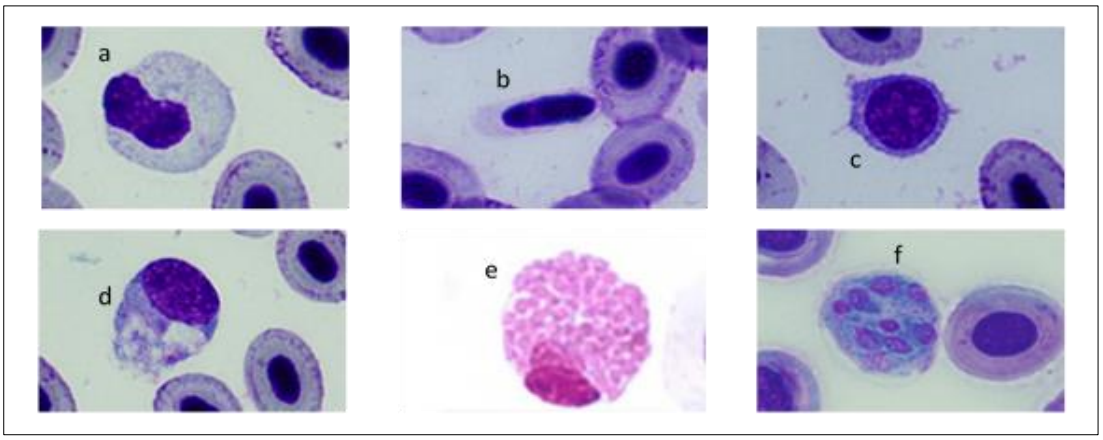

Fig. 5: Morphology and coloration of white blood cells, thrombocyte and parasite blood stages. (a) Neutrophil, (b) thrombocyte, (c) lymphocyte, (d) monocyte, (e) eosinophil (reproduced from Piačková et al. 2014), (f) *S. molnari* blood stage.

3.2.7 Data processing

The measured and calculated numbers on absolute differential cell counts, haematocrit, haemoglobin and MCV were statistically processed. For each blood parameter of every experimental fish, the arithmetic mean with standard deviation was calculated per day. These results were statistically analysed in STATISTICA 12.

The non-parametric comparison of two independent samples by Mann Whitney U test was performed to evaluate changes between infected and control group of fish, on each day of the experiment.

For visualization, the arithmetic means, standard deviations and significance levels were depicted by bar charts in Microsoft Excel.

4 RESULTS

4.1 Haematocrit (Fig. 6)

The haematocrit values of infected fish were determined to fluctuate insignificantly over time, however, a slight decrease on 2 and 42 days post infection (dpi) was noted. Unexpectedly, the haematocrit values of control fish dropped on days 4 and 7 dpi. The Mann Whitney U test determined a significant difference between control and infected fish for days 7 (p=0.0339) and 56 (p=0.0495) dpi.

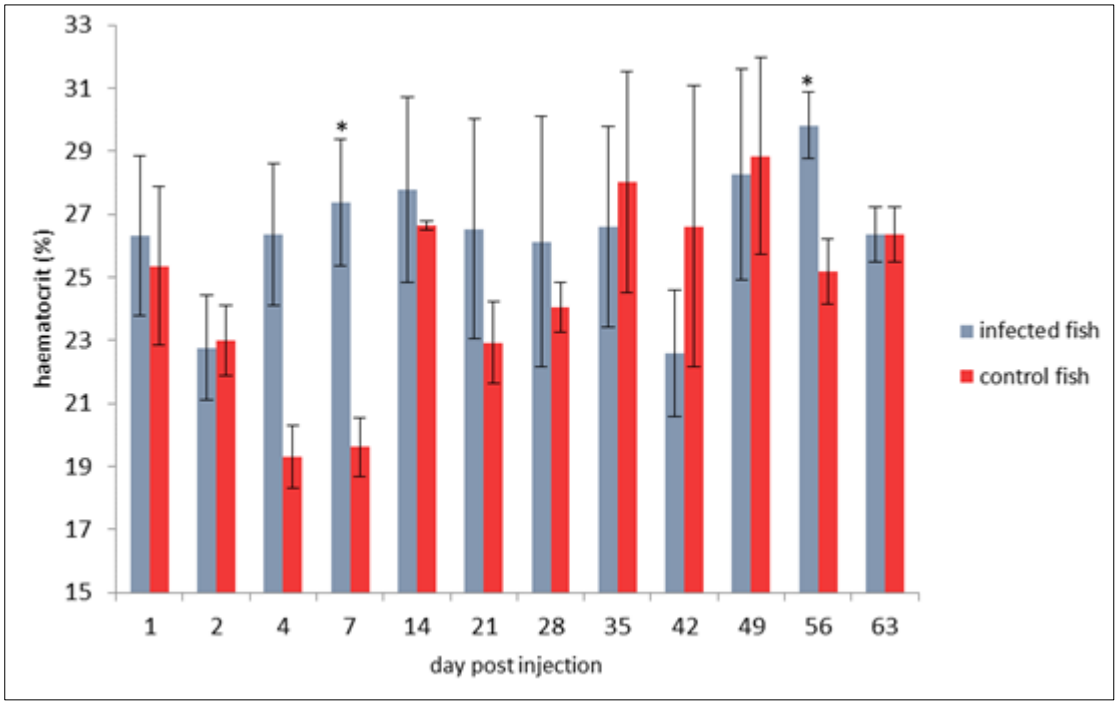

Fig. 6: The changes of haematocrit values during experimental infection of common carp with *S. molnari* blood stages, showing infected and control fish. Graph shows arithmetic means with standard deviations on 12 sampling points post infection. Asterisks determine the significance levels between control and infected fish, based on Mann Whitney U test. (*) $0.01 \leq p < 0.05$.

4.2 Haemoglobin (Fig. 7)

Haemoglobin concentration in carp blood remained stable in infected fish, during the whole experiment. Similar to haematocrit values, there is a decline in haemoglobin concentration in control fish on 4 and 7 dpi. A statistical difference between control and infected fish was determined on 4 (p=0.0253) and 21 (p=0.0253) dpi.

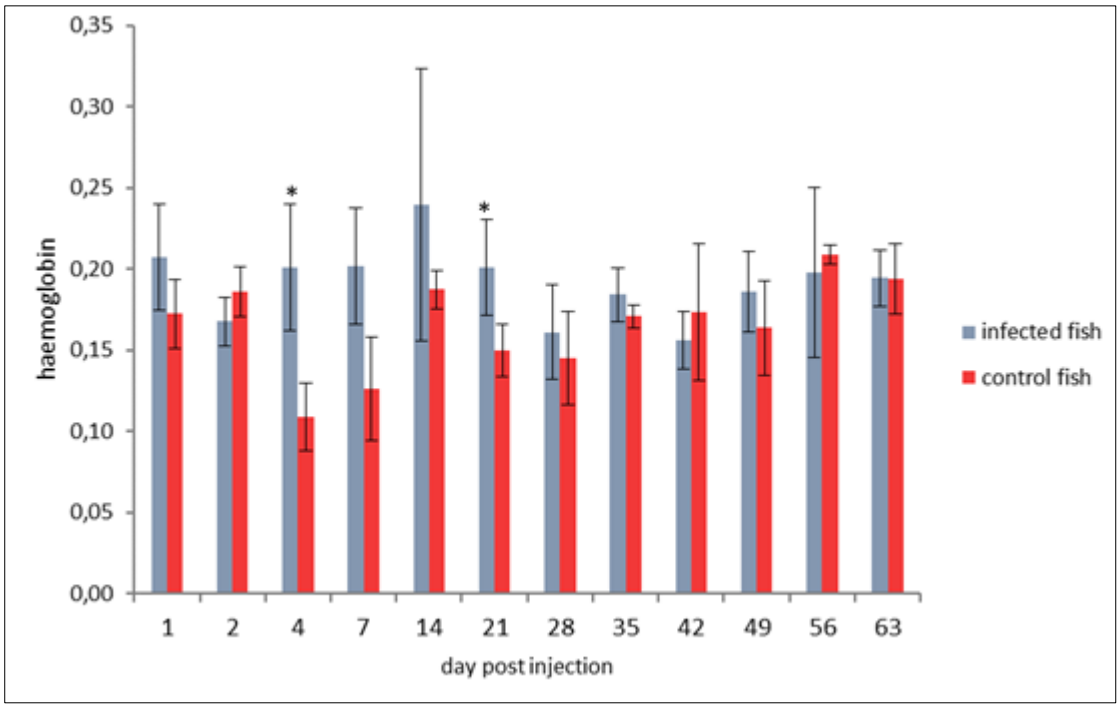

Fig. 7: Changes of haemoglobin concentration during the infection of common carp with *S. molnari* blood stages, showing infected and control fish. Arithmetic means with standard deviations for 12 sampling points post infection are shown. Asterisks determine the significance levels of the difference between control and infected fish, based on Mann Whitney U test. (*) $0.01 \leq p < 0.05$.

4.3 Erythrocytes (Fig. 8)

In infected fish, the number of red blood cells was shown to fluctuate very slightly. Once again, there is a notable drop of erythrocyte numbers in control fish on 4 and 7 dpi, similar as for haemoglobin and haematocrit. The statistical analysis determined that the values for control and infected fish differ significantly on 7 (p=0.0253) and 56 (p=0.0495) dpi.

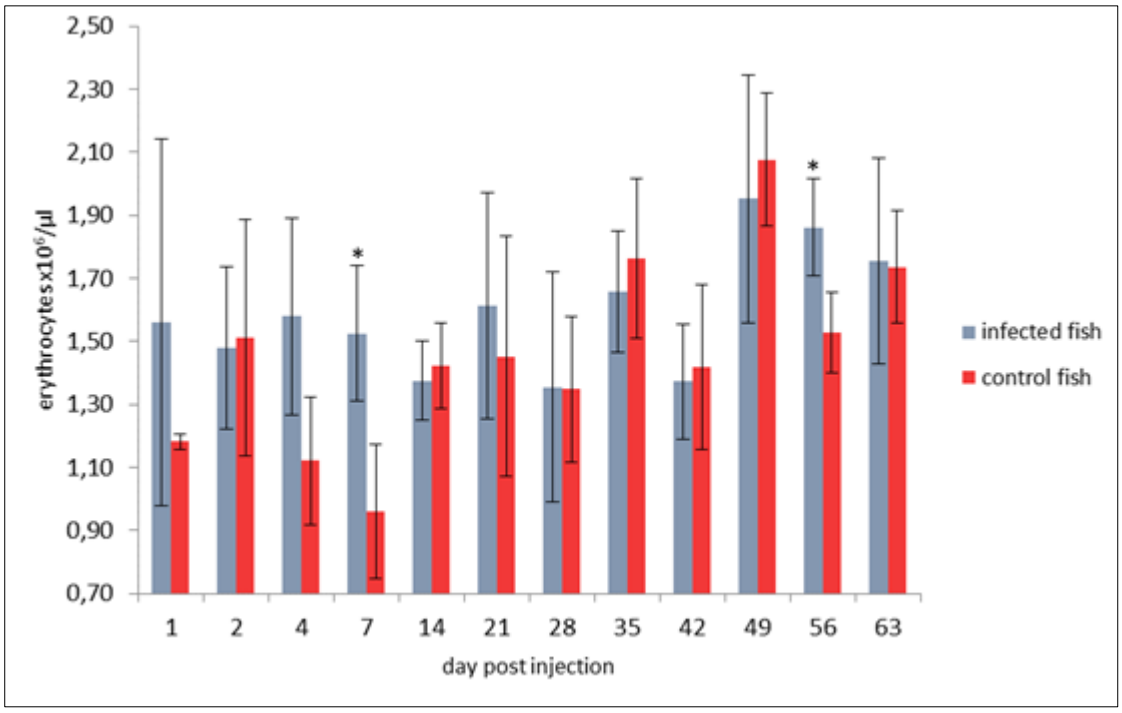

Fig. 8: Changes of erythrocyte numbers ($x10^6$/µl of blood) during the infection of common carp with *S. molnari* blood stages. Arithmetic means with standard deviations are shown for 12 sampling points post infection, for infected and control fish. Asterisks demonstrate the significance levels of the difference between control and infected fish, calculated using the Mann Whitney U test. (*) $0.01 \leq p < 0.05$.

4.4 Mean cell volume (Fig. 9)

The numbers of mean cell volume of erythrocytes (MCV) seem to be stable during the experiment. In control fish the MCV was higher 1 dpi and on 49 dpi there was a very small drop. Infected fish numbers of MCV were increased 14 and 28 dpi. According to the statistical test the numbers of MCV did not change significantly.

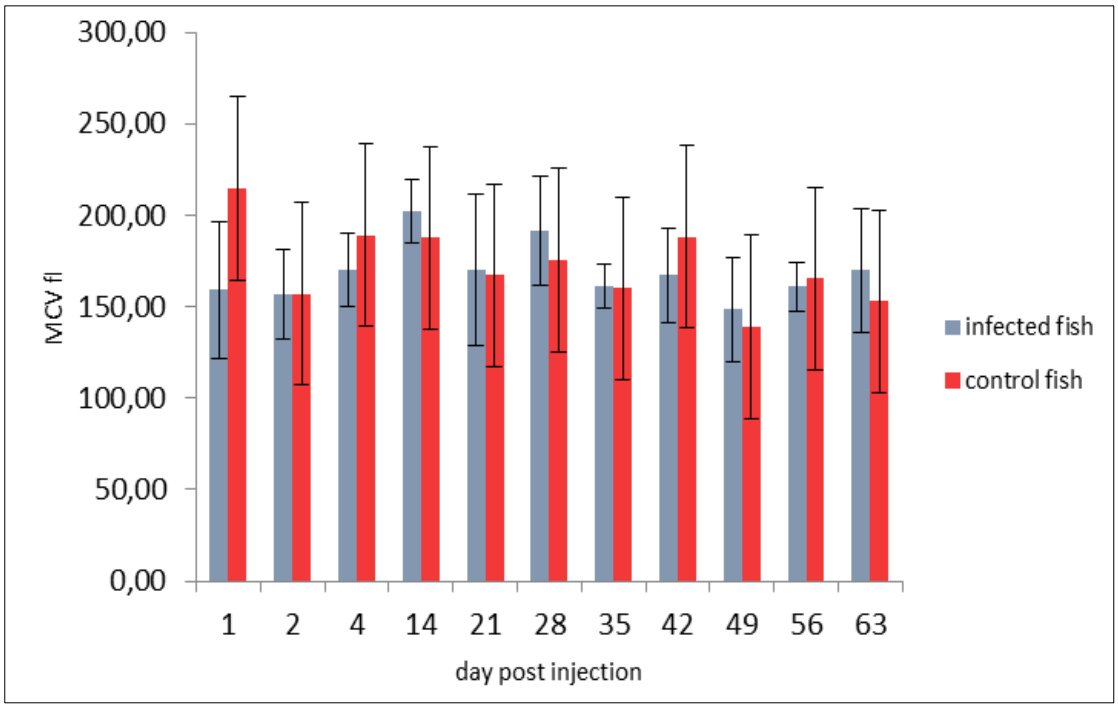

Fig. 9: Changes of MCV numbers (fl) during the infection of common carp with *S. molnari* blood stages. Arithmetic means with standard deviations are shown for 12 sampling points post infection, for infected and control fish.

4.5 Thrombocytes (Fig. 10)

Thrombocyte numbers of infected fish were found to rise gradually during the infection with *S. molnari*. The data show a significant increase from 35 dpi onwards and numbers reach their peak 56 dpi. There is a decrease 42 dpi, a trend which is also seen in other blood cell parameters. The control group numbers drop 7 and 21 dpi. The statistic test, comparing control and infected fish, determined a significant difference 42 (p=0.0253) and 49 (p=0.0253) dpi.

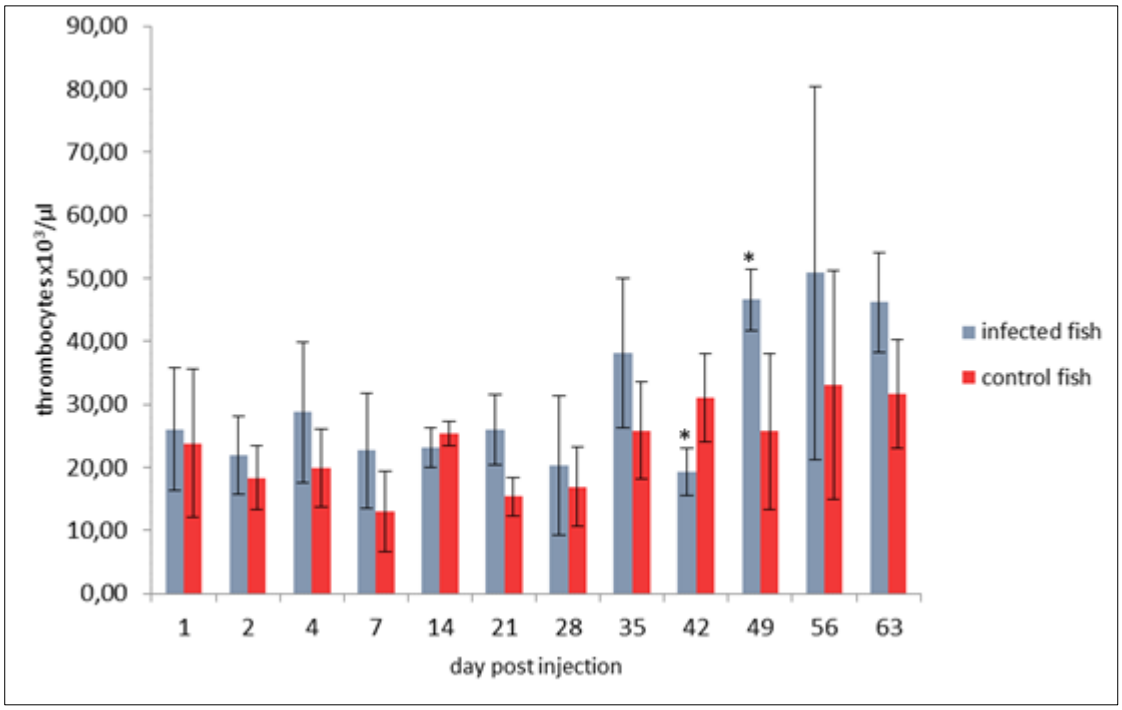

Fig. 10: Changes of thrombocyte numbers ($x10^3$/µl of blood) during the infection of common carp with *S. molnari* blood stages. Arithmetic means with standard deviations are shown for 12 sampling dates post infection. Asterisks demonstrate the significance levels of the difference between control and infected fish, determined by Mann Whitney U test. (*) $0.01 \leq p < 0.05$.

4.6 Leukocytes (Fig. 11)

The numbers of white blood cells of infected fish increased distinctly during the infection with *S. molnari*, reaching approximately 10-fold concentrations of those determined in uninfected fish. The numbers begin to rise 21 dpi and reach two peaks, 35 dpi and 56 dpi. There is a decrease 42 dpi. The numbers of leukocytes in the control group remain stable. According to the statistical analysis, significant difference between infected and control fish is seen 28 (p=0.0339), 35 (p=0.0253), 49 (p=0.0253), 56 (p=0.0495) and 63 (p=0.0339) dpi.

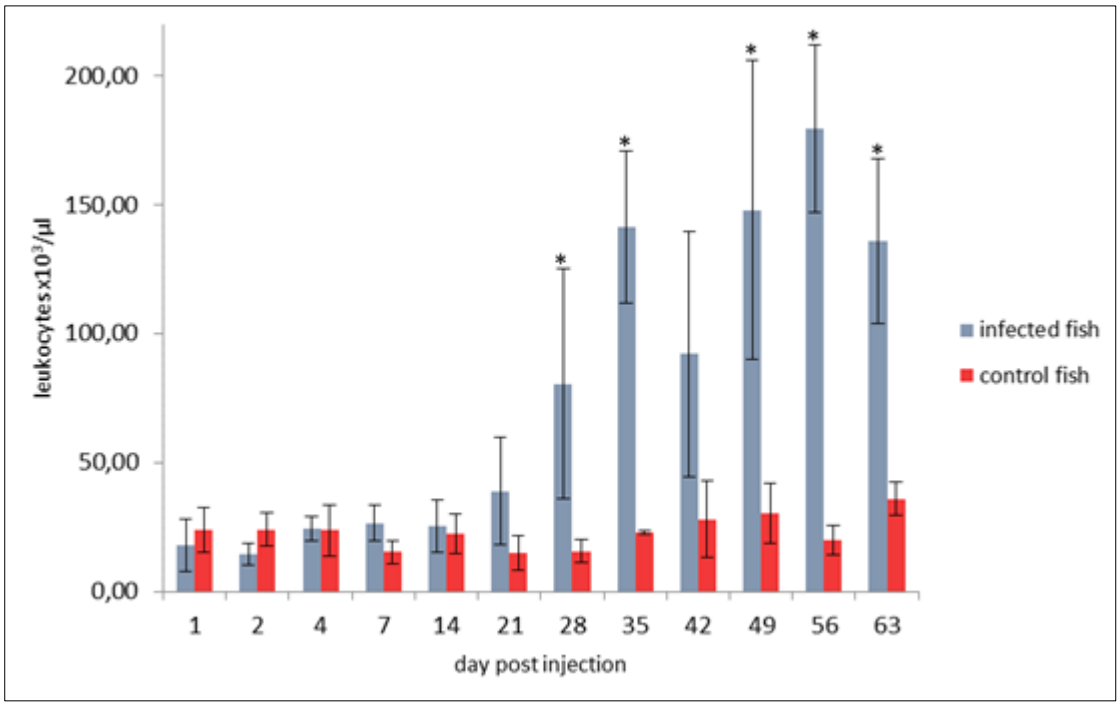

Fig. 11: Changes of leukocyte numbers in ($x10^3$/µl of blood) during experimental infection of common carp with *S. molnari* blood stages. Arithmetic means with standard deviations are shown for 12 sampling points post infection. Asterisks demonstrate the significance levels of the difference between control and infected fish, determined by Mann Whitney U test. (*) $0.01 \leq p < 0.05$.

4.7 Lymphocytes (Fig. 12)

The results from the lymphocyte numbers basically correspond with leukocytes, so the cell number responsible for the changes observed in leukocytes are lymphocytes. The numbers of lymphocytes start increasing 21 dpi and reach a peak 56 dpi. A statistical difference between control and infected fish was determined 35 (p=0.0253), 49 (p=0.0253), 56 (p=0.0495) and 63 (p=0.0339) dpi.

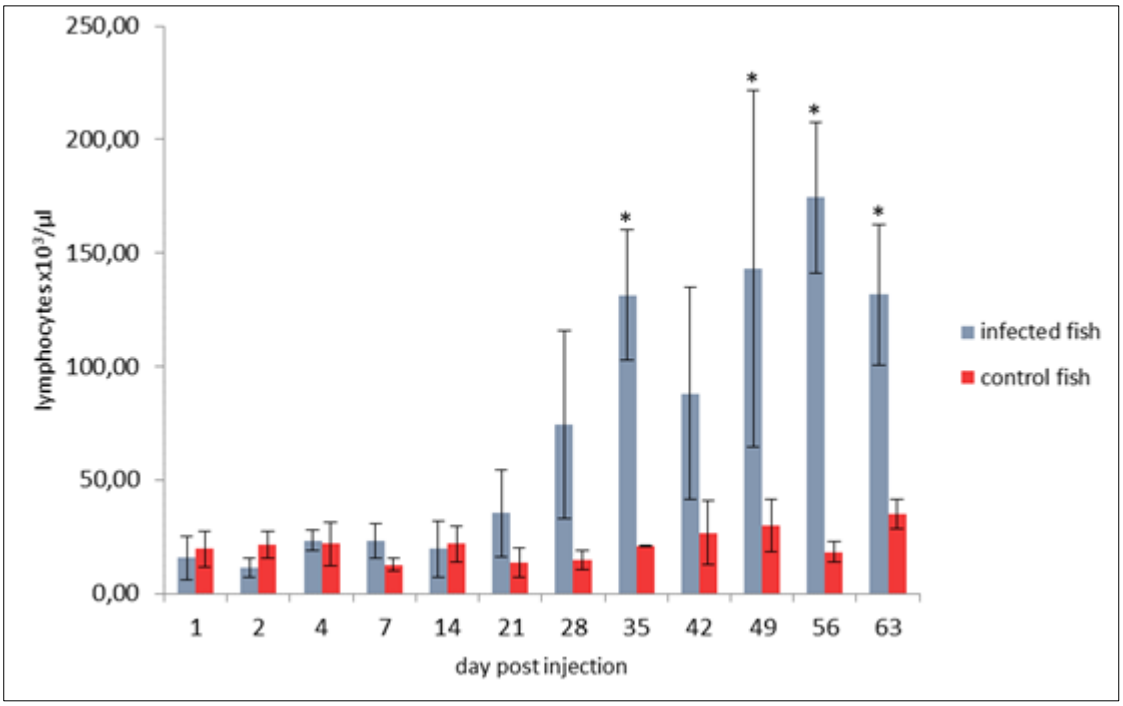

Fig. 12: Changes of lymphocyte numbers ($x10^3$/µl of blood) during experimental infection of common carp with *S. molnari* blood stages. Arithmetic means with standard deviations are shown for 12 sampling points post infection. Asterisks determine the significance levels of the difference between control and infected fish, as a result of the Mann Whitney U test. (*) $0.01 \leq p < 0.05$.

4.8 Monocytes (Fig. 13)

A rise of monocyte numbers in infected fish is evident from 7 to 35 dpi. Thereafter, on 42 and 49 dpi there is a sharp drop. On 56 dpi the numbers increase again and reach their maximum. Sixty three dpi the numbers decline once again. The statistical analysis determined that the monocyte numbers of control and infected fish significantly differ 1 (p=0.0253), 14 (p=0.0253), 28 (p=0.0339) and 56 (p=0.0495) dpi.

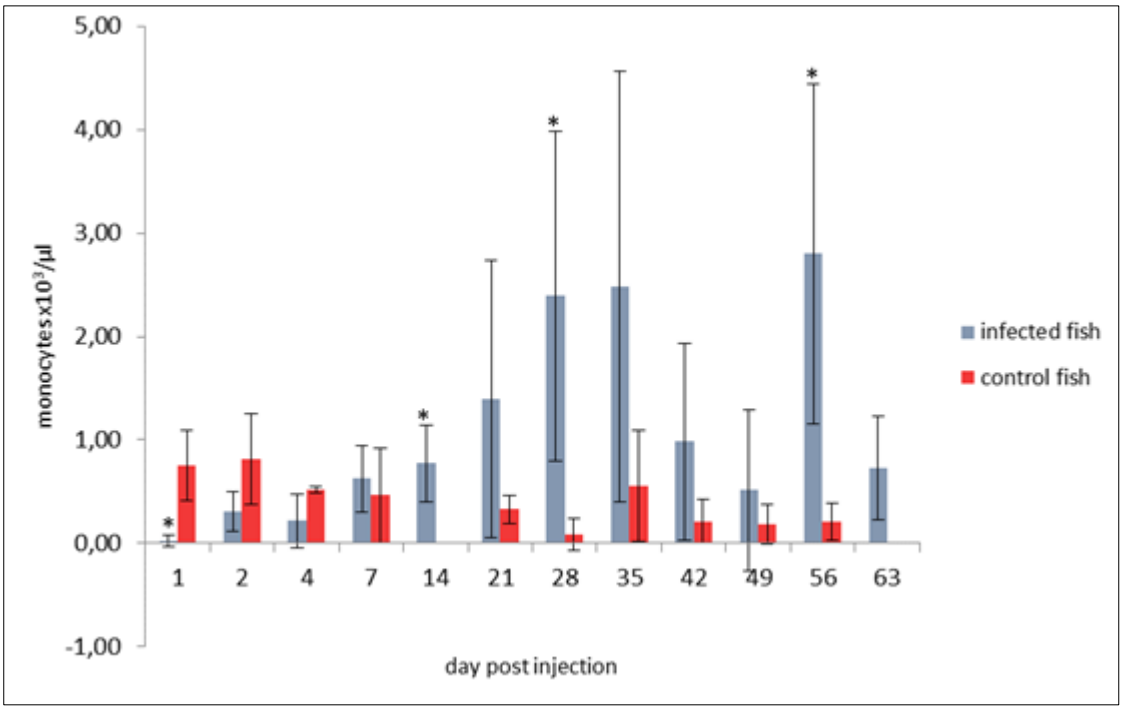

Fig. 13: Changes of monocyte numbers (x10^3/µl of blood) during experimental infection of common carp with *S. molnari* blood stages. Arithmetic means with standard deviations are given for 12 sampling points post infection. Asterisks demonstrate the significance levels of the difference between control and infected fish, as determined by the Mann Whitney U test. (*) $0.01 \leq p < 0.05$.

4.9 Neutrophils (Fig. 14)

It is evident that the number of neutrophils fluctuates during the whole experimental period. Higher numbers of neutrophils in infected fish are observed from 14 dpi onwards and they reach their peak 35 dpi. According to the statistical analysis, a significant difference is seen 35 (p= 0.0253), 49 (p= 0.0253) and 63 (p=0.0339) dpi.

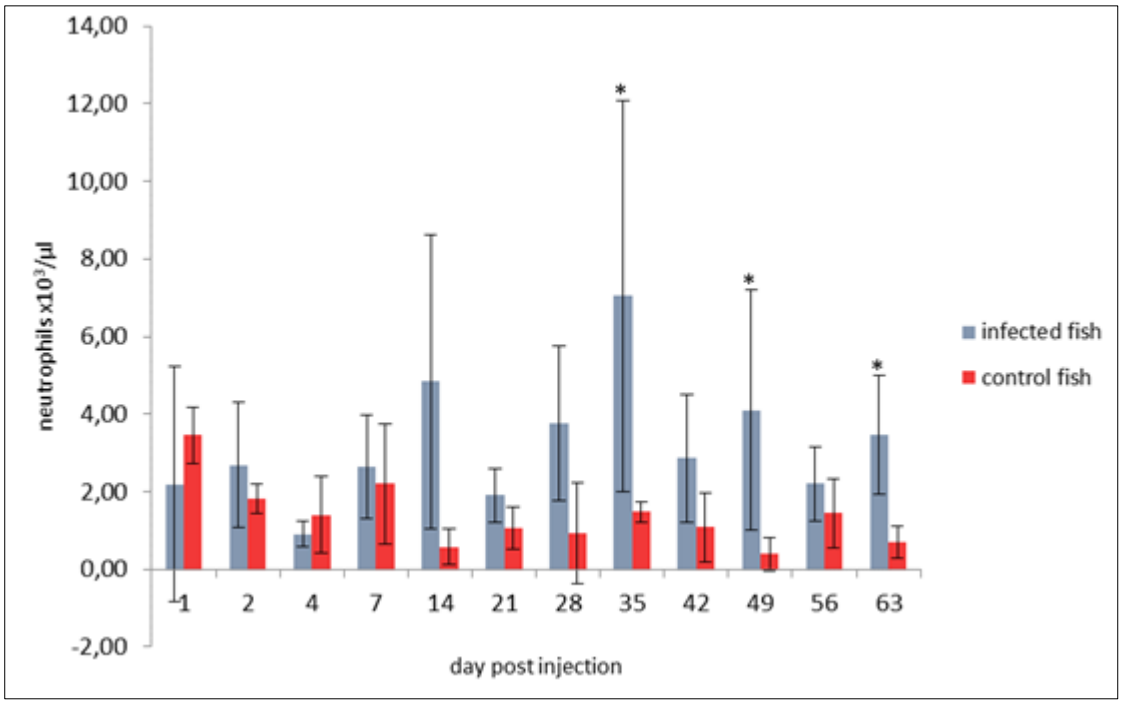

Fig. 14: Changes of neutrophil numbers ($x10^3$/µl of blood) during experimental infection of common carp with *S. molnari* blood stages. Arithmetic means with standard deviations are given for 12 sampling dates post infection. Asterisks determine the significant difference between control and infected fish, as determined by Mann Whitney U test. (*) $0.01 \leq p < 0.05$.

In summary, we observed a haematological and immunological response to *S. molnari* infection in common carp, only after an initial period of latency. Cellular components of immunity rise 14 dpi and a strong increase in lymphocyte numbers is visible from 28 dpi onwards. The host response manifests itself as a massive proliferation of lymphocytes which reached approximately 10-fold concentrations of those of control fish. Monocytes and neutrophils as well as thrombocytes also increased their number in experimentally infected fish, but to a much lower intent. In contrast to leukocytes, parameters related to red blood cells, haemoglobin and haematocrit did not change significantly.

5 DISCUSSION

The purpose of this thesis was to perform a controlled infection experiment with proliferative blood stages of the myxozoan parasite *S. molnari* and to understand the haematological changes of carp as a response to infection over time. Significant haematological and immunological changes were determined only after a latency period of three weeks, 21 - 28 dpi with *S. molnari* blood stages. This delay in host response agrees with the detection of the parasite in different organs, for the first time 21 days after infection (2 out of 5 fish infected; Jedličková 2017, Fig. 15). This may be explained by initial proliferation of *S. molnari* in a site, where it cannot be recognized by the immune system, for example intracellularly. Support for this interpretation can be found in early infection stages of *Myxobolus cerebralis*, where the first cell divisions and initial development in fish only minutes or hours after infection occur intracellularly in the epithelial cells and the subcutis of rainbow trout (El-Matbouli et al. 1995, Daniels et al. 1976). In heavily naturally infected fish from carp ponds, we often noticed a significant decrease of red blood cells and hence haematocrit (personal observation). However, in the experimental infection study, the numbers of red blood cells, haematocrit, haemoglobin and MCV values did not change significantly during the infection by *S. molnari* in common carp. Nevertheless, red blood cells parameters changes are common during parasitic infections in fish, e.g. rainbow trout affected by *T. bryosalmonae* develops a chronic microcytic anaemia with distinct poikilocytosis (Hoffmann and Lommel 1984, Palíková et al. 2016). While we are unable to tell if other factors are co-responsible for the anaemia observed in *S. molnari*-infected carp from ponds, it may well be that this condition only occurs during chronic infection and after the 63 days of study in the present experiment. In *T. bryosalmonae* anaemia was observed between September and October in fish exposed to *T. bryosalmonae*-enzooic waters in July (Palíková et al. 2016), hence no earlier than 2 months after initial infection. Alternatively, the dose of the parasite given (single injection, 14 500 stages per fish) may be much lower than a continuous exposure to water-borne actinospores over weeks or months, in ponds.

In the experimental infection of common carp with *S. molnari*, leukocyte numbers were found to change considerably over time, demonstrating a strong immunological response of carp to proliferation of *S. molnari*. In order to be able to interpret changes in immune cell composition, these need to be analysed in the light of parasite proliferation and localisation.

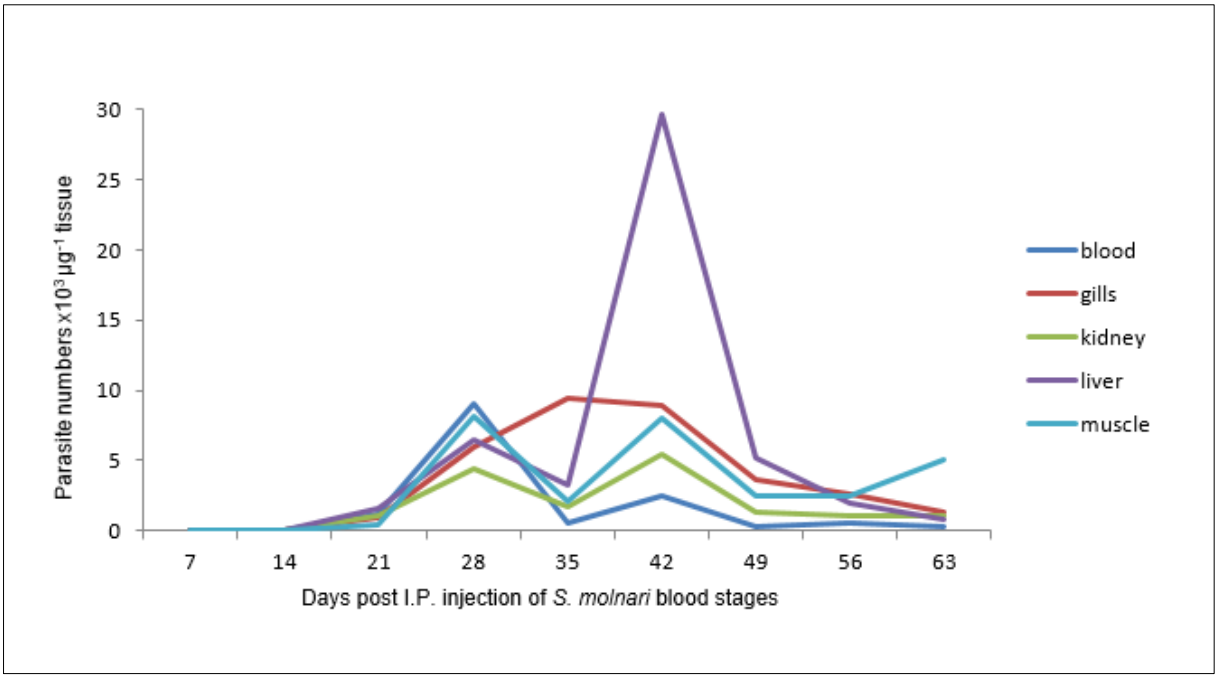

Fig. 15: Quantification and localisation of *S. molnari* developmental stages in carp after I.P. injection with stages derived from the blood (data from present experiment, modified after Jedličková 2017).

For our experiment, this was performed by Jedličková (2017; Fig. 15) who detected *S. molnari* for the first time, in blood of carp, 21 dpi. In the blood, parasite numbers reach two peaks, 28 dpi (highest peak) and 42 dpi (lower peak). On 28 dpi the increase in parasite numbers in other organs is caused by the presence of blood stages in capillaries. 35 dpi, parasite numbers suddenly drop and 42 dpi the parasites invade tissues, predominantly the liver. Then the numbers of parasites decrease in all tissues and the blood. The acute infection changes into a chronic low level one. As a response to the infection, firstly, the cellular mediators of innate immune responses, phagocytically active monocytes and neutrophils increase from 14 dpi. These cells are responsible for non-specific recognition of antigens and stimulation of other immune components. Their numbers peak 35 dpi which is when the first peak of parasites that occurred 28 dpi is effectively eliminated from the host, showing strongly decreased parasite numbers (Fig. 15). Thrombocytes which may contribute with their phagocytic activity to the immune response (Thuvander et al. 1987, Nagasawa et al. 2014) also increase their numbers on 35 dpi. 42 dpi, the number of monocytes, neutrophils and thrombocytes decreases again, as parasite numbers peak in the liver. A decrease of lymphocytes, mediators of acquired immunity can also be noted on the same day. This may be related to an escape of *S. molnari* from immune surveillance by translocation to the liver, after initial specific detection of

parasite antigens. A similar strategy is observed during pre-erythrotic stages of the infection of *Plasmodium* in mammals few hours after entering the host. After being injected by mosquito into the host, some sporozoites migrate to lymph nodes where they stimulate the activation of cytotoxic T cells. Others migrate via blood to liver, where they develop in hepatocytes and hide from immune response (Bertolino and Bowen 2015). Because of its function the liver is predisposed to be a targeted organ of pathogens. This organ filters blood from gut and takes up pathogens and products of metabolism. In the liver a local and controlled inflammation against dietary and commensal molecules occurs and at the same time co-inhibitory receptors and immunosuppressive mediators are secreted. Antigens of these pathogen molecules are presented by MHC on antibody presenting cells which can induce immunological tolerance. These mechanisms prevent a massive inflammation and damaging of the tissue but also help the pathogens e.g. *Plasmodium* to develop infection (Robinson et al. 2016, Protzer et al. 2012). When *Plasmodium* sporozoites reach the liver sinusoids they enter the perisinuisoidal space via Kupffer cells, which can induce tolerance and defend the parasite against immune responses (Frevert et al. 2005, Frevert et al. 2006). Before reaching the final hepatocytes, the parasite stages penetrate several hepatocytes where a red herring in form of circumsporozoite protein (CSP) and other proteins are left behind, which help to divert cytotoxic T cells (Frevert et al. 2005, Bertolino and Bowen 2015).

In *S. molnari* infection in carp, lymphocytes, mediators of acquired immunity, start to rise 21 dpi. 35 dpi lymphocytes in infected fish reach the first peak and the numbers display approximately 10-fold concentrations in comparison with uninfected fish. Further specifying the lymphocyte cell type, according to flow cytometry, on day 49 of infection, 34% of all WBC are IgM^+ B cells (unpublished data). Compared with 12-15% of B cells in control fish (unpublished data), this indicates that a considerable percentage of leukocytes are represented by B cells that mediate adaptive immunity. A similar immune response to that in common carp against *S. molnari* is observed in hosts of *T. bryosalmonae*. A massive increase of lymphocytes is observed in interstitial tissue of the kidney and lymphocytes form the major cell population in peripheral blood during PKD (Chilmonczyk et al. 2002). This increase of B cell numbers can be caused by polyclonal activation. During such an activation, B cells produce non-specific antibodies against several antigens. Some of these antibodies can be targeted against the parasite. A second option of B cell activation is by specific antigens of the parasite. B cells differentiate to mature memory B cells, which are important for a secondary immune response e.g. during reinfection when specific antibody secreting cells can cause a faster or more

pronounced specific response (Schroeder and Cavacini 2010, Secombes and Wang 2012). As it was mentioned, activated B cells stimulate phagocytes and activate the complement pathway (Bruce and Brown 2017) and help to develop more intensive immune response. Based on the data obtained in the present study, we are unable to differentiate if the immune response *to S. molnari* is specific or polyclonal. In the future, this could be clarified by reinfection experiments, by exposure of parasites to sera from different days of infection or by running parasite lysates on SDS gels and blotting with sera from infected fish and applying anti carp IgM antibody. The latter could elucidate immunogenic proteins in parasite lysates and determine which sera are able to detect these.

6 CONCLUSIONS

- A controlled infection experiment by IP injection of blood stages of *Sphaerospora molnari* into *Cyprinus carpio* was performed.
- The cellular and haematological response was observed after 28 days post infection.
- A massive proliferation of lymphocytes with two peaks on days 35 and 56 post infection specify the host immune response against the parasite.
- Monocytes and neutrophils increased from day 14 post infection and thrombocytes on day 35 post infection as a non-specific immune response to the parasite.
- Lymphocytes rise from day 21 post infection and on day 49 onwards they mediate adaptive immunity as a response to the parasite.
- Red blood cells, haemoglobin and haematocrit did not change significantly during the experiment.

7 LIST OF ABBREVIATIONS

APC - Antigen-presenting cell

ASC – Antibody secreting cell

BCR – B cell receptor

CLR – C-type lectin receptor

CMC – Cell-mediated cytotoxicity

CSP – Circumsporozoit protein

dpi – day post infection

GALT – Gut associated lymphoid tissue

GIALT – Gill associated lymphoid tissue

Ig – Immunoglobulin

MALT – Mucosa-associated lymphoid tissue

MCV – Mean cell volume

MHC – Major histocompatibility complex

NCC – Non-specific cytotoxic cell

NET – Neutrophil extracellular trap

NIALT – Nasopharynx-associated lymphoid tissue

NK cell – Natural killer cell

NLR – NOD-like receptor

PAMP – Pathogen-associated molecular pattern

PGRP – Peptidoglycan recognition protein

PKD – Proliferative kidney disease

PRR – Pattern-recognition receptors

SALT – Skin-associated lymphoid tissue

SDS – Sodium dodecyl sulphate

SPF – Specific pathogen free

TCR – T cell receptor

TLR – Toll-like receptor

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