RESEARCH ARTICLE

Biology Contravenes Taxonomy in the Myxozoa: New Discoveries Show Alternation of Invertebrate and Vertebrate Hosts

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Myxozoans are small, multicellular, spore-forming parasites that are currently accorded the rank of phylum in the subkingdom Protozoa (*1*). Two classes of myxozoans are recognized: Myxosporea, which occur almost exclusively in cold-blooded vertebrates (predominantly fish), and the morphologically different Actinosporea, which occur in invertebrates (notably annelid worms). Actinosporea and Myxosporea have both been known for 80 years; however, even though developmental stages have been described in their respective hosts, complete life cycles—particularly the infectious stages—have eluded identification.

Abstract. *For 80 years the infectivity of salmonid whirling disease has eluded discovery. New findings now show that this myxosporean disease of fish is initiated by what is regarded as an actinosporean produced in a tubificid oligochaete. Experimental results provide evidence that, instead of being considered as representatives of separate classes in the phylum Myxozoa, the myxosporean and actinosporean are alternating life forms of a single organism.*

In this article we report that, in at least one situation, an actinosporean and a myxosporean are not separate entities, but are alternating life stages of a single organism. We tracked the complete life cycle of the myxosporean that causes whirling disease in salmonid fish. We further demonstrated, under controlled laboratory conditions, a previously unrecognized identity that is shared by a myxosporean and an actinosporean. In essence, whirling disease in the fish results in the formation of myxosporean spores that are incapable of infecting other salmonids but which, when released into the environment, initiate an infection in an oligochaete of the family Tubificidae. Infection in the tubificid results in the production of an actinosporean that is incapable of infecting other worms, but which is the long-sought infective agent of salmonid whirling disease.

Our explanation of the myxozoan life cycle should not be considered radical, for the use of different hosts and the occurrence of comparable changes in morphology are well established in parasitology. Such alternations or transformations are common among the helminths and universal in malarial organisms.

These findings suggest that a reappraisal of myxozoan taxonomy should be considered. They also provide a model for investigating life cycles of other myxosporeans and actinosporeans and open new avenues for controlling myxosporean diseases of fish.

Background Biology of Myxosporea

Whether histozoic or coelozoic, most myxosporean infections are well tolerated by the host fish—as, for example, *Myxosoma cerebralis* is in its original host, the European brown trout (*Salmo trutta*). In contrast, in a new host, the rainbow trout (*Salmo gairdnerii*), the parasite produces a virulent infection termed "whirling disease" (*2, 3*).

Whirling disease derives its name from the tail-chasing behavior of afflicted salmonids. Histozoic *M. cerebralis* attacks cartilage, and, if the organs of equilibrium are involved, motor control is deranged. Spores of *M. cerebralis* are universally present in salmonids with whirling disease. However, fresh spores of *M. cerebralis* are not infectious in fish (*3–5*). Instead, spores must first be "aged" in an aquatic environment (3 to 4 months) before infectivity is produced (*4*). However, it has not been shown that the spores per se are infectious after aging. And, although the nature of the infectivity has been diligently sought, it has eluded identification.

We have found that whirling disease infectivity for fish does not develop endogenously in the spores of *M. cerebralis*. To arrive at this conclusion, we aged spores in aquatic environments consisting of springwater over inert substrates such as sand, glass wool, or pasteurized or sterilized aquatic soil and then added rainbow trout. In all trials the spores failed to yield infectivity. In contrast, rainbow trout that were held in containers of pond soil from fish hatcheries where whirling disease was enzootic readily acquired the infection. Because these trout developed whirling disease after being fed worms, tubificid oligochaetes were, by virtue of their abundance, implicated as an intermediate host.

Conclusive support of this premise was obtained by populating each of a number of containers of pasteurized trout pond soil with a different species of tubificid oligochaete and then adding fresh spores of *M. cerebralis* (*5*). After 4 months we added susceptible rainbow trout fry. Whirling disease occurred only among fry in containers that had received tubificids from a fish hatchery where the disease was epizootic or in containers that had received normal tubificids plus spores of *M. cerebralis* (*5*).

Background Biology of Actinosporea

Actinosporea have been known since 1899, when A. Stolc described a representative organism from a tubificid oligochaete (*6*). The postulated life histories of several actinosporeans of the genus *Triactinomyxon* have been described and illustrated in detail (*7–9*). However, this research was based on tubificids taken from nature: the elapsed time of development was not known and the authors were not able to identify the initial stage of the developmental cycle. Various investigators have assumed that the Actinosporea are infectious for the tubificids in which they are found; however, such infectivity has never been demonstrated.

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Genesis of an Actinosporean

Having implicated tubificid oligochaetes in the so-called aging process of *M. cerebralis* (*5*), we next sought to identify the nature of the infectivity of whirling disease. This phase of the investigation led to the discovery that, when spores of *M. cerebralis* were kept in the presence of tubificids for several months, a previously unrecognized stage in the myxosporean life cycle appeared, an actinosporean of the genus *Triactinomyxon* (Fig. 1).

Known numbers of spores of *M. cerebralis* were added to quantified populations of tubificids and the two were kept together for periods as long as several months (*10*). A stock of about 180,000 worms was placed in a container holding axenic aquatic soil; spores were then added in the amount of 100 per worm. To assess the effect of contact time, we removed half the worms after 10 days, thoroughly washed them, and transferred them to a second container of soil. After 20 days, one-sixth or about 30,000 worms were similarly transferred to a third container. Two containers, each with 30,000 worms but no spores, served as negative controls. All containers received a slow flow of springwater at 12.5°C. During the ensuing several months, samples of worms were crushed for microscopic examination and others were fixed for later scrutiny and histological examination.

On day 104, but not earlier, crushed tubificids from the first container showed a previously unrecognized organism that we identified as an actinosporean of the genus *Triactinomyxon* (Fig. 1). The organism was present in all later samples from the first container and from containers that held tubificids that had been in contact with *M. cerebralis* spores for only 10 or 20 days. The *Triactinomyxon* could not be found in the tubificids that had had no contact with the myxosporean.

Features of the *Triactinomyxon*

Morphology. The *Triactinomyxon* is anchor-shaped and is topped by three polar capsules. The cavity of the anterior end or epispore is about 36 μm long and contains 32 to 50 spherical sporozoites (Fig. 2). The sporozoites reacted to bisbenzamide stain and were positive for DNA (*11*). Beneath the short column of sporozoites, the style extends about 90 μm, and the tapering arms project about 170 μm further.

Relatedness to M. cerebralis. The epispore reacted strongly with fluorescein isothiocyanate–conjugated rabbit antiserum to *M. cerebralis* (Fig. 3) (*12, 13*). The gossamer structural material of the style and arms did not react with conjugated antiserum.

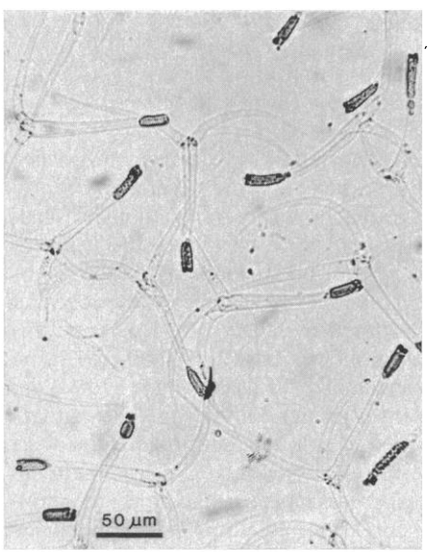
Production dynamics. The *Triactinomyxon* proved to be waterborne as well as present within tubificids. Three weeks after we found the organisms in the tubificids, effluent from the first container (containing about 60,000 tubificids) yielded *Triactinomyxon* at rates of 2,400 to 3,100 per minute at a flow of 120 ml/min. Production declined during the following month but continued at a detectable level for 7 months. *Triactinomyxon* never appeared in the effluent from the negative control containers.

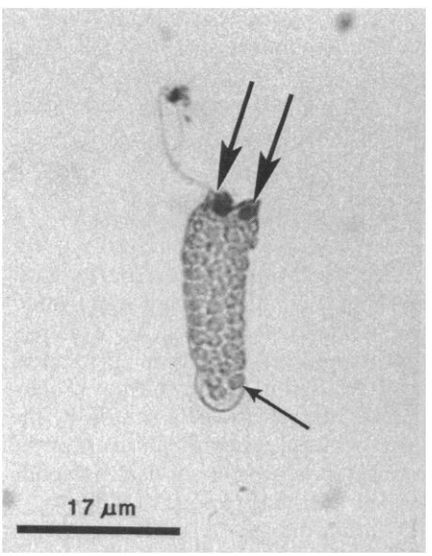
The *Triactinomyxon* readily passed screen mesh of 200 μm but was retained by 50-μm mesh, in agreement with earlier results showing that the major peak of the then-unknown infectivity of whirling disease was trapped on 50-μm screen (*5*). The finding that *Triactinomyxon* is waterborne agrees with our earlier observation that whirling disease occurs among trout fry held solely in the water column as well as among fry having access to the soil (*5*).

Triactinomyxon and Whirling Disease

Data from three sets of experiments conducted separately provide evidence that the *Triactinomyxon* initiates the clinical condition known as salmonid whirling disease and that within the vertebrate host a morphological change results in the development of a myxosporean of the genus *Myxosoma* (Fig. 4).

In the first trial, 20 rainbow trout fry were simply exposed to *Triactinomyxon* (*14*). The test fry developed whirling disease, whereas the nonexposed source stock of fish remained healthy. At 19





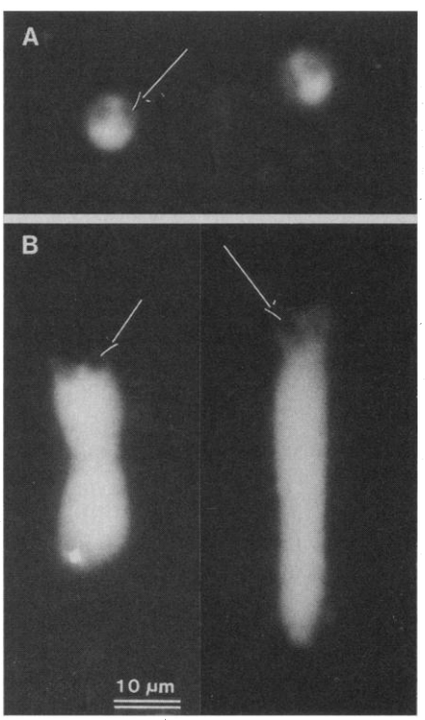


Fig. 1 (left). Mature waterborne *Triactinomyxon* produced in tubificid oligochaetes after exposure to *M. cerebralis*. Fig. 2 (center). Epispore of *Triactinomyxon* showing two of the three pyriform polar capsules (large arrows) and the numerous internal sporozoites (small arrow). Fig. 3 (right). Comparative response of epispore of a mature *Triactinomyxon* and of *M. cerebralis* to rabbit antiserum prepared against *M. cerebralis* and conjugated with fluorescein isothiocyanate. (A) Positive reaction of mature spores of *M. cerebralis* to the homologous antiserum. (B) Comparable reactivity of triactinomyxons to antiserum against *M. cerebralis*. Arrows point to nonreactive polar capsules.

weeks after exposure, a group of 12 fry was processed by the pepsin-trypsin-dextrose (PTD) centrifugation method of myxosporean spore release and concentration (*15*). The affected fry harbored 680,000 myxosporean spores each.

In the second experiment signs of whirling disease appeared in a group of 20 fry held for 6 days (*16*) on 50-μm mesh screen that was collecting experimentally produced waterborne *Triactinomyxon*. Whirling disease also developed in another 20 fry placed for 6 days directly in a container of tubificids that were producing the actinosporean. Both groups of fish also produced spores of *M. cerebralis* (Table 1). Measured numbers of *Triactinomyxon* administered to other groups of 20 fry by mouth or by intraperitoneal injection (*17*) resulted in the production of spores of *M. cerebralis* but not in signs of whirling disease. Intramuscular injection of *Triactinomyxon* produced neither the disease nor myxosporean spores. Negative controls remained free of *M. cerebralis* spores (Table 1).

In the third trial, duplicate negative controls, each consisting only of tubificids (100 g, or about 14,700), failed to produce *Triactinomyxon*, and showed no infectivity for rainbow trout fry. Positive controls also consisted of 100 g of tubificids, but *M. cerebralis* spores were added at the rate of 40 per worm in one case and 400 per worm in the other. Both containers of worms and myxosporean spores produced *Triactinomyxon*, but the differential was twofold instead of tenfold. *Triactinomyxon* from the more productive container was tested in duplicate; both of two groups of 20 trout fry that were exposed for 3 days developed whirling disease.

Table 1. Occurrence of whirling disease and spores of *M. cerebralis* in groups of 20 rainbow trout fry variously exposed to spores of an actinosporean of the genus *Triactinomyxon*. Numbers in parentheses indicate sample size.

Treatment	Signs of whirling disease	Average number of *M. cerebralis* spores per fish at	
		4 months	4.5 months
Exposure (6 days) to tubificids producing *Triactinomyxon*	Yes	245,400 (3)	(0)*
Exposure (6 days) to waterborne *Triactinomyxon*	Yes	94,200 (3)	400,000 (4)
Intraperitoneal injection of 14,200 *Triactinomyxon*	No	1,000 (3)	11,000 (11)
Intubation of 7,100 *Triactinomyxon*	No	300 (3)	26,400 (9)
Intramuscular injection of 7,100 *Triactinomyxon*	No	0 (3)	0 (3)
None (contact with *Triactinomyxon* prevented)	No	0 (3)	0 (1)†

*Accidental loss. †Sole survivor of accidental loss.

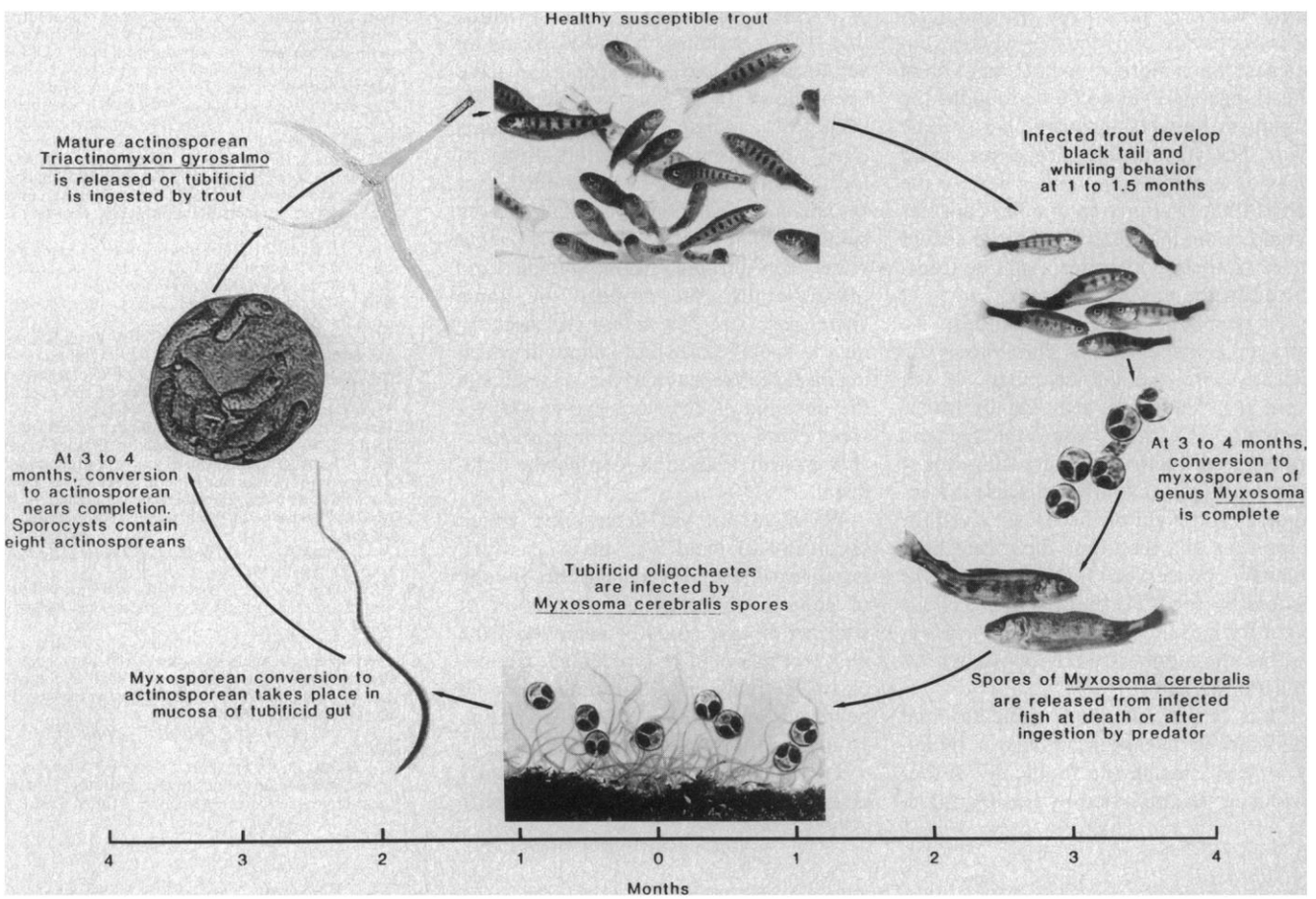


Fig. 4. Biphasic life cycle of a myxozoan. Clockwise from bottom center: Spores of *M. cerebralis* infect tubificid oligochaetes and initiate the actinosporean phase. At 12°C and 3 to 4 months later conversion to the actinosporean is nearly complete as multiple parasite cysts mature in the worm gut. Young salmonid fish ingest worms or waterborne *Triactinomyxon* infects fish via the gut or a branchial route and the myxosporean phase begins (top center). At 1 to 1.5 months the fish show signs of whirling disease; at 3 to 4 months the myxosporean phase is completed with maturation in the cartilage of spores of *M. cerebralis*. The myxosporean phase is not capable of infecting other fish, nor can the actinosporean phase infect oligochaetes.

Tubificid Resistance to *Triactinomyxon*

We challenged tubificids with *Triactinomyxon* but were unable to show autoinfection. A population of about 14,700 worms in axenic soil was exposed on eight occasions during a 3-week period. The *Triactinomyxon* were not quantified; the challenge consisted of organisms collected during 2- to 5-day periods and simply added to the container of worms. Four months after this challenge, 140 groups of five worms each were crushed and examined microscopically. No *Triactinomyxon* were found. In addition, three groups of about 800 worms each were crushed in a mortar and the coarse material was allowed to settle. The supernatant material was centrifuged for 10 minutes at 600*g*; it too was devoid of *Triactinomyxon*. The tubificids did not produce whirling disease in trout fry, and when the fish were digested by the PTD method no spores of *M. cerebralis* were found.

Form and Function of *Triactinomyxon*

Janiszewska (*8*) postulated that the great tapering processes of *Triactinomyxon* provide buoyancy. We agree, but suggest that a more important function of the elongate valves could be to lodge the organism between gill lamellae of host fish. The anchor-like processes could provide a temporary hold whereby the long-unknown function of polar capsules would come into play for a more secure hold, so that sporozoites could be transferred to the vascular system.

To evaluate these possibilities, we sprayed a suspension of *Triactinomyxon* directly into the gill chambers of ten trout fry, held them individually for 20 seconds, and then rinsed the fry and transferred them to springwater for 4 months. Signs of whirling disease did not appear, and eight of the fry survived for 4 months, at which time they were individually processed for *M. cerebralis* spores by the PTD method. Five of the eight fry harbored spores of *M. cerebralis*, but the numbers were low—only 600 to 3100 per fish.

Thus far, all evidence indicates that the whirling disease infectivity is, initially at least, inside the tubificid. Further evidence was obtained by feeding infection-bearing tubificids to rainbow trout fry. In duplicate, ten fry were fed thoroughly washed tubificids in four consecutive feedings. Duplicate groups of ten other fry were fed tubificids that had been thoroughly washed and externally decontaminated in 40 parts per million sodium hypochlorite for 2 minutes; the chlorine was then neutralized with sodium thiosulfate. Decontamination was terminated before the worms were dead and discolored. Palatability was not affected, for the fry avidly ate them.

After 4 months at 12.5°C, the four lots of fry were killed and processed by the PTD method. All yielded *M. cerebralis* spores.

Conclusion

Fresh spores of *M. cerebralis* have never been shown to infect fish; neither have *Triactinomyxons* been shown to infect their annelid hosts. The explanation is that *M. cerebralis* infects the tubificid and does so in a matter of 10 days or less. A new development cycle occurs and results in a *Triactinomyxon* that infects the trout.

In this particular case, the life forms that have previously been known as an actinosporean and a myxosporean have been shown to be simply alternate life stages of a single organism. It seems likely that comparable alternations of form and host will be found among other Myxozoa. Indeed, Janiszewska (*8*) noted similarities of spore formation in what were then termed Actinomyxidia and Myxosporidia. We propose the name *Triactinomyxon gyrosalmo* for identifying the specific cause of salmonid whirling disease. We leave to the systematists the decision as to which name—*Myxosoma cerebralis* or *Triactinomyxon gyrosalmo*—will be used to identify the organism.

We have not yet determined which species of tubificid is involved in whirling disease (*5*). Considering the biology of aquatic oligochaetes, prevention of whirling disease could be achieved if the host worms could be eradicated. Chemicals selectively lethal for annelids could achieve eradication, but such compounds are not yet known.

Two investigators have independently claimed to have infected trout with whirling disease by using *M. cerebralis* spores simply aged in water (*18, 19*). In neither study were details of the work sufficient to judge whether tubificids were involved.

The tubificids we used do not permit us to equate our system with pure culture techniques that are basic to microbiology and virology. Accordingly, we cannot claim to have fulfilled Koch's postulates for whirling disease. We have, however, offered the only explanation we know of that is supported by experimental data.

References and Notes

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10. Aquatic soil and resident tubificids were taken from the Ridge, West Virginia, trout hatchery, which is free of whirling disease. The worms (average weight, about 1.5 mg) were repeatedly washed in springwater that is free of fish pathogens. Before use, soil was depopulated at 70°C for 1 hour, cooled, and then also washed in pathogen-free water. Test containers were 9-liter glass jars 19 cm in diameter with a screened outlet at the 6-liter level. The containers held axenic soil to a depth of 6 to 8 cm; worms were fed granular trout ration. The spores of *M. cerebralis* were harvested from infected fingerling rainbow trout by simple maceration screening followed by centrifugation gentle enough to preserve viability.
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13. When reacted in direct tests with 11 different fish myxosporeans, the conjugated rabbit antiserum to *M. cerebralis* showed heterologous activity only against another member of the genus *Myxosoma*.
14. Fry were kept in a 3-liter container of aerated springwater to which was added a 48-hour-long (but otherwise unquantified) harvest of *Triactinomyxon* from the initial container of 60,000 tubificids. At 2-day intervals, the procedure was repeated twice with fresh water and *Triactinomyxon*.
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16. After the 6 days of exposure, fry were moved to containers that received pathogen-free springwater.
17. Fry given *Triactinomyxon* orally or by intramuscular injection each received 7,100 organisms; those injected intraperitoneally each received 14,200 organisms. All three groups were placed in pathogen-free springwater.
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