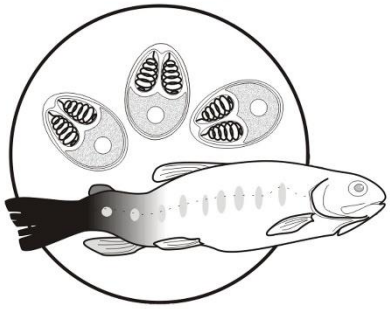
Comparative studies on the host specificity of myxozoan parasites (Myxozoa)

Final report



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1. Summary

Without detailed knowledge on host adaptation and development of myxozoan fish parasites (Cnidaria, Myxozoa), we cannot understand epidemics, and an effective protection of fish against these parasites cannot be developed. In Hungary, especially carp breeders suffer from multiple disease outbreaks in their fish ponds proven or thought to be caused by myxozoans. The detailed examination of host specificity of such pathogens is crucial in this context, since it is well known that parasites causing severe disease outbreaks and mortality in offspring are mainly invasive species originating from different habitats. Therefore, the project aimed at gaining essential knowledge on the nature of myxozoan host specificity. We investigated the genetic background of host susceptibility for myxozoan species having wide host-range. For the first time, we found evidence for host-shift, and for the process of speciation for myxozoan parasites. We proved that blood and the host's recirculation system has major role in the development of myxozoans, even though this role could be rather different for various myxozoan parasites. While examining the role of host blood in the dissemination of *Myxobolus* spp., we provided clear evidence that myxozoans' host specificity is not determined during the early, intrapiscine development involving the vascular system. Furthermore, as a part of international collaboration, we gained important knowledge on the biodiversity of *Sphaerospora* species, and on the motility of sphaerosporid blood stages, in relation to their phylogenetic position and evolutionary origin.

2. Összefoglalás

A nyálkaspórás halparaziták (Cnidaria, Myxozoa) gazdához való adaptálódásának és egyedfejlődésének részletes ismerete nélkül nem érthetjük meg azok járványtanát. Továbbá ezen ismeretek nélkül nem fejleszthető ki hatékony védekezési módszer a parazitafertőzés ellen. Hazánkban leginkább a pontytenyésztők érintettek bizonyított vagy vélt nyálkaspórások által okozott többféle kórforma kialakulásában. Ezen kórokozók gazdakörének és a gazdaválasztás specifikusságának részletes vizsgálata ebben az összefüggésben döntő fontosságú, mivel jól ismert tény, hogy az ivadékok súlyos betegségeit és mortalitását okozó paraziták általában más élőhelyekről származó invazív fajok. Ezért a projekt célja az volt, hogy alapvető ismereteket szerezzünk a nyálkaspórás paraziták gazdafajlagosságáról. Vizsgáltuk a gazdafajlagosság genetikai hátterét széles gazdakörű (több gazdafajt fertőzni képes) nyálkaspórás fajok esetében. Elsőként találtunk kísérleti bizonyítékot a gazdaváltásra és a fajképződés folyamatára a nyálkaspórások között. Bebizonyítottuk, hogy a vér és a gazda keringési rendszere fontos szerepet töltenek be a nyálkaspórások fejlődésében, bár ez a szerep meglehetősen különböző lehet az egyes parazita fajok esetében. Miközben vizsgáltuk a vér *Myxobolus* fajok terjesztésében betöltött szerepét, egyértelmű bizonyítékot szolgáltattunk arra vonatkozóan, hogy a nyálkaspórások gazdafajlagossága a halon belüli fejlődés, keringési rendszert is érintő, korai stádiumában még nem jelenik meg. Továbbá nemzetközi együttműködés részeként fontos tudásanyagot gyűjtöttünk a *Sphaerospora* fajok biológiai sokféleségéről, és a sphaerosporid véralakok motilitásáról, filogenetikai helyzetükkel és evolúciós eredetükkel összefüggésben.

3. Project-related publication activity

The outcome of the project was presented on international and national scientific conferences, and published in prestigious, peer-reviewed journals. In total, four presentations and six papers were published with the cumulative impact factor of 18.123. The principal investigator was the first/last author of the majority of publications (4 of 6 papers and 4 of 4 presentations). Furthermore, a BSc thesis related to the project was prepared with the supervision of the principal investigator and participant researcher.

4. Results

In the course of the project, we clarified several aspects of the development of myxozoan model species, exploring the background of host specificity and susceptibility, besides examining the host invasion mechanisms of the parasite.

The project involved two main topics, (i) the host specificity of myxozoans originating from natural habitats (4.1.), and (ii) experimental studies on myxozoan parasites, which enabled us to study the nature of host susceptibility and myxozoans' host specificity in details (4.2.).

4.1. Studies on the host specificity of fish parasites collected from different geographical regions

In the course of the host specificity studies on the myxozoan parasite fauna of naturally infected fish, we primarily aimed to isolate and compare morphologically similar parasite spores from the same tissues of closely related fish species. The survey was focused on species developing in different fish hosts of various age-groups. We compared morphologically similar parasite spore isolates originating from different habitats. Furthermore, the exceptional intrapiscine development and host adaptation of *Sphaerospora* spp. was studied as a part of international collaboration.

Host specificity and biodiversity of *Sphaerospora* species (Holzer et al. 2014, and Patra et al. 2018)

In cooperation with colleagues from the Institute of Parasitology, Ceske Budejovice, Czech Republic, the etiology and host specificity of economically relevant *Sphaerospora* species were studied. Swim bladder inflammation (SBI) is an important disease of common carp fingerlings in Central Europe. In the 1980s, its etiology was ascribed to multicellular proliferative stages of the myxozoan parasite *Sphaerospora dykovae* (formerly *S. renicola*). *S. dykovae* was reported to proliferate in the blood and in the swim bladder prior to the invasion of the kidney, where sporogony takes place. Due to the presence of emerging numbers of proliferative myxozoan blood stages at different carp culture sites in recent years, we analysed cases of SBI, for the first time, using molecular diagnostics, to identify the myxozoan parasites present in diseased swim bladders. Large multicellular myxozoan swim bladder stages characterised heavy SBI cases were identified as *S. dykovae*, however, blood stages were predominantly represented by *Sphaerospora molnari*, whose numbers were greatly increased in carp with mild and heavy SBI, compared with SBI-free fish. *S. molnari* was found to invade different organs and cause inflammatory changes also in the absence of *S. dykovae*. We provided evidence that the etiology of SBI can vary in relation to culture site and disease severity, and that emerging numbers of *S. molnari* in the blood represent an important co-factor or precondition for SBI.

Sphaerospora spp. are extraordinary due to their extremely long and unique insertions in the variable regions of their SSU and LSU rDNA, and due to the formation of motile proliferative stages in the hosts' blood. To date, small number of species have been characterized at a DNA level, and information on the patterns responsible for their phylogenetic clustering was limited. Therefore, the study aimed at the investigation of sphaerosporid biodiversity both in natural habitats and fish farms. Multiple phylogenetic analyses were performed to explore phylogenetic relationships and evolutionary trends within the *Sphaerospora* sensu stricto (s.s.) clade, by matching host and habitat features. Our study considerably increased the number of SSU rDNA sequence data for *Sphaerospora* (s.s.) by sequencing 17 new taxa. Phylogenetic analyses showed that sphaerosporids cluster according to their vertebrate host order and habitat, but not

according to geography. The study revealed the coevolutionary adaptation of sphaerosporids, and the host-driven species diversification. The new findings significantly contributed to our knowledge of the biodiversity and evolutionary history of the members of the *Sphaerospora* (s.s.) clade.

Characterization of *Sphaerospora molnari* blood stages (Hartigan et al. 2016)

As a part of the international collaboration, the proliferating blood stages of *Sphaerospora molnari* causing gill sphaerosporosis in common carp fingerlings, was studied in details. We described the peculiar movement of *Sphaerospora molnari*, a myxozoan parasite with proliferating blood stages in its host, common carp. *S. molnari* blood stages have developed a unique "dancing" behaviour, using the external membrane as a motility effector to rotate and move the cell. The movement is exceptionally fast compared to other myxozoans, non-directional and constant. Our findings revealed that the movement is based on two cytoplasmic actins that are highly divergent from those of other metazoans. Cellular motility is essential for microscopic parasites, it is used to reach the host, migrate through tissues, or evade host immune reactions. Our study showed that this new type of motility holds key insights into the evolution of cellular motility and associated proteins.

Aspects of host specificity for *Myxobolus pseudodispar* in natural habitats (Forró & Eszterbauer 2015, 2016)

Myxobolus pseudodispar is capable of infecting and developing mature myxospores in several cyprinid species. However, *M. pseudodispar* isolates from different fish show up to 5% differences in the SSU rDNA sequences. This is an unusually large intraspecific difference for myxozoans, and only some of the muscle-dwelling myxozoan species possess such a high genetic variability. Therefore, we studied the correlation between the host specificity and the phylogenetic relationship of parasite isolates. SSU rDNA-based phylogenetic analyses performed on dozens of *M. pseudodispar* isolates originated from naturally infected fish show that the isolates tend to cluster according to their fish host species mainly. However, few exceptions could be observed. Most of the parasite isolates originated from natural infections in Lake Balaton, Hungary. Lake Balaton, as the largest natural freshwater lake in Central Europe, has special epizootiological importance due to its hydrological conditions and diverse fish fauna. All cyprinids which are common hosts of *M. pseudodispar* are native in the lake, just as the oligochaete fauna susceptible for the most common myxozoans in Hungary. Previous studies indicated that co-infection by several myxozoan species might take place in oligochaetes from such a parasite-rich habitat. Oligochaetes are the definitive hosts of many freshwater myxosporeans, thus the sexual reproduction occurs in the worm. Our theory is that *M. pseudodispar* lineages (i.e. genetically distinct clones) are able to recombine in the worm in the course of reproduction, which could highly increase the genetic diversity among isolates of *M. pseudodispar*.

Besides the well-known host species of *M. pseudodispar*, we managed to detect the infection in new host species as well. Our findings showed that asp, *Aspius aspius* and European chub, *Squalius cephalus* were also able to get infected with the parasite species originally described from common roach, *Rutilus rutilus*. Although parasite isolates from asp and chub did not differ in spore morphology and tissue tropism from the isolates studied so far, the molecular characterization clearly show their distinction from the known isolates. Phylogenetic analysis suggested that the isolates from both fish hosts represent new phylogenetic clades, but having

common ancestor with the isolates from common bream, *Abramis brama* and white bream, *Blicca bjoerkna*.

4.2. *In vivo* life cycle experiments to study the susceptibility of fish hosts and the nature of myxozoans' host specificity

To study the host specificity of myxozoans under experimental conditions, two parasite model species were used. The differential developmental characteristics of *Myxobolus pseudodispar*, a common myxozoan parasite with wide host range, and *Myxobolus cerebralis*, the most pathogenic myxozoan species, were compared, and factors influencing their host specificity were examined.

Host susceptibility to *Myxobolus cerebralis*, in relation to the level of allelic diversity of fish (Eszterbauer et al. 2015)

Whirling disease, caused by the myxozoan parasite *Myxobolus cerebralis*, has high economical and ecological importance worldwide. Susceptibility to the disease varies considerably among salmonid species. In brown trout (*Salmo trutta* m. *fario*), the infection is usually subclinical with low mortality, which increases the risk of parasite dissemination, especially when farm fish are used for stocking natural habitats. The influence of intraspecific genetic differences (especially the level of homozygosity) on susceptibility is unknown. Therefore, we examined the possible correlations between parental genetic diversity and offspring susceptibility of brown trout stocks to whirling disease. Two brown trout brood stocks from a German and a Hungarian fish farm were genetically characterized using microsatellite and lineage-specific genetic markers. The individual inbreeding coefficient *f* and pairwise relatedness factor *r* were estimated based on eight microsatellite markers. Brood stock populations were divided into groups according to low and high *f* and *r* value estimates, and subjected to selective fertilization. The offspring from these separate groups were exposed to *M. cerebralis* actinospores, and the infection prevalence and intensity was measured and statistically analysed. The analysis of phylogeographic lineage heritage revealed high heterogeneity in the Hungarian brood stock since > 50% of individuals were Atlantic-Danubian hybrids, while only pure Atlantic-descending specimens were detected in the German population. Based on f_{msat} and r_{msat} estimations, classified non-inbred (NIB), inbred (IB) and a group of closely related fish (REL) were created. The susceptibility of their offspring varied considerably. Although there was no significant difference in the prevalence of *M. cerebralis* infection, the mean intensity of infection differed significantly between NIB and IB groups. In REL and IB groups, a high variability was observed in infection intensity. No external clinical signs were observed in the exposed brown trout groups. Our findings indicate that the allelic diversity of brown trout brood stock may constitute a significant factor in disease susceptibility, i.e. the intensity of parasite infection in the subsequent generation.

Host specificity of myxozoans during intrapiscine development (Forró et al. 2015; Bali 2015; Eszterbauer et al. 2017; Sipos et al. 2018a,b)

Besides the host specificity study of natural isolates of *Myxobolus pseudodispar*, we intended to find experimental proof to the wide host range of the parasite, with cross-infection experiments and genetic analyses based on SSU rDNA. Putative host-shift was studied under *in vivo* experimental condition using different cyprinid host species (fish exposure trials), including the type host *Rutilus rutilus*. Furthermore, fish-to-worm transmission experiments (oligochaetes exposure trials) were performed with the parasite's myxospores obtained from

natural infections to analyze the infectivity of the examined isolates. The experimental findings distinguished 'primary' and less-susceptible 'secondary' hosts. Host-shift is a gradual change of a wild host range when parasite species distinguish between primary and secondary hosts. The primary host is the one, to which the parasite is well adapted, and it is the one that supports the survival of the parasite. The parasites are less adaptive to the secondary host and their reproductive success is less dependent on them. Between primary and secondary hosts, the parasite can carry out cross-infections and a prior primary host can change to secondary host or even become unsusceptible. Our findings suggest, that host-shift may occur for *M. pseudodispar*. It is considered a multi-host parasite species, and previous and current findings suggest that its primary host is common roach, which is also the type host. The host-shift may explain the unexpected or uncertain phylogenetic position of certain parasite isolates, which clustered with different host species and not with the ones they were isolated from. They might have already started to shift to a new host while genetically they still cluster with other isolates from their former primary host species. The parasite speciation in progress seems to explain the high genetic diversity among isolates which are morphologically indistinguishable.

One of the main aim of the project was to take a step forward in the understanding of the nature of myxozoan host specificity. For doing so, we investigated the early development of two closely related myxozoan parasites, the highly pathogenic *Myxobolus cerebralis*, the causative agent of the whirling disease in salmonids, and *Myxobolus pseudodispar*, a common, non-pathogenic parasite of cyprinids. The aim of the study was to examine under *in vivo* laboratory conditions whether fish blood is involved in the intrapiscine development of the two parasite species, and investigate if there is dissimilarity between the parasite infection intensity in blood and if it varies in terms of host susceptibility and parasite pathogenicity. Highly susceptible, less susceptible and non-susceptible hosts were involved. Blood samples were taken 1 day, 1 week and 1 month post exposure to *M. cerebralis* and *M. pseudodispar*, respectively. The prevalence and infection intensity was estimated by parasite-specific quantitative real-time PCR. Although previous findings assumed that *M. cerebralis* might escape from host immune system by migrating via peripheral nerves, our experimental results demonstrated that *M. cerebralis* is present in blood during the early stage of intrapiscine development. For the non-pathogenic *M. pseudodispar*, the highest infection prevalence was found in the original host, common roach *Rutilus rutilus*, whereas the highest infection intensity was detected in rudd *Scardinius erythrophthalmus*, a "dead-end" host of the parasite. The presence of *M. pseudodispar* developmental stages in the blood of both susceptible and non-susceptible cyprinids suggests that the susceptibility differences remain hidden during the early stage of infection. When compared, the infection dynamics of the two species examined, showed remarkable differences. While the infection intensity of *M. cerebralis* rapidly declined, *M. pseudodispar* infection remained more or less constant throughout the entire experiment. The observed differences in the time trend may be related to their dissimilar pathogenicity, the discrepancies in the adaptation to blood immune factors, and/or the site of spore formation. As *M. pseudodispar* is non-pathogenic, the selective pressure on resistance development by the host should be rather low. It is likely that hosts did not acquire adaptive immunity against the parasite. This is probably not the case for *M. cerebralis*, that could be another reason why the declining infection intensity was observed in the blood. Our findings, based on qPCR analysis, supplied essential details on the host specificity of the examined histozoic *Myxobolus* species. We experimentally proved for the first time that *M. cerebralis* does enters the bloodstream in the early stage of infection. Studies from the early 2000s assumed that myxozoan parasites were able to invade susceptible host species only. We proved that it was not the case, and our findings supply clear evidence that host specificity is not determined the early, intrapiscine development involving the vascular system.

5. Project-related presentations and publications

Bali K (2015): Patogén és apatogén nyálkaspórás (Myxozoa) fajok halon belüli fejlődésének összehasonlító vizsgálata [in Hungarian with English summary]. BSc thesis. Veterinary Medical Faculty, Szent István University, Budapest. (Supervisors: Edit Eszterbauer and Barbara Forró).

Eszterbauer E, Forró B, Tolnai Z, Guti CF, Zsigmond G, Hoitsy Gy, Kallert DM (2015): Parental genetic diversity of brown trout (*Salmo trutta* m. *fario*) brood stock affects offspring susceptibility to whirling disease. PARASITES AND VECTORS 8: Paper 141. 9 p.

Eszterbauer E, Ursu K, Sipos D, Forró B, Dán Á (2017): Susceptibility-related differences in the quantity of developmental stages of *Myxobolus* spp. (Myxozoa) in fish blood. In: 18th EAFP International Conference on Diseases of Fish and Shellfish. Belfast, UK, 2017.09.04-2017.09.08. Paper No. 039-P.

Forró B, Bali K, Eszterbauer E (2015): New insights in the host specificity of myxozoans: the role of fish blood in the early development of two *Myxobolus* species. In: 9th International Symposium on Fish Parasites. Valencia, Spain, 2015.08.31-2015.09.04. Paper O-067.

Forró B, Eszterbauer E (2015): A *Myxobolus pseudodispar* nyálkaspórás halparazita gazdafajlagosságának kísérletes vizsgálata [in Hungarian]. Akadémiai Beszámolók. Budapest, 2015. január 28.

Forró B, Eszterbauer E (2016): Correlation between host specificity and genetic diversity for the muscle-dwelling fish parasite *Myxobolus pseudodispar*: examples of myxozoan host-shift? FOLIA PARASITOLOGICA 63: Paper 019. 8 p.

Hartigan A, Estensoro I, Vancová M, Bílý T, Patra S, Eszterbauer E, Holzer AS (2016): New cell motility model observed in parasitic cnidarian *Sphaerospora molnari* (Myxozoa:Myxosporea) blood stages in fish. SCIENTIFIC REPORTS 6: Paper 39093. 12 p. (2016)

Holzer AS, Hartigan A, Sneha P, Peckova H, Eszterbauer E (2014): Molecular fingerprinting of the myxozoan community in common carp suffering Swim Bladder Inflammation (SBI) identifies multiple etiological agents. PARASITES AND VECTORS 7: Paper 398. 9 p.

Patra S, P Bartošová-Sojková, H Pecková, I Fiala, E Eszterbauer, AS Holzer (2018): Biodiversity and host-parasite cophylogeny of *Sphaerospora* (sensu stricto) (Cnidaria: Myxozoa). PARASITES AND VECTORS 11: Paper 347. 24 p.

Sipos D, Ursu K, Dán Á, Eszterbauer E (2018a): Hal fajok fogékonyságával összefüggő különbségek a vérben előforduló *Myxobolus* spp. (Cnidaria, Myxozoa) parazita fejlődési alakok mennyiségében [in Hungarian]. Akadémiai Beszámolók, Budapest, 2018. január 24.

Sipos D, K Ursu, Á Dán, D Herczeg, E Eszterbauer (2018b): Susceptibility-related differences in the quantity of developmental stages of *Myxobolus* spp. (Myxozoa) in fish blood. PLOS ONE 13:(9) Paper e0204437. 15 p.