

THE ROLE OF COPEPODS (*CYCLOPS* SPP.) IN ELIMINATING THE ACTINOSPORE STAGES OF FISH-PARASITIC MYXOZOANS

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The actinospore consumption of copepods (*Cyclops* spp.) was demonstrated by laboratory observations. It was observed that in experimental dishes the number of actinospores floating in the water decreased, or such actinospores were completely eliminated, in the presence of copepods. The ingestion of actinospores by copepods and their further fate were monitored by fluorescent staining and by conventional histological techniques. The actinospores were observed to have got caught on the filters of *Cyclops* spp. Two and a half hours after the copepods had been placed into water containing actinospores, their digestive tract was found to contain spores that had extruded their filaments from the polar capsules. After copepods having ingested the actinospores of the species *Myxobolus pseudodispar* had been fed to roaches, no muscle infection developed in the fish host. It is likely that *Cyclops* spp. can filter out actinospores floating in the water also from natural waters, thus decreasing the chance of development of myxosporean infections.

Key words: Myxozoa, actinospore stages, elimination, copepods, *Cyclops* spp.

The development of numerous species of Myxozoa has become known since Wolf and Markiw (1984) proved that actinosporeans did not belong to a distinct taxonomic entity but represented the developmental stages of fish-parasitic myxosporeans evolving mostly in oligochaete hosts. The number of myxospore-actinospore pairs identified in laboratory experiments markedly increased in the past two decades. In their summary of the research done in this field, Kent et al. (2001) reported 27 developmental cycles taking place in fish and in oligochaete alternate hosts. Of the factors affecting the intensity of *Myxobolus cerebralis* infection, El-Matbouli et al. (1999) and Blazer et al. (2003) studied the influence of substrate and water temperature, while Stevens et al. (2001) made observations on the effect exerted by the quantity of infective myxospores on intraoligochaete development. The last mentioned authors studied the susceptibility or resistance of oligochaete strains originating from different geographic regions to myxosporean in-

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fections. The correlations of the intensity of infection developing in the fish host during experimental infection with water temperature and the quantity of spores used for infection have been studied by Liyanage et al. (2003) for the species *Thelohanellus hovorkai* Achmerov, 1960 and by Székely et al. (2001) for the species *Myxobolus pseudodispar* Gorbunova, 1936.

The elimination of parasitic helminths by aquatic invertebrates has been documented by Sudarikov and Shigin (1975) as well as Kazakov and Zatravkin (1987), but similar observations on fish-parasitic myxosporeans have been reported only in a single paper (Chepurnaya, 1992). This latter report discusses elimination of the myxospores of *Myxobolus pavlovskii* Achmerov, 1954, as the author could not know the actinospore stage of that species, which was demonstrated for the first time by Ruidisch et al. (1991).

A substantial proportion of copepods acquire their food by filtration. According to some notions this filtration is fully mechanical, which means that during a continuous flow of water all particles exceeding a certain size get stuck on the comb-like teeth formed by the maxillula and the maxilla, irrespective of whether or not they are food particles. Other authors believe that copepods actively hunt for food and select the food to be filtered with the sensory bristles of their antennula by palpation or in response to the effect of the water current or chemical stimuli. Observations show that predatory cyclopoids most often prey on animal protozoans (primarily ciliates), rotifers, oligochaetes, small branch-horned and shell-covered crustaceans (Cladocera and Ostracoda) and small insect larvae, but they also ingest other oar-footed crustaceans (Copepoda) as well as their nauplii and copepodits (Fryer, 1957; Einsle, 1964; Dévai, 1977).

In recent years, we have performed life cycle experiments with numerous species of Myxozoa in our laboratory (El-Mansy and Molnár, 1997a, 1997b; El-Mansy et al., 1998; Molnár et al., 1999a, 1999b; Székely et al., 1998, 1999, 2001, 2002; Eszterbauer et al., 2000; RácZ et al., 2004) and detected the actinospore stages of the given myxozoans. In addition to the successful experiments, experimental reproduction of the life cycle failed in numerous cases, even though the technique used for the experiment was the same. On several occasions we observed that in experiments in which copepods multiplied in large numbers, no or only few actinospores appeared. At the same time, experimental reproduction of the life cycle was successful, i.e. floating actinospores were detected, in parallel experiments of the same set-up in which *Cyclops* spp. did not propagate in large numbers. This suggested the possibility that with their incidental predatory or filtering activity *Cyclops* spp. might be responsible for the failure of the experiments in question.

The objective of the experiments reported here was to demonstrate whether copepods indeed eliminate actinospores floating in the water. We also wished to determine whether actinospores ingested by the copepods maintain their infectivity for the fish host.

Materials and methods

The *Myxobolus pseudodispar* triactinospores used in the experiments were produced in 500-ml plastic cups as described by Székely et al. (1999, 2001). Actinospores floating in the water were collected by filtering water from the dishes through a fine mesh of 21 µm pore size, in the last drops of water remaining in the filter. The copepods used for the experiments were obtained from a duck-rearing pond and from pet shops. According to identification to the genus level only, *Cyclops*, *Eucyclops* and *Acanthocyclops* species were used in the experiments; however, further on all of them will be referred to collectively as cyclops. Before starting the experiments, numerous cyclops specimens were examined on a slide, squashed under a coverslip, to confirm their actinospore-free status.

Experiment 1

(A) In this experiment, 6–10 cyclops specimens each were placed into 2-ml wells of a 24-well cell culture plate. The water in the wells contained a specific number of actinospores (26–31 actinospores in one drop of water). The number of floating actinospores was again determined by examining the entire water content of the individual wells, drop by drop, on a slide under a light microscope 22 h after the cyclops specimens had been placed into the wells. The control group was constituted by some wells into which only actinospores but no cyclops specimens had been placed. The cyclops-containing and the control groups were kept and examined under identical conditions, at a temperature around 13 °C.

(B) In a further experiment, 20 cyclops specimens and at least 200 triactinomyxon (TAM) specimens were placed into each well, then 18 h later the number of remaining actinospores was estimated by examination under a stereomicroscope. To serve as a control group, minimum 200 TAMs per well, but no cyclops specimens, were placed into the wells.

Experiment 2

In the second experiment, actinospores were labelled with fluorescent 5(6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) by the method of Yokoyama and Urawa (1997). The staining solution was diluted to 10 mM in 100% dimethyl sulphoxide (DMSO) and kept at 4 °C until used. Ten µl of this solution was added to 1 ml of the suspension containing minimum 2000 spores. The suspension was shaken and then allowed to stand at room temperature for 15 min. To remove the dye granules, the suspension was repeatedly filtered and washed with ion-exchanged water. Subsequently, cyclops specimens were placed into the diluted suspension containing fluorescent spores. Periodically, one cyclops each was removed from the suspension and washed on a 250 µm mesh to

remove any actinospores potentially adhering to the cyclops. The cyclops specimens thus cleaned were fixed in 1% formalin, placed into glycerol or Macrogol-400 dropped onto a glass slide, and examined in UV light under a microscope.

The other half of the spores was stained with Giemsa, and then cyclops specimens were placed into the suspension containing the stained spores. (Some of the dye granules remaining undissolved in the spore suspension were intentionally not removed even after multiple cycles of washing and filtration, as they are easily discernible by microscopy and thus indicate whether or not the cyclops has already filtered into itself some material from the suspension). Two and a half hours after having been placed into the suspension, the cyclops specimens were filtered through a nylon mesh, washed, and then examined on a slide under a light microscope. During examination under microscope, the digestive tract of the copepods flattened under a coverslip was examined in its entire length for the presence of actinospores.

Experiment 3

In this experiment, laboratory-reared SPF roach (*Rutilus rutilus*) fingerlings 4 to 6 cm in size were infected with experimentally produced *M. pseudodispar* triactinospores by two different methods.

(A) Five roach specimens were infected with TAMs floating in the water by keeping the fish in 2 litres of water containing approximately 7000 actinospores over a period of 7 h. Subsequently the fish were transferred into 10-litre aerated aquaria with filtered water and kept at room temperature (20–23 °C). The fish were fed dry fish food during the experiment. The first roach was dissected on postinfection (PI) day 85 while the remaining fish specimens together on PI day 138. The entire musculature of the fish was examined in squash preparations under a light microscope for the presence of *M. pseudodispar*.

(B) Five roach specimens were fed cyclops containing TAMs in their intestinal tract. In this case the roaches were fed cyclops specimens that had been previously kept in a suspension containing approximately 7000 actinospores for 16 h. Sixteen hours later some drops of the spore suspension were examined by light microscopy. The cyclops specimens were washed using a 250 µm mesh to remove any actinospores possibly adhering to their body surface. The roaches were placed into 1-litre plastic dishes one by one, and 30 cyclops specimens were added to the water of each dish. Two hours later it was checked whether the fish had ingested the infected cyclops. Subsequently the roach fingerlings were kept in the manner described above, until PI day 138 when they were dissected as described previously.

Results

Experiment 1

(A) In 15% of the water drops taken from the cyclops-containing wells after 22 h, light microscopic examination revealed only one actinospore each, while the remainder did not contain spores at all. In the control group, the initial 26 TAMs/drop value had decreased to 15 TAMs/drop by the end of the experiment.

(B) In the stereomicroscopic examination, 18 h after the addition of cyclops a few actinospores were observed only in a single well of the plate, while in the other wells the cyclops had ingested all the actinospores. A reduction in the number of TAMs was not observed in any of the wells of the control plate.

Experiment 2

In the second experiment, the TAMs labelled with CFSE were well visible at the filtering apparatus of the copepods 1–1.5 h (Figs 1 and 2) and in the content squeezed out from the digestive tract 4.5 h (Fig. 3) after the copepods had been placed into the spore suspension. When the cyclops were placed into a Giemsa-stained spore suspension containing also dye granules, the dye granules were discernible in the digestive tract already 2.5 h later (Fig. 4). The spore bodies of TAMs were easy to recognise in the content of the digestive tract, and especially the polar capsules and the their shot-out filaments, as well as the damaged caudal processes were well discernible (Fig. 5).

Experiment 3

(A) Of the roaches infected with floating TAMs, the single roach specimen dissected on PI day 85 had no detectable myxosporean infection, but the muscles of the further four fish dissected on PI day 138 were infected by plasmodia containing *M. pseudodispar* spores.

(B) Before feeding the cyclops to the fish in the feeding experiment, the cyclops-containing spore suspension was checked after 16 h (Fig. 6). It was found that the number of TAMs had decreased markedly as compared to the control (Fig. 7). Thus, we can be absolutely certain that the cyclops had ingested the TAMs. Despite this fact, when the roaches fed with actinospore-containing cyclops were dissected on PI day 138, their musculature was not infected with *M. pseudodispar* plasmodia.

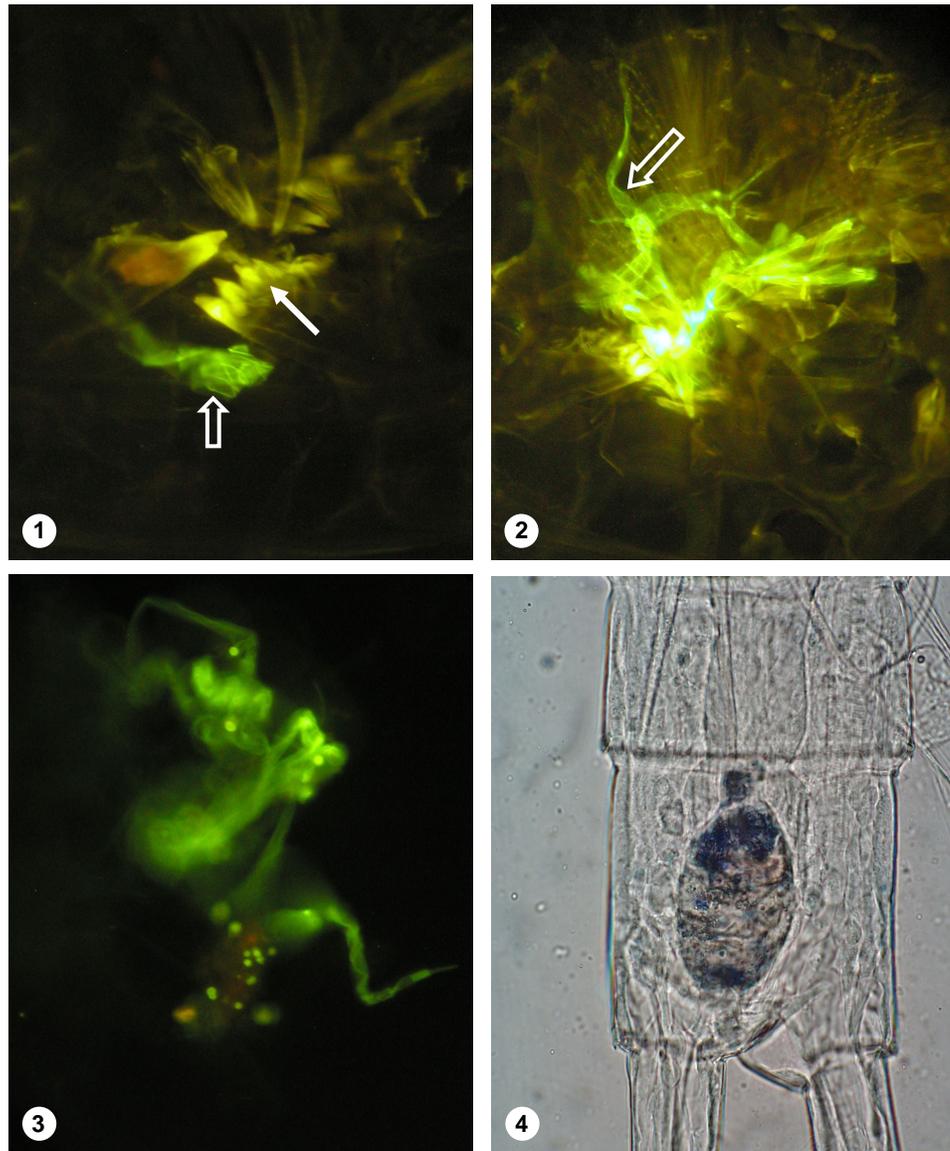


Fig. 1. Damaged processes of triactinomyxons (TAM) labelled with fluorescent dye and showing greenish fluorescence (empty arrow) at the filtering apparatus (arrow) of cyclops, 1 h after placing the cyclops into actinospore suspension (magnification: $\times 80$). *Fig. 2.* One and a half hours after placing the cyclops into the actinospore suspension, several fluorescent TAMs (arrow) can be observed at the filtering apparatus of the cyclops (magnification: $\times 80$). *Fig. 3.* Fluorescent TAMs in the squeezed-out digestive tract content of the cyclops 4.5 h after placing the cyclops into the actinospore suspension (magnification: $\times 160$). *Fig. 4.* Discoloration of the intestinal content of the cyclops is well discernible 2.5 h after placing the cyclops into a spore suspension containing Giemsa-stained TAMs and dye granules (magnification: $\times 250$)

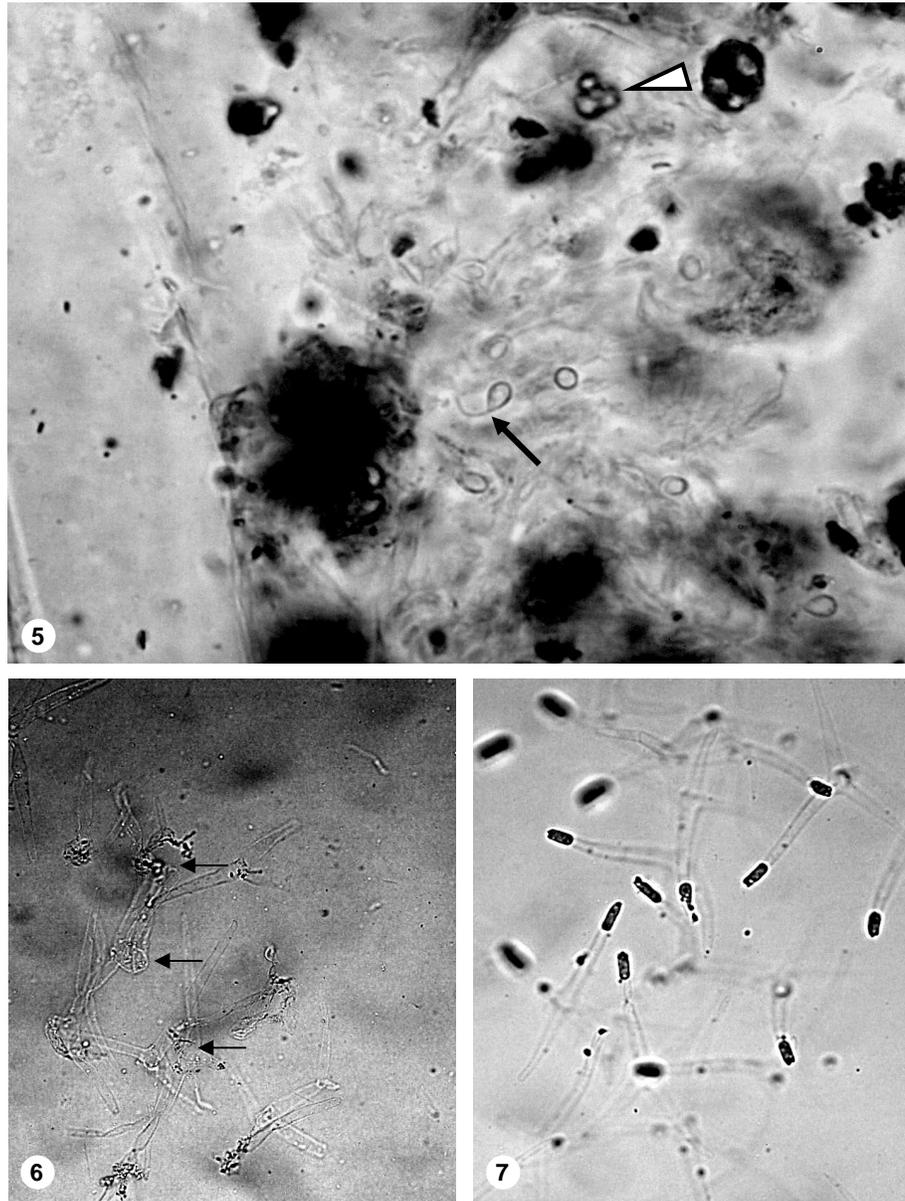


Fig. 5. Two and a half hours after placing the cyclops into the Giemsa-stained TAM suspension, the polar capsules with their extruded spore filaments (arrow) and the spore bodies in upper view (arrowhead) with the three polar capsules surrounded by a mass of damaged processes and styles of the TAMs are well discernible (magnification: $\times 1000$). *Figs 6 and 7.* Spore suspensions originally containing identical numbers of TAMs 16 h after placing in the cyclops (*Fig. 6*), with a few damaged TAMs (arrows), and the control spore suspension not containing cyclops after the same period of time (*Fig. 7*), with unaffected TAMs (magnification: $\times 100$)

Discussion

Up to the present, only a single paper (Chepurnaya, 1992) has reported the elimination of myxosporean species by invertebrate aquatic organisms. The author performed experiments with *M. pavlovskii*, a gill parasite of the silver carp and bighead. At that time, Chepurnaya (1992) did not know yet that *M. pavlovskii* had an actinospore stage developing in tubificids, as was later demonstrated by Ruidisch et al. (1991). Chepurnaya (1992) assumed that the fish were infected directly with the myxospores which left the intestinal tract of the tubificids unaffected. She regarded *Tubifex* as a carrier host facilitating the spread of myxosporean species, which can carry myxospores from the lower sediment layers up to the higher regions where fish can have access to the spores more easily. Chepurnaya (1992) studied the myxospore ingestion of rotifers (Rotatoria), branch-horned crustaceans (Cladocera), oar-footed crustaceans (Copepoda), shell-covered crustaceans (Ostracoda) and mollusc species (Mollusca). Of the invertebrates studied, she considered the two snail species and the tubificids unsuitable for reducing the number of myxospores and thus for lowering the chance of infection, as the spores left these species in unaffected condition, with intact, unopened spore valves and with their filaments not having been extruded. She had the same opinion about the tubificids included in the study. At the same time, according to her studies in the other aquatic organisms listed above, and thus also in the copepods (*Cyclops* spp.), two hours after the ingestion of myxospores the spores had extruded their filament and in several cases their spore valves had also opened. This is confirmed by the findings of our own experiments, in which we found that the actinospores had already extruded their spore filaments 2.5 h after having been ingested by the cyclops.

The results of our experiments prove that the actinospores ingested by copepods will not cause *M. pseudodispar* infection after getting into fish. The absence of infection can be explained in two ways. Firstly, it may be supposed that the actinospores ingested by copepods and having extruded their spore filaments have lost their infectivity. Secondly, it is also possible that the point of attack of this myxosporean species is actually not in the intestine of the fish host. This is supported by the results of the experiment conducted by Székely et al. (2001) with *M. pseudodispar*, when successful infection could be produced only with floating TAMs but not by feeding actinospore-infected tubificids.

Chepurnaya (1992) studied the *M. pavlovskii* infection of silver carp fingerlings of the same lake also under natural conditions. She compared the infection of fish with the zooplankton density of the water, and found that the zooplankton density and the gill infection of fish were inversely proportional to each other. By way of explanation, she suggested that at a low zooplankton density the fish fingerlings change over to detritophagy, and thus ingest a larger number of myxospores from the detritus. It is possible that the lower intensity of gill in-

fection observed by Chepurnaya (1992) at a high zooplankton density could also be explained by the more intensive actinospore consumption of copepods.

Our experimental studies have confirmed the actinospore consumption of copepods. The actinospore consumption of cyclops (and in all likelihood also that of other similar aquatic organisms) may offer possibilities that can be utilised not only in laboratory experiments but even in the fish farms. Thus, e.g. the reduction of fish infections caused by certain myxosporean species could be achieved by maintaining a high zooplankton biomass in the fish ponds.

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References

- Blazer, V. S., Waldrop, T. B., Schill, W. B., Densmore, C. L. and Smith, D. (2003): Effects of water temperature and substrate type on spore production and release in eastern *Tubifex tubifex* worms infected with *Myxobolus cerebralis*. *J. Parasitol.* **89**, 21–26.
- Chepurnaya, A. G. (1992): Role of aquatic invertebrates in the elimination of spores of *Myxobolus pavlovskii* (Achmerov, 1954) (Myxozoa, Myxobolidae), parasite of silver carp. *Ecol. Parasitol.* **1**, 150–153.
- Dévai, I. (1977): Key to the identification of copepods (Calanoida and Cyclopoida) (in Hungarian). *Vízügyi hidrobiológia* **5**, 220.
- Einsle, U. (1964): Larvalentwicklung von Cyclopiden und Photoperiodik. *Naturwissenschaften* **51**, 345–346.
- El-Mansy, A. and Molnár, K. (1997a): Extrapiscine development of *Myxobolus drjagini* Akhmerov, 1954 (Myxosporea, Myxobolidae) in oligochaete alternative hosts. *Acta Vet. Hung.* **45**, 427–438.
- El-Mansy, A. and Molnár, K. (1997b): Development of *Myxobolus hungaricus* (Myxosporea: Myxobolidae) in oligochaete alternate hosts. *Dis. Aquat. Org.* **31**, 227–232.
- El-Mansy, A., Molnár, K. and Székely, Cs. (1998): Development of *Myxobolus portucalensis* Saraiva et Molnár, 1990 (Myxosporea: Myxobolidae) in the oligochaete *Tubifex tubifex* (Müller). *Syst. Parasitol.* **41**, 95–103.
- El-Matbouli, M., McDowell, T. S., Antonio, D. B., Andree, K. B. and Hedrick, R. P. (1999): Effect of water temperature on the development, release and survival of the triactinomyxon stage of *Myxobolus cerebralis* in its oligochaete host. *Int. J. Parasitol.* **29**, 627–641.
- Eszterbauer, E., Székely, Cs., Molnár, K. and Baska, F. (2000): Development of *Myxobolus bramae* (Myxozoa: Myxobolidae) in oligochaete alternate host, *Tubifex tubifex*. *J. Fish Dis.* **23**, 19–25.
- Fryer, G. (1957): The food of some freshwater cyclopoid copepods and its ecological significance. *J. Anim. Ecol.* **26**, 263–268.
- Kazakov, B. E. and Zatravkin, M. N. (1987): Role of some hydrobionts in elimination of larvae of fish Monogenea (in Russian). *Papers of the Laboratory of Helminthology, USSR Acad. Sci. (Trudi Gelmintol. Lab. Acad. Nauk. USSR)* **35**, 41–45.

- Kent, M. L., Andree, K. B., Bartholomew, J. L., El-Matbouli, M., Desser, S. S., Delvin, R. H., Feist, S. W., Hedrick, R. P., Hoffmann, R. W., Khattra, J., Hallett, S. L., Lester, R. J. G., Longshaw, M., Palenzuela, O., Siddall, M. E. and Xiao, C. (2001): Recent advances in our knowledge of the Myxozoa. *J. Eukaryot. Microbiol.* **48**, 395–413.
- Liyanage, Y. S., Yokoyama, H. and Wakabayashi, H. (2003): Dynamics of experimental production of *Thelohanellus hovorkai* (Myxozoa: Myxozoa) in fish and oligochaete alternate hosts. *J. Fish Dis.* **26**, 575–582.
- Molnár, K., El-Mansy, A., Székely, Cs. and Baska, F. (1999a): Development of *Myxobolus dispar* Thelohan, 1895 (Myxosporea: Myxobolidae) in an oligochaete alternate host *Tubifex tubifex* (Müller). *Folia Parasitol.* **46**, 15–21.
- Molnár, K., El-Mansy, A., Székely, Cs. and Baska, F. (1999b): Experimental identification of the actinosporean stage of *Sphaerospora renicola* Dykova and Lom, 1982 (Myxozoa: Sphaerosporidae) in oligochaete alternate hosts. *J. Fish Dis.* **22**, 1–11.
- Rácz, O. Z., Székely, Cs. and Molnár, K. (2004): Intraoligochaete development of *Myxobolus intimus* (Myxozoa: Myxobolidae), a gill Myxozoan of the roach (*Rutilus rutilus*). *Folia Parasitol.* **51**, 199–207.
- Ruidisch, S., El-Matbouli, M. and Hoffmann, R. W. (1991): The role of tubificid worms as an intermediate host in the life cycle of *Myxobolus pavlovskii* (Achmerov, 1954). *Parasitol. Res.* **77**, 663–667.
- Stevens, R., Kerans, B. L., Lemmon, J. C. and Rasmussen, C. (2001): The effects of *Myxobolus cerebralis* myxospore dose on triactinomyxon production and biology of *Tubifex tubifex* from two geographic regions. *J. Parasitol.* **87**, 315–321.
- Sudarikov, V. E. and Shigin, A. A. (1975): On importance of components of aquatic biocoenoses in Trematoda elimination (in Russian). *Papers of the Laboratory of Helminthology, USSR Acad. Sci. (Trudi Gelmintol. Lab. Acad. Nauk. USSR)* **30**, 140–202.
- Székely, Cs., El-Mansy, A., Molnár, K. and Baska, F. (1998): Development of *Thelohanellus hovorkai* Achmerov, 1960 and *Thelohanellus nikolskii* Achmerov, 1955 (Myxosporea: Myxozoa) in oligochaete alternate hosts. *Fish Pathol.* **33**, 107–114.
- Székely, Cs., Molnár, K., Eszterbauer, E. and Baska, F. (1999): Experimental detection of the actinospores of *Myxobolus pseudodispar* (Myxosporea: Myxobolidae) in oligochaete alternate hosts. *Dis. Aquat. Org.* **38**, 219–224.
- Székely, Cs., Molnár, K. and Rácz, O. (2001): Complete developmental cycle of *Myxobolus pseudodispar* (Gorbunova) (Myxozoa: Myxobolidae). *J. Fish Dis.* **24**, 461–468.
- Székely, Cs., Rácz, O., Molnár, K. and Eszterbauer, E. (2002): Development of *Myxobolus macrocapsularis* (Myxosporea: Myxobolidae) in an oligochaete alternate host, *Tubifex tubifex*. *Dis. Aquat. Org.* **48**, 117–123.
- Wolf, K. and Markiw, M. E. (1984): Biology contravenes taxonomy in the myxozoa: new discoveries show alternation of invertebrate and vertebrate hosts. *Science* **225**, 1449–1452.
- Yokoyama, H. and Urawa, S. (1997): Fluorescent labelling of actinospores for determining the portals of entry into fish. *Dis. Aquat. Org.* **30**, 165–169.