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Experimental examination of intrapiscin and intraoligochaete developmental stages of fish parasitic myxosporeans

Summary of Ph.D. thesis

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Frequently used abbreviation

CFSE	5(6)-carboxyfluorescein diacetate succinimidyl-ester
H&E	haematoxylin and eosin
PKD	Proliferative Kidney Disease
plate	cell-well plate
SPF	Specific Pathogen Free (here: free from myxosporean infection)
SPSS	Statistical Package for Social Sciences
TAM	triactinomyxon
TEHAG	temperate water fish farm
QP 2.0	Quantitative Parasitology 2.0

1. INTRODUCTION AND AIMS OF THE STUDY

Myxozoans (Phylum: Myxozoa) represent an economically important group of microscopic fish-parasites. Approximately 1350 species of 52 genera belong to the phylum, of which some species cause extreme infections especially in Western Europe and the USA. *Myxobolus cerebralis*, the causative agent of whirling disease, and *Tetracapsuloides bryosalmonae*, the cause of PKD, are responsible for high mortality in trout and other salmonid populations. In Hungary, the most pathogen myxosporean parasites are *M. cyprini*, *Sphaerospora molnari* and *S. renicola*, the causative agent of malignant anaemia, gill-sphaerosporosis and swimbladder inflammation in common carp, respectively.

Besides their economical importance myxoporeans are studied worldwide because of two reasons. It was only not too long ago derived that myxosporeans develop by two alternate hosts, in most cases a fish and an oligochaeta. Because of the morphological variability of spores within a species (myxospores from fish and actinospores from oligochaetes) and the simply body shape, the taxonomy of this peculiar parasite is still uncertain. Recently it was proved that myxosporeans can not be regarded as protozoans and they belong to metazoan organisms, closely related to Cnidaria and Bilateralia. That is why the myxosporean-research is one of the fastest developing fields in fish parasitology. Since the first success in experimentally reproducing the developmental cycle of *Myxobolus cerebralis*, the intrapiscine and intraoligochaete (and occasionally the intrapolychaete) development of numerous myxosporean species has become known. Because of the long duration and difficult execution of the experiments, however, in most cases only the intraoligochaete stage of the developmental cycle was studied and, of the about 25 life cycles known at present, the complete developmental cycle of 4 species has only been elucidated. The Hungarian research group was among the first teams that started life cycle studies in myxosporean research.

The aims of the study was

- to continue the life-cycle experiments on the most common myxosporeans of Hungarian fish species. Meanwhile detect the actinosporean stages of some species with experimental infection of SPF oligochaete stock and reproduce complete developmental cycle by infecting SPF fish with actinospores obtained from oligochaetes.
- The second aim was to follow the dependence of intraoligochaete development upon the myxospore density, temperature and other factors.
- Finally to collect data of the occurrence of actinosporean stages in oligochaetes from natural waters and fish farm.

2. MATERIALS AND METHODS

Experimental infections. Spores of *Myxobolus macrocapsularis* and *M. intimus* were collected from the gills of common bream (*Abramis brama*) and roach (*Rutilus rutilus*), respectively. Oligochaetes *Tubifex tubifex* and *Limnodrilus hoffmeisteri* were collected from a muddy pool where no fishes live. Definite number of worms were placed into a plastic dish. Calculated number of myxospores collected from the fish were added into the dish and the worms were supplied with heat-sterilized mud and water. Water from the dishes was filtered through a fine mesh of 21 µm pore size every week. When actinospores were observed, the worms were washed out from the mud and placed into wells of plates one by one and kept at low temperature (4°C). In some experiments, after the cessation of actinospore release, the worms were kept on plates at 4°C until their death. However, in some experiments after cessation of actinospore release for a few days, the worms were placed back from the plates into sterilized mud kept at room temperature, and were provided with food. The actinospores released from the worms and floating in the water were photographed and recorded on videotape for subsequent measurement with the help of the IMAGO® computer programme. Drawings were also made of the spores and their dimensions were recorded. Some of the infected worm

specimens were fixed for histoligal and semithin sections. In order to reproduce the complete developmental cycle of *M. intimus* and *M. pseudodispar*, the muscle parasite of roach, SPF fish fry were infected with actinospores by two methods. Either SPF fingerlings were placed into water containing floating actinsopores or infected worms were fed to SPF fingerlings.

Effect of different factors on the actinospores production. We examined on plates the diminishing of actinospores in water in the presence of *Cyclops* spp. by compound and light microscope. Later TAMs were labelled with fluorescent dye CFSE. *Cyclops* spp. were added into the water which contained labelled TAMs. After a definite time cyclops specimens were removed from the water and examined using a fluorescence microscope. Finally we made an attempt to produce infection in SPF roach fingerlings by feeding *Cyclops* spp. which consumed *M. pseudodispar* actinospores. Roach fingerlings infected with floating actinospores served as control.

Using replicated laboratory experiment we examined the effect of water temperature and myxospore density on the *M. pseudodispar* actinospore production from *T. tubifex*. The experiment followed a split-plot design, with 3 temperatures (19, 23 and 27°C) and 2 myxospore density (50 and 470 myxospores/*T. tubifex*). Ten replicates of 100 *T. tubifex* were randomly assigned to the 6 treatments. The groups of 100 worms were kept in mud in a plastic dish. When TAMs were observed, the worms were immediately washed out from the mud and placed into wells of plates one by one. The plates were kept at 4°C. The following infection parameter were evaluated for each group: average first day of TAM release, duration of TAMs release, average number of TAMs released per infected worm (intensity), total number of TAMs released by each dish and worm infection rate (prevalence). Kruskal-Wallis and Mann-Whitney *U*-test (SPSS 11.0) were used to determine whether the total number of TAMs differed between the six groups. The prevalence, median and mean intensity were analysed by QP 2.0 programme. After cessation of actinospore release for a few days, the worms were placed back from the plates into sterilized mud

kept at room temperature. The water above the oligochaetes was filtered regularly to detect the re-emergence of floating actinospores. The released actinospores were not only checked but also counted regularly. The experiment was concluded on 337 days post exposure.

Occurrence of actinosporean stages from oligochaetes. The occurrence and morphology of actinosporean stages of myxosporeans were studied in Estonia and Hungary. Oligochaetes were collected from two sampling sites in Hungary, in TEHAG and in the River Tisza, and from four sampling sites in Estonia (two biotopes of Lake Võrstjärv, and one biotope from Lake Peipsi and River Emajõgi, respectively). In addition to oligochaetes collected from natural Estonian waters, 15 oligochaete species, which had been kept and cultured in aquaria for several years, were also examined. These worms had been kept many years as separate micropopulations and supplied with mud from Lake Võrstjärv.

3. RESULTS

Experimental infections. We have reproduced experimentally the intraoligochaete development of *Myxobolus macrocapsularis* and *M. intimus*. In all experiments, typical triactinospores developed in *Tubifex tubifex* specimens but no infection was found in *Limnodrilus hoffmeisteri*. In their morphological characteristics the TAMs of two species released from the worms differed from the actinospores of known *Myxobolus* species originating from cyprinids. In the three experiments with *M. macrocapsularis*, TAMs floating in the water were first filtered on days 66, 69 and 85, respectively, after initial exposure to myxospores. In the two experiments with *M. intimus*, the presence of floating TAMs was first observed in the filtrate of water from the experimental dish on day 37 and 58, respectively, after the first exposure to myxospores. In the first experiment, performed with *M. intimus*, after transferring the worms into plates, TAMs released for 15 days starting from the first day of individual examination. Spore release was synchronous. In the course of the 2-month period of observation on

the plates kept at 4°C, no new spore release could be observed. In the second experiment, after the cessation of TAMs release on 4°C, oligochaetes placed back from the plates into the dishes supplied with mud, a new TAMs release was observed, which continued the following 11 months. In histological and semithin sections mature pansporocysts, each harbouring eight TAMs and early developmental stages were easily distinguishable in the gut epithelium of the worms. Pansporocysts were segregated from the lumen of the worm's intestine by only a thin layer of the ectoplasm of the infected epithelial cells. In the case of *M. pseudodispar* we performed reverse developmental cycle experiment, by infecting fish with floating TAMs obtained from oligochaetes. Myxospores collected from experimentally infected roach initiated a new development in *T. tubifex* and the resulted TAMs infected roach. Infection of the fish occurred only as a result of invasion by floating TAMs and attempts to produce infection by feeding *T. tubifex* specimens infected with mature TAMs failed. No plasmodial development was found either in roach or in other cyprinids experimentally infected by *M. intimus* actinospores.

Effect of different factors on the actinospores production. Microscopical examination revealed that the amount of TAMs diminished shortly afterwards that *Cyclops* spp. were placed into the water. Using fluorescent labelled TAMs the spores were clearly visible at the filtering apparatus of copepods 1-1,5 h, in the digestive tract 4,5 h after the cyclops were placed into the TAMs suspension. The extruding of polar filaments of the actinospores took place 2,5 h. Infection of the fish occurred only as a result of invasion by floating actinospores and attempts to produce infection by feeding *Cyclops* spp. which consumed *M. pseudodispar* actinospores failed.

In the dishes maintained 19, 23 and 27°C, higher temperatures induced earlier releases of TAMs. Duration of TAMs release decreased as temperature increased. The worms infected with higher myxospore density released TAMs longer period than the worms infected with lower myxospore density. The total number of TAMs released per groups did not differ among temperatures and myxospore density. The

prevalence were higher among the worms infected at lower temperature with higher myxospore density, than lower myxospore density. At higher temperature the prevalences did not differ among the two myxospore density. The prevalence were higher among the worms infected with higher myxospore density at lower temperature, than at higher temperature. The prevalence did not differ among the worms infected with lower myxospore density at the two temperature. The worms released more TAMs (the *intensity* were higher) infected with lower myxospore density at higher temperature, than lower temperature. The total number of TAMs released by the 6 group over the duration of release showed that the worms infected with higher myxospore density released more TAMs at each temperature. The total number of TAMs were the highest at the group, which were infected at the highest temperature. We detected proliferation (worms produced about 1,5 TAMs for each myxospore ingested – assuming all myxospores were ingested) in the group infected with lower myxospore density at the highest temperature.

Occurrence of actinosporean stages from oligochaetes. It was the first occasion that actinosporean stage of myxosporean parasite was described from Estonian waters. Three types of triactinomyxon were detected. One of the three types differed from all triactinomyxon forms hitherto described. Of the 15 cultured oligochaetes examined, only a stock of *T. tubifex* proved to be infected. We described 14 actinospore types (4 triactinomyxon, 4 neoactinomyxum, 3 aurantiactinomyxon, 1-1 guyenotia, raabeia and antonactinomyxon) from a Hungarian fish farm (TEHAG) and 4 types (2 triactinomyxon, 1-1 aurantiactinomyxon and guyenotia) from River Tisza close to Tiszafüred.

4. CONCLUSIONS

Experimental infections.

- One of the experiment performed with *M. intimus*, the intraoligochaete development took place 37 days, indicating that the

actinospore development of this species can be accomplished within an extremely short time at the optimum temperature.

- We proved that the lower temperature is obviously unfavourable for the development of myxosporeans parasitizing cyprinids that require a higher temperature range in the summer vegetation period, and this is why they stopped their spore release in the tubificid worms at 4°C.
- At the same time, our experiments also demonstrated that the early actinospore stages do not die in the oligochaetes under adverse temperature conditions; they just interrupt or slow down their development and will start to develop again and the release of actinospores will accelerate when favourable temperature conditions are resumed.
- Two consecutive complete developmental cycles of *M. pseudodispar* were successfully reproduced by the use of SPF oligochaete and fish hosts, which suggested that *M. pseudodispar* infection will be a suitable laboratory model in the future.
- Infection of the fish occurred only as a result of invasion by floating actinospores. This suggests that in the roach-oligochaete *M. pseudodispar* model the infective cells of actinospores presumably invade the fish through the skin and gills, rather than through the gut wall, and reach the site of final development, the skeletal muscle cells, by a subsequent migration.
- We could not infect fish with *M intimus* with actinospores obtained in experiments. In the roach population studied in Lake Balaton, *M. intimus* infection was observed only in 2-year-old or older fish; therefore, it is not impossible that development can be accomplished successfully only in more mature fish rather than in fingerlings used in the present experiments.

Effect of different factors on the actinospores production.

- We suppose that *Cyclops* spp. and other aquatic invertebrates are suitable organisms for diminish the myxosporean infection in fish farm and natural waters.

- We suppose that the intraoligochaete development is less synchronous in the case of infection with higher myxospore density.
- We proved that the statistical analysis of prevalence, mean and median intensity are more suitable descriptors to quantify myxosporean infection in oligochaetes than the total number of TAMs per infected group.
- We suppose that the difference between the prevalences were the results of heat-stress beside the higher infectious of worms due to the higher myxospore density.
- From the results of statystical analysis of intensity we assume that in worms infected with lower myxospore density at higher temperature the proliferation caused higher TAMs production per infected worm.

Occurrence of actinosporean stages from oligochaetes.

- The fact that only *T. tubifex* stock proved to be infected with actinospores among 15 oligochate stocks maintained in Estonia under identical condition indicated big differences among the susceptibility of oligochaete species to myxosporean infection.
- Research in Hungary based on the morphological characterisation resulted in finding some new actinospores and some actinospore types resembling earlier described types or actinosporean stages of known myxosporean species.
- Our actinosporean stage, which composed of eight spores is the third in the antonactinomyxon collective group in the world. Regarding the linkage of the actinosporean type found by us differs from the earlier described two antonactinomyxon types. Based on this character one can even question our decision to regard this form as antonactinomyxon.

5. LIST OF PUBLICATION

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