



Efficacy of fumagillin and TNP-470 in preventing experimentally induced whirling disease in rainbow trout, *Oncorhynchus mykiss*  
by Linda Sue Staton

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in  
Biological Sciences  
Montana State University  
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**Abstract:**

Fumagillin, an antibiotic derived from the fungus *Aspergillus fumigatus*, and TNP-470, a super active analog of fumagillin, were tested for efficacy to prevent *Myxobolus cerebralis* (Mc) infection in rainbow trout. Six trials (2 laboratory and 4 field trials) were conducted in which fumagillin or TNP-470 medicated feed was fed to juvenile rainbow trout (~36 mm) exposed to Mc triactinomyxons. Fish were fed medicated feed at various dosages, at various times with respect to the time of exposure, and for varying lengths of time. Fumagillin was either top-dressed on feed or incorporated in feed. Presence and level of Mc infection was determined by polymerase chain reaction, operculum blots, histology, spore counts, and electron microscopy evaluation conducted 90-240 d post-exposure. Hematology samples were also collected to evaluate potential toxic effects of treatment. Fumagillin and TNP-470 were top-coated treatment was not efficacious in preventing or reducing Mc infection. Although incorporated fumagillin and TNP-470 administered for 10 d or 26 d did reduce the level of Mc infection, results were not significantly different from positive controls. No treatment group was effective in preventing Mc infection. Although fumagillin treatment and TNP-470 fed for 10 d did not appear to negatively impact fish performance, toxicity was observed in fish fed TNP-470 for 26 d. These fish became lethargic 30 d post exposure, and blood samples revealed low hematocrits, severely decreased lymphocytes, and reduced numbers of blast cells. Electron microscopy revealed spore deformations resulting from both to fumagillin and TNP-470 treatment. Fumagillin affected the polar capsules and vacuolated the sporoplasm, and TNP-470 inhibited proper spore valve shell production. In general, results were very inconsistent, both within and between studies. Fumagillin and TNP-470 were not effective in the prevention or control of Mc infection in rainbow trout.

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MONTANA STATE UNIVERSITY-BOZEMAN  
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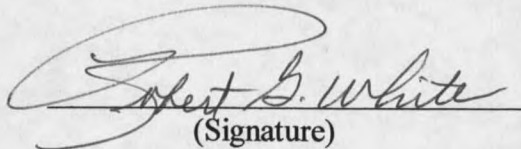
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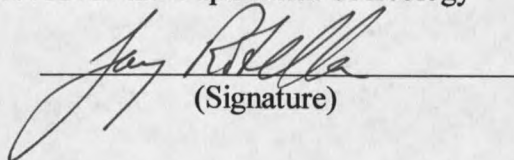
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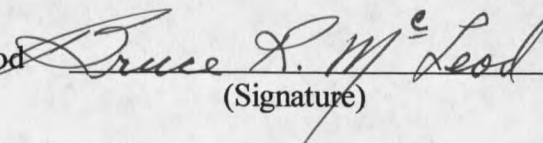
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## ABSTRACT

Fumagillin, an antibiotic derived from the fungus *Aspergillus fumigatus*, and TNP-470, a super active analog of fumagillin, were tested for efficacy to prevent *Myxobolus cerebralis* (*Mc*) infection in rainbow trout. Six trials (2 laboratory and 4 field trials) were conducted in which fumagillin or TNP-470 medicated feed was fed to juvenile rainbow trout (~36 mm) exposed to *Mc* triactinomyxons. Fish were fed medicated feed at various dosages, at various times with respect to the time of exposure, and for varying lengths of time. Fumagillin was either top-dressed on feed or incorporated in feed. Presence and level of *Mc* infection was determined by polymerase chain reaction, operculum blots, histology, spore counts, and electron microscopy evaluation conducted 90-240 d post-exposure. Hematology samples were also collected to evaluate potential toxic effects of treatment. Fumagillin and TNP-470 were top-coated treatment was not efficacious in preventing or reducing *Mc* infection. Although incorporated fumagillin and TNP-470 administered for 10 d or 26 d did reduce the level of *Mc* infection, results were not significantly different from positive controls. No treatment group was effective in preventing *Mc* infection. Although fumagillin treatment and TNP-470 fed for 10 d did not appear to negatively impact fish performance, toxicity was observed in fish fed TNP-470 for 26 d. These fish became lethargic 30 d post exposure, and blood samples revealed low hematocrits, severely decreased lymphocytes, and reduced numbers of blast cells. Electron microscopy revealed spore deformations resulting from both to fumagillin and TNP-470 treatment. Fumagillin affected the polar capsules and vacuolated the sporoplasm, and TNP-470 inhibited proper spore valve shell production. In general, results were very inconsistent, both within and between studies. Fumagillin and TNP-470 were not effective in the prevention or control of *Mc* infection in rainbow trout.

## INTRODUCTION

Since 1893, *Myxobolus cerebralis* (*Mc*), the causative agent of whirling disease has been known to infect rainbow trout, *Onchorynchus mykiss* (Hoffman 1990; Hedrick 1998; El-Matbouli et al. 1995). This parasite is thought to have developed as a non-pathogenic organism associated with brown trout *Salmo trutta* in central Europe and Northern Asia. It has since spread worldwide to over 21 countries due to the stocking of fish, discarding non-consumable carcass parts, or from avian droppings (Hoffman 1990; Taylor and Lott 1978). The parasite was first found in the United States in Pennsylvania waters in 1956, and is currently one of the most serious threats to wild and captive salmonids throughout the country (Rognile and Knapp 1998). The disease has now spread to 22 other states. Recent rainbow trout population declines in Colorado and Montana blue ribbon streams have been attributed to *Mc* (Vincent 1996).

Living organisms are continually faced with a barrage of antagonistic invaders that continually challenge their immune defense system. Unfortunately, with respect to many invaders, including *Mc*, immunological responses often occur too late to combat infection and prevent disease. Hence, without treatment intervention abnormal behavior, lesions, morbidity, and/or mortality occurs (Hedrick et al. 1999). In a *Mc* infection, a number of factors play a role in the initiation and management of infection. Numerous studies have concentrated on the worm host, evaluating such parameters as genetics, life stage, environment, temperature, etc. (Wolf et al. 1986; Hamilton and Canning 1988; El-Matbouli and Hoffman 1998; El-Matbouli et al. 1999; Antonio et al. 1999). Other studies have concentrated on the fish host, examining such factors such as age susceptibility,

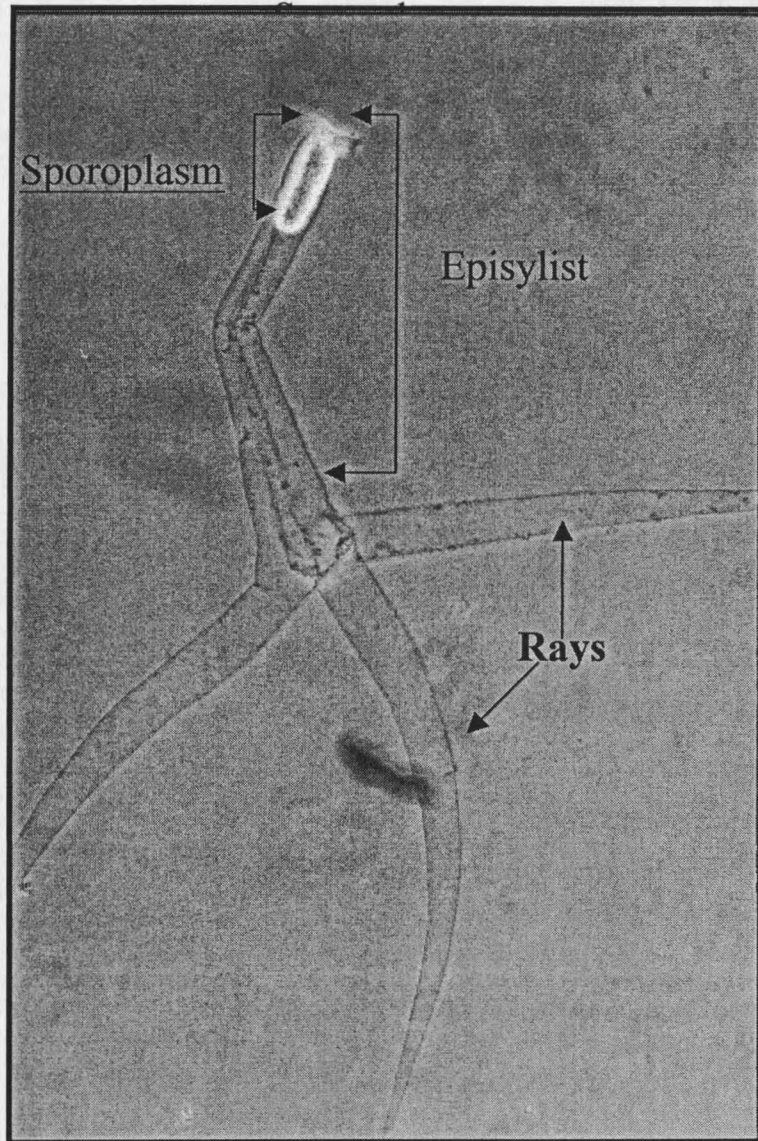
species susceptibility, and parasite exposure level in an attempt to find the weak link in the parasite's life cycle (El-Matbouli and Hoffman 1991; Halliday 1974; Baldwin et al. 2000; Thompson et al. 1999). The goal of my study was to experimentally induce whirling disease in rainbow trout and to evaluate the efficacy of two antimicrobial therapeutants, fumagillin and TNP-470 (a superactive analog of fumagillin) to prevent or control infection.

### *Myxobolus cerebralis* - Taxonomy, Life Cycle and Parasitic Characteristics

The phylum *Cnidaria* is characterized by metazoan organisms which have both somatic and reproductive cells, complex life cycles and cellular organelles. This phylum includes oceanic hydras, sea anemones, corals and jellyfish. The Myxozoa, including *Mc* is taxonomically grouped with these organisms (Rognlie and Knapp 1998). *Myxobolus cerebralis* has a two host life cycle consisting of the oligochaetae worm *Tubifex tubifex* and a salmonid fish. It also has two intermediate water borne life stages, the spore and the triactinomyxon (TAM) (Hedrick et al. 1998). The spore, which is the encysted stage of the parasite, is ingested from a decaying infected fish or filtered from the substrate and consumed by the *T. tubifex*. The spore embeds in the intestinal epithelium of the worm and rapidly proliferates. In the intestinal epithelium, the spore replicates into numerous TAMs which are shed by the *T. tubifex* either through the digestive tract or by death of the worm (El-Matbouli and Hoffman 1998). Triactinomyxons are free floating organisms that consist of three rays and an epistylis (El-Matbouli and Hoffman 1998; Hedrick et al. 1998). Triactinomyxoans remain viable in the water column for 7 d at 7° C (Hedrick et

al. 1998). The epistylis harbors the polar capsules and the infective sporoplast. The polar capsules anchor the TAM to the fish host; usually via the epidermis, buccal cavity, or the respiratory epithelium. The infective sporoplast fires the sporoplasm into the fish tissue (Figure 1). It has been reported that TAMs are drawn to salmonid fishes by chemical and mechanical stimuli (El-Matbouli et al. 1999). Within 5 minutes of TAM attachment, the sporoplasm begins to migrate through dermal tissue and 4 to 24 d post exposure the parasite reaches the nervous system. Once in nervous tissue, *Mc* again proliferates, transforms into a precartilaginous vegetative state, and migrates toward cartilaginous tissue. After at least 20 d in nervous tissue, *Mc* migrates toward cartilaginous tissue where it transforms into a trophozoite and begins feeding on the cartilage. The majority of trophozoites are found in the cranial cartilage, where they feed and reproduce until their eventual transformation into spores. The spore remains in the cartilage cavity surrounded by bone until death of the fish host. Decay or digestion by a predator returns the spore to the environment where it is available for ingestion by *T. tubifex* to perpetuate the parasitic life cycle (Hedrick et al. 1998). Spores may remain viable for years (Lom 1995).

The level and severity of *Mc* infection has been reported to be dose and temperature dependant (Markiw 1992; El-Matbouli et al. 1999; Hedrick et al. 1999). At 15 °C an optimal relationship appears to exist between *T. tubifex* and *Mc*. However, at 25 °C the development and release of mature TAMs is hindered and production ceases after 96 h. At 5 °C TAM production occurs, but at a slower rate of maturation (El-Matbouli et al. 1999).



**Figure 1.**-A wet mount of a triactinomyxon (TAM). At the apical end of the epistylist is the sporoplasm containing 64 sporoplasts. The remaining three structures are rays that aid in buoyancy. Picture compliments of Beth MacConnell.

*Myxobolus cerebralis* is species specific and reportedly only affects salmonid hosts. Rainbow trout appear to be the most susceptible, while arctic grayling and lake trout the most resistant (Hedrick et al. 1998). However, experiments with a variety of salmonid species and different strains within a species have produced varying results with respect to susceptibility to the parasite. Based on current information, species susceptibility from least susceptible to most susceptible is as follows: lake trout (*Salvelinus namaycush*), arctic grayling (*Thymallus arcticus*), reciprocal splake (male lake trout X female brook trout), splake (female lake trout X male brook trout), coho salmon (*Oncorhynchus kisutch*), brown trout (*Salmo trutta*), atlantic salmon (*Salmo salar*), chinook salmon (*Oncorhynchus tshawytscha*), steelhead (*Oncorhynchus mykiss*), brook trout (*Salvelinus fontinalis*), cutthroat trout (*Oncorhynchus clarki*), golden trout (*Salmo aquabonito*), sockeye salmon (*Oncorhynchus nerka*) and rainbow trout (*Oncorhynchus mykiss*) (O'Grodnick 1979; Hoffman 1990; Markiw 1992; Wagner et al. 2000; Hedrick et al. 1998).

Susceptibility of fish to *Mc* infection is also age dependant. Larval fish (<12 weeks) are more susceptible to infection because *Mc* attacks the cartilage. As growth occurs, the cartilage becomes ossified into bone which cannot support the parasite. Also, with increasing age the immune system is better able to combat infection. Therefore, in general terms, the older a fish is before it is exposed to *Mc*, the greater the chance of survival (Hedrick et al. 1998; Wolf and Markiw 1982; Molnar 1991; Hoffman and Byrne 1974).

Fish severely infected with *Mc* often exhibit clinical signs of whirling disease. These signs include whirling behavior, black tail, shortened snout and operculum, exophthalmia and scoliosis. The occurrence of clinical signs increases susceptibility to predation due to color changes, decreased body condition, whirling behavior and impaired movement.

### Management Strategies

Whirling disease has spread rapidly throughout the western United States (Rognlie and Knapp 1998). The development of an effective therapeutant control for whirling disease would be of great benefit to salmonid fisheries programs dependent upon stock supplementation. It would also indirectly benefit all salmonid fisheries programs by decreasing the number of spores liberated into the system and thereby reducing overall system infectivity. To date, there are no known effective management strategies for controlling whirling disease, particularly with respect to incidence in wild populations. However, there is little doubt that stocking of infected fish exacerbates the problem. If whirling disease is to be controlled or managed, it is imperative that stocking of infected fish be terminated. The development of a drug or therapeutant treatment would benefit the management of whirling disease in both captive and wild fisheries. Preliminary studies have indicated that fumagillin may be such a compound, or possibly, the super active analog of fumagillin, TNP-470 (El-Matbouli and Hoffman 1991; Higgins and Kent 1998).

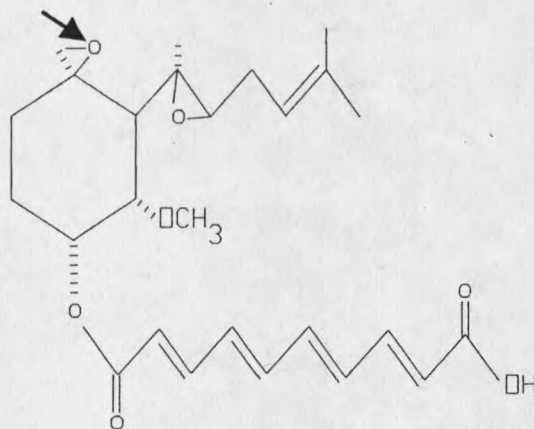
### Therapeutants – Fumagillin and TNP-470

Fumagillin is a natural occurring water insoluble antibiotic produced by the fungus *Aspergillus fumigatus*. *A. fumigatus* was first researched by Georg Fresenius in the mid 1800's, but was not described until 1948 (Schmidt 1998). Fumagillin has broad therapeutic potential as a result of its potent antimicrobial properties. To date, fumagillin has been found to be efficacious in treating a number of Microsporidia infections, including *Encephalitozoan cuniculi*, *Encephalitozoan hellum* and *Nosema apis* (Coyle et al. 1998; Guyonnet et al. 1995; Liu 1973; Katsnelson and Jamieson 1952). Fumagillin has also been used to treat AIDS patients, and as an experimental cancer drug (Stock 1966). TNP-470 was developed as a superactive analog of fumagillin. The drug reportedly has a higher bioreactivity and lower toxicity than fumagillin when used at the same dosages (Coyle et al. 1998). Although a complete understanding of the mechanism of action of fumagillin and TNP-470 is unknown, there is evidence of inhibition of RNA synthesis and angiogenesis of tumors (Jaronski 1972; Sin et al. 1997).

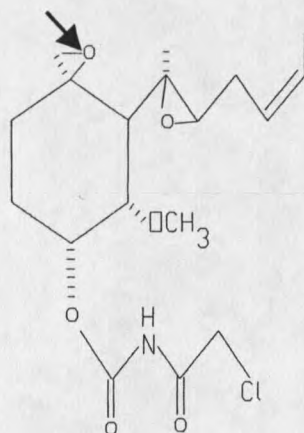
The organic structure of fumagillin has two active epoxide groups which have been shown to covalently bind to cellular proteins and hinder protein synthesis (Figure 2a) (Sin et al. 1997). The binding of the epoxide groups causes the endothelial cell cycle to stop in the later stages of the first growth phase of mitosis. Fumagillin binds irreversibly to the protein methionine amino peptidase that hinders angiogenesis (Sin et al. 1997; Griffith et al. 1998). Angiogenesis is important in promoting wound healing and combating infection. In addition, fumagillin has been shown to decrease pathology of

Karposi's sarcomas, diabetic retinopathy and rheumatoid arthritis in humans (Griffith et al. 1998). Molecules of TNP-470 differ from fumagillin at the number 1 carbon side chain on the cyclohexane (Figure 2b).

a.



b.



**Figure 2.** a. Organic chemical structure of Fumagillin DCH. b. Organic chemical structure of TNP-470. Arrow denotes the epoxide that is the probable reactive site of drug/pathogen interaction based on human cellular research (Sin et al. 1997; Griffith et al. 1998 ; Yoshida et al. 1998).

In recent studies, TNP-470 was found to be relatively nontoxic at extremely high doses in mice compared to fumagillin, and more effective in tumor treatment (Sin et al. 1997; Griffith et al. 1998; Yoshida et al. 1998). Therefore, it was hypothesized that this super active analog may be more efficacious in combating *Mc* infection than fumagillin.

Recent investigations have shown that fumagillin may have relatively broad potential for use in aquaculture against microsporidian and myxosporean parasitic infections. Fumagillin medicated feed treatment has been reported to be efficacious in the treatment of *Pelistophora* infection in eels when administered at the time of exposure at 5, 7.2 to 14.4 and 50 mg/kg/d for 20, 30 and 60 d (Kano et al. 1992). These authors reported no toxic side effects related to fumagillin treatment. Rhee et al. (1993) reported fumagillin at 3.95 mg/500g body weight (BW)/d for 30 d at 45 d pe was efficacious for the treatment and prevention of *Thelohanellus kitauei* in Israel carp. Molnar et al. (1987) reported fumagillin at 1 g/kg feed was efficacious in the prevention and treatment of the myxosporean, *Sphaerospora renicola* in common carp if fed prior to or immediately after exposure.

Fumagillin has also been reported to be effective in the treatment of other myxosporean diseases including proliferative kidney disease, ceratomyxosis, and whirling disease in salmonids. Hedrick et al. 1988 reported that the oral administration of fumagillin at 0.5 g/kg feed for 6 weeks was effective in controlling an experimental infection of proliferative kidney disease in chinook salmon. Although these authors found no toxic effects of fumagillin treatment using the above-described treatment regimen, they did report that treatment with fumagillin at 1.0 g/kg feed for 7 weeks

resulted in a depletion of hematopoietic tissue in the kidney. Similar results regarding the treatment of proliferative kidney disease (PKD) using fumagillin treatment have been reported for coho salmon and rainbow trout (Higgins and Kent 1996; le Gouvello et al. 1998). Sockeye salmon treated with 0.1 or 1.0 mg/kg fish/d TNP-470 for 5 weeks had no sign of PKD, however the higher dosed fish exhibited atrophy of the renal interstitial hematopoietic tissue (Higgins and Kent 1998). Ibarra et al. (1990) reported that the oral administration of fumagillin at 0.25 and 0.50 g/kg feed at 2% BW/d was not efficacious in protecting rainbow trout exposed to ceratomyxosis. They also reported that extended feeding of fumagillin resulted in increased mortality. El-Matbouli and Hoffman (1991) reported that fumagillin treatment at 2.2 mg/kg BW/d for 50 and 130 d was efficacious in controlling whirling disease infection in rainbow trout exposed to *Mc*.

To date, only a few therapeutants have been examined for potential use against *Mc*. Furazolidone, acetarsol, clamoxyquin and proguanil hydrochloride have been tested for efficacious properties against *Mc*, but have been reported to not be effective (Taylor et al. 1973; Aldermann 1986). As described above, El-Matbouli and Hoffman (1991) reported that fumagillin treatment of fish exposed to *Mc* was efficacious, and reduced both parasite density in cranial cartilage and clinical signs of whirling disease.

Fumagillin is the only therapeutant that has been reported to be efficacious against *Mc* infection. Although the mode of action of fumagillin in controlling whirling disease is not well understood, a reduction in spore numbers and changes in spore morphology suggest an effect on DNA or RNA synthesis (Janorsky 1972). Spore deformities in fumagillin treated fish are visible using light and electron microscopy. Commonly

observed deformities include vacuolization of the spore interior and abnormal polar capsules. These deformities cause the spore to be nonviable. Therefore, the primary objectives of my study were to:

1. Determine the efficacy of fumagillin and TNP-470 in preventing experimentally induced whirling disease in rainbow trout.
2. Determine the efficacy of fumagillin in preventing naturally induced whirling disease in rainbow trout, chinook salmon and steelhead trout at Colorado and Idaho state hatcheries.
3. Evaluate the effect of the time of application of fumagillin and TNP-470 treatment (prior to, upon, or following exposure), dosage and duration of treatment on the level of *Mc* infection.

## STUDY AREAS

Six trials were conducted at five test sites with varying environmental conditions (i.e. temperature, flow, turbidity, infectivity, etc.) to evaluate the efficacy of fumagillin and TNP-470 to control whirling disease in fish exposed to *Mc*. Trial sites included Pahsimeroi State Fish Hatchery (SFH), ID; Chalk Cliffs SFH, CO; Eagle SFH, ID; Willow Creek, MT and the Wild Trout Research Laboratory (WTRL), MT (Figure 3). Rainbow trout, steelhead trout and chinook salmon were selected for efficacy evaluations (Table 1).



**Figure 3.**-Location of trials: Pahsimeroi SFH, Ellis ID; Eagle SFH, Eagle ID; Chalk Cliffs SFH, Nathrop CO; Willow Creek, Harrison MT; and the Wild Trout Research Laboratory, Bozeman MT.

Table 1. Summary of methodology for all six efficacy trials comparing individual trials start and end dates; fish species and age; type, dose and duration of exposure; dose, time and duration of treatment, number of replicates, fish per test unit; and water temperature.

Trial Sites	Pahsimeroi SFH	Chalk Cliffs SFH	Eagle SFH	Montana (Willow Crk)	Wild Trout Research Lab (1998)	Wild Trout Research Lab (2000)
Start Date	05/29/1998	11/08/1998	09/04/1998	10/06/1998	08/31/1998	02/16/2000
Completion Date	12/10/1998	06/27/1999	05/06/1999	06/02/1999	04/28/1999	07/19/2000
Species & Age	SHT, SCS 10 wk	RBT 8 wk	RBT 8 wk	RBT 6 wk	RBT 8 wk	RBT 12 wk
Type of Exposure	Pahsimeroi/Salmon River Water	Chalk Creek Water	Boise River Water	Willow Creek Water	Controlled Dose	Controlled Dose
Exposure Dose	Unknown	Unknown	Unknown	Unknown	1900 TAMs/fish	1000 TAM's/fish
Duration of Exposure (d)	180	240	10	3 or 7	2 h	2 h

Table 1(continued). Summary of methodology for all six efficacy trials comparing individual trials start and end dates; fish species and age; type, dose and duration of exposure; dose, time and duration of treatment, number of replicates, fish per test unit; and water temperature.

Trial Sites	Pahsimeroi SFH	Chalk Cliffs SFH	Eagle SFH	Montana (Willow Crk)	Wild Trout Research Lab (1998)	Wild Trout Research Lab (2000)
Fumagillin Treatment Dose (mg/kg BW/d)	3.75 & 7.5	7.5	7.5	7.5	3.75 & 7.5	7.5*
Time of Treatment (d pe)	14	0	10	-5, 3, 7	0, 14	0
Duration of Treatment (d)	10	10	14	10	10	10 or 26**
Number of Test Units	18	6	6	14	18	35
Number of Replications	3	3	3	2	3	5
Number of Fish/Test Unit	100	100	150	60	60	38
Water Temperature	0 ->15 °C	10.1-11 °C	12.7-15.1 °C	6.6-15.9 °C	13 °C	13 °C

\* TNP-470 in the WTRL 2000 trial was administered at 0.75 mg/kg BW/d

\*\* All fumagillin treatments were treated for 10 d and TNP-470 treatments were fed for 10 or 26 d pe

## METHODS

Feed Production and Administration

Fumagillin dicyclohexylamine (DCH) salt used in all top-coated feed trials was obtained from Sanofi Santé Nutrition Animale (Z.I. La Ballastiere, 33501 Libourne Cedex, France). The "Sanofi" fumagillin DCH was a pure powder that contained approximately 65% fumagillin. Rangen feed crumbles, size #1, were top-coated with a mixture of Sanofi fumagillin DCH dissolved in ETOH and then overlaid with vegetable oil (le Gouvello, personal communication). The top-coated feed was prepared immediately prior to the experiment start date and stored at 4° C in the dark throughout the experiment to maintain consistent biological activity. Sanofi fumagillin DCH, Chinoin Pharmaceutical fumagillin DCH (Hungary), and TNP-470 were incorporated into Rangen size #1 crumbles for the Wild Trout Research Laboratory (WTRL) 2000 experiment by Mansour El-Matbouli at the University of Munich, Germany. The incorporated medicated feed pellets were sent via air express to Bozeman, MT and stored at 4° C in the dark. Fish were fed Sanofi fumagillin top-coated feed at a concentration of 3.75, and/or 7.5 mg/kg BW/d, and Sanofi or Chinoin fumagillin incorporated feed at 7.5 mg/kg BW/d (note: 3.75 mg fumagillin/kg BW  $\approx$  0.25 g/kg feed at 1-2 % BW/d). TNP-470 incorporated feed was fed at 0.75 mg/kg BW/d.

Time of treatment relative to time of exposure was -5 (prophylactic), 0, 3, 7, 10 and 14 d post-exposure (pe), which varied between trials. Fish were fed medicated feed for 10, 14 or 26 consecutive days. Control fish received non-medicated Rangen #1

crumbles throughout the duration of the trial. Feed was weighed out for individual tanks daily and fish were fed 3 - 4 times/d. Feed amounts were increased monthly to compensate for fish growth. Fish were converted to larger crumbles as their size increased. Fish were reared at density and flow indexes as recommended by Piper et al. (1992).

### Field Trial Exposures

#### Pahsimeroi State Fish Hatchery

Pahsimeroi A type steelhead trout (SHT) and Sawtooth strain spring chinook salmon (SCS) 10 weeks old were continuously exposed to infected surface water for 24 d at the Pahsimeroi State Fish Hatchery (SFH). Fish were held in 30 gallon plastic garbage cans that had been converted into aerated rearing units and placed in hatchery raceways. One hundred fish were randomly selected for each of three replicates per treatment. Blinding and randomization of treatment designation was conducted. Treatment groups included positive controls (exposure, no treatment), 3.75 mg Sanofi fumagillin/kg BW/d, and 7.50 mg Sanofi fumagillin/kg BW/d. In all treated groups fumagillin treatment was initiated 14 d pe, and fish were fed medicated feed for 10 d. Following exposure and treatment at the Pahsimeroi SFH, fish were transported to the Sawtooth State Fish Hatchery (Note: Sawtooth SFH water supply was also *Mc* positive). Abnormal behavior, clinical signs and mortalities were noted daily in each replicate of each test group. Five fish from each replicate were sampled at 45 d pe (frozen heads for PCR), at 90 d pe (whole head histology), and at 120 d pe (operculum blots, histology and

spore counts). Prior to trial termination the fish were moved back to the Pahsimeroi SFH. At 180 d pe the trial was terminated with operculum blots, histology and half head spore count samples obtained from five fish in each replicate. An additional five fish were sampled individually for whole head spore counts, and all remaining fish in each replicate were pooled in groups of five and sampled for whole head spore counts (Table 2). All fish sampled were euthanized with MS-222, weighed, measured for total length and evaluated for clinical signs of whirling disease.

**Table 2.**-Sampling collection schedule and procedures for Pahsimeroi State Fish Hatchery, ID; Chalk Cliffs State Fish Hatchery, CO; Eagle State Fish Hatchery, ID; Willow Creek, MT and Wild Trout Research Laboratory, MT (1998) trials.

Time Post-Exposure	Treatments Sampled	Fish Per Treatment	Sample Collection Procedures
45d	All	5	Whole fish frozen for PCR.
90 d	All	5	Whole fish in Davidson's fixative for histology.
120 d	All	5	Head cut sagittally; one half for spore counts; from other half, operculum sampled for operculum blot and then fixed in Davidson's for histology.
180 d	All	5	Head cut sagittally; one half for spore counts; from other half, operculum sampled for operculum blot and then fixed in Davidson's for histology.
240 d	All	All	First five fish: Head cut sagittally; one half for spore counts; from other half, operculum sampled for operculum blot and then fixed in Davidson's for histology. Second five fish, whole heads for spore counts. Any fish remaining pooled in five head samples for spore counts.

### Chalk Cliffs State Fish Hatchery

Eight week old Erwin strain rainbow trout were continuously exposed to infected surface water during the duration of the trial. Thirty gallon plastic garbage cans that were converted into aerated rearing units were placed in a hatchery raceway. One hundred fish were randomly selected for each of three replicates per treatment. Blinding and randomization of treatment designation was conducted. Treatment groups included positive controls (exposure, no treatment), and 7.50 mg Sanofi fumagillin/kg BW/d. Fumagillin treatment was initiated 0 d pe, and fish were fed medicated feed for 10 d. Abnormal behavior, clinical signs and mortalities were noted daily in each replicate of each test group. Five fish from each replicate were sampled at 45 d pe (frozen heads for PCR), at 90 d pe (whole head histology), and at 120 and 180 d pe (operculum blots, histology and spore counts). At 240 d pe the trial was terminated with operculum blots, histology and half head spore count samples obtained from five fish in each replicate. An additional five fish were individually sampled for whole head spore counts, and all remaining fish in each replicate were pooled in groups of five and sampled for whole head spore counts (Table 2). All fish sampled were euthanized with MS-222, weighed, measured for total length, and evaluated for clinical signs of whirling disease.

### Field/Laboratory Combination Trial Exposures

#### Eagle State Fish Hatchery

Eight week old Kamloop strain rainbow trout were placed in a live-car in the Boise River and exposed to TAMs for 10 d. After exposure, the fish were transported to

the Eagle SFH, ID and reared in TAM free water until the completion of the trial. Treatment groups included positive controls (exposure, no treatment), and 7.50 mg Sanofi fumagillin/kg BW/d. Fumagillin treatment was initiated 10 d pe, and fish were fed medicated feed for 14 d. Each treatment had 150 fish per tank with three replicates. Abnormal behavior, clinical signs and mortalities were noted daily in each replicate of each test group. The sampling regime consisted of five fish from each replicate at 45 d pe (frozen heads for PCR), at 90 d pe (whole head histology), and at 120 and 180 d pe (operculum blots, histology and spore counts). An additional five fish per replicate were also collected at 120 d pe (whole head histology) because of poor preservation of the 90 d pe samples. At 240 d pe the trial was terminated with operculum blots, histology and half head spore count samples obtained from the five fish in each replicate. An additional five fish were individually sampled for whole head spore counts, and all remaining fish in each replicate were pooled in groups of five and sampled for whole head spore counts (Table 2). All fish sampled were euthanized with MS-222, weighed, measured for total length and evaluated for clinical signs of whirling disease.

#### Montana (Willow Creek)

Erwin strain rainbow trout (RBT) were reared at the Ennis National Fish Hatchery prior to *Mc* exposure. Five days before *Mc* exposure, one test group was fed Sanofi fumagillin at 7.5 mg/kg BW/d. Six cages containing 6 week old RBT were placed in Willow Creek at Harrison, MT for a 3 or 7 d exposure to TAMs. After exposure, the fish were transported to the WTRL and reared in TAM free water until the completion of the trial. Treatment groups included negative control (no exposure, no treatment), positive

controls (3 and 7 d exposure, no treatment), 7.50 mg Sanofi fumagillin/kg BW/d 5 d prophylactically and at 3 d pe; 7.50 mg Sanofi fumagillin/kg BW/d 5 d prophylactically and at 7 d pe; 7.50 mg Sanofi fumagillin/kg BW/d at 3 d pe; 7.50 mg Sanofi fumagillin/kg BW/d at 7 d pe. Post-exposure medicated feed was administered for 10 d in all treatment groups. Each treatment was conducted in duplicate with 60 fish per tank. Abnormal behavior, clinical signs and mortalities were noted daily in each replicate of each test group. The sampling regime consisted of five fish from each replicate at 45 d pe (frozen heads for PCR), at 90 d pe (whole head histology), and at 120 and 180 d pe (operculum blots, histology and spore counts). At 240 d pe the trial was terminated with operculum blots, histology and half head spore count samples obtained from five fish in each replicate. An additional five fish were individually sampled for whole head spore counts, and all remaining fish in each replicate were pooled in groups of five and sampled for whole head spore counts (Table 2). All fish sampled were euthanized with MS-222, weighed, measured for total length and evaluated for clinical signs of whirling disease.

### Laboratory Trials

#### Wild Trout Research Laboratory 1998

This trial was a laboratory study that involved 8 week old Erwin strain rainbow trout from Ennis National Fish Hatchery. Based on procedures developed at the University of California, Davis, fish were exposed to a known concentration of TAMs in 4 L plastic buckets. Triactionmyxons were obtained from *T. tubifex* cultures maintained at the Bozeman Fish Health Center. Fish were exposed to 1900 TAMs per fish for 2 h.

Each bucket (1 bucket/replicate) contained 2 L of source water. Treatment groups included negative controls (no exposure, no treatment), positive controls (exposure, no treatment), 3.75 mg Sanofi fumagillin/kg BW/d initiated at 0 d pe; 3.75 mg Sanofi fumagillin/kg BW/d initiated at 14 d pe; 7.50 mg Sanofi fumagillin/kg BW/d initiated at 0 d pe ; and 7.50 mg Sanofi fumagillin/kg BW/d initiated at 14 d pe. Medicated feed was administered for 10 d pe in all treatment groups. Following the treatment period, all fish received the same non-medicated diet for the remainder of the trial. Each treatment group was conducted in triplicate, with 60 fish per replicate. Following exposure to TAMs, fish were immediately transferred to individual 38 L aquaria. Blinding and randomization of treatment designation was conducted. Fish were maintained on recirculating water at a constant 13 °C. Abnormal behavior, clinical signs and mortalities were noted daily in each replicate of each test group. The sampling regime consisted of five fish from each replicate at 45 d pe (frozen heads for PCR), at 90 d pe (whole head histology), and at 120 and 180 d pe (operculum blots, histology and spore counts) (Table 2). At 240 d pe the trial was terminated with operculum blots, histology and half head spore count samples obtained from five fish in each replicate. An additional five fish were individually sampled for whole head spore counts, and all remaining fish in each replicate were pooled in groups of five and sampled for whole head spore counts. All fish sampled were euthanized with MS-222, weighed, measured for total length, and evaluated for clinical signs of whirling disease.

Wild Trout Research Laboratory 2000

This trial was a laboratory study that involved 12 week old Arlee strain rainbow trout reared at the Bozeman Fish Technology Center. Similar to procedures used in the WTRL 1998 trial, fish were exposed to a known concentration of TAMs in 4 L plastic buckets. Triactionmyxons were obtained from *T. tubifex* cultures maintained at the Bozeman Fish Health Center. Fish were exposed to 1000 TAMs per fish for 2 h. Each bucket (1 bucket/replicate) contained 2 L of source water. Treatment groups included negative controls (no exposure, no treatment), positive controls (exposure, no treatment), 7.50 mg Sanofi fumagillin top-coated/kg BW/d, 7.50 mg Sanofi fumagillin incorporated/kg BW/d, 7.50 mg Chinoïn fumagillin incorporated/kg BW/d, and 0.75 mg TNP-470 incorporated/kg BW/d (note: two TNP-470 treatment groups). Medicated feed treatment was initiated to all treated groups 24 h pe to TAMs. All treated groups received medicated feed for 10 d, with the exception of a single TNP-470 treatment group that was fed for 26 d. Following the treatment period, all fish received the same non-medicated diet for the remainder of the trial. Five replicate tanks of fish were used for each treatment group, with 38 fish/replicate. Following exposure to TAMs, fish were immediately transferred to individual 38 L aquaria. Blinding and randomization of treatment designation was conducted. Fish were maintained on recirculating water at a constant 13 °C. Abnormal behavior, clinical signs and mortalities were noted daily in each replicate of each test group. The EM sampling was performed on two fish from two replicates of each treatment group at 5 min, 1, 2, 4, 6, 10 and 26 d pe. Electron microscopy samples were also collected from two fish from each TNP-470 26 d

treatment replicate at 30 and 61 d pe and from two fish from all tanks at 150 d pe. Histological samples were collected 6, 10, and 26 d pe from two fish from two replicates of all treatments. Histological samples were also collected from two fish per tank from all TNP-470 26 d treatment replicates on 30 and 61 d pe. Histological samples were collected from 10 fish per tank from all tanks at 150 d pe. Hematology consisting of blood smears and hematocrits was conducted 30 and 61 d pe on two fish from all TNP-470 26 d treatment replicates. At 150 d pe 20 fish from each tank were sampled for hematology, length, weight and clinical signs (Table 3). Half heads from 5 of these 20 fish were evaluated for spore counts. All fish sampled were euthanized with MS-222.

#### Sample Analysis

##### TAM Collection, Enumeration, and Calculation

Triactinomyxons were collected from a laboratory culture of *Mc* infected *T. tubifex* maintained at the Bozeman Fish Health Center (USFWS, Bozeman, MT). Water was siphoned off of the *T. tubifex* cultures, settled for 10 minutes and filtered with a 20-micron mesh Nytex screen. The TAMs were then rinsed off the screen and collected in a volumetric container (Hedrick et al. 1999). A 50 ul aliquot was placed on a microscope slide and coverslipped. The total number of viable TAMs was determined, and this number multiplied by 20 to quantify TAMs/ml. The procedure was repeated three times and the number of TAMs/ml averaged. The total number of TAMs collected from the worm cultures was calculated by multiplying the mean number of TAMs/ml by the total

Table 3.-Sampling collection schedule and procedures for WTRL 2000 trial at Bozeman, MT

Time Post-Exposure	Treatments Sampled	Fish Per Treatment	Sample Collection Procedures
5 min	All <sup>1</sup>	4*	Whole fish in Modified Karnofski's fixative (MKF) for EM.
1d	All	4*	Caudal, dorsal, and pectoral fins cut in small pieces with scapel blade and placed in MKF for EM.
2d	All	4*	Caudal, dorsal, and pectoral fins cut in small pieces with scapel blade and placed in MKF for EM.
4d	All	4*	Caudal, dorsal, and pectoral fins cut in small pieces with scapel blade and placed in MKF for EM.
6d	All	4*	Fish cut sagittally with spinal column left intact; half with spinal column cut into small pieces perpendicular to spine and placed in MKF; other half fixed intact with Davidson's fixative for histology.
10d	All	4*	Fish cut sagittally with spinal column left intact; half with spinal column cut into small pieces perpendicular to spine and placed in MKF; other half fixed intact with Davidson's fixative for histology.
26 d	TNP-470 10d	4*	Fish cut sagittally with spinal column left intact; half with spinal column cut into small pieces perpendicular to spine and placed in MKF; other half fixed intact with Davidson's fixative for histology.
	TNP-470 26d	4*	
	positive control	4*	
30 d	negative control	4	Fish fixed with Davidson's for histology. Hematology samples were collected for blood smears and hematocrit. Kidneys dissected out and placed in MKF for EM.
	TNP-470 26d	10	
61d	negative control	2	Fish fixed with Davidson's for histology. Hematology samples were collected for blood smears, hematocrit and plasma proteins. Kidney blots were made.
	TNP-470 26d	10	
150d	All <sup>2</sup>	100	Head cut sagittally; one half for spore counts; from other half, 5 pieces of operculum near pseudobranch and low brain collected and placed in MKF for EM, and remaining tissue fixed in Davidson's for histology. Hematology samples were collected for blood smears, hematocrit and plasma proteins.

<sup>1</sup> excluding negative control

<sup>2</sup> 4 fish per replicate for EM, 10 for spore counts and histology.

\* 2 fish per replicate and only 2 replicates per treatment

ml of TAM water. The millimeters of TAM solution added to each bucket was calculated using the following equation:

$$\text{TAM soln. (ml)} = \frac{[\text{number of fish/exposure}] \times [\text{TAMs per fish (i.e. exposure dose)}]}{\text{TAMs/ml}}$$

All exposures were conducted using a single group of TAMs.

### Spore Extraction

Spores were extracted from fish tissues and enumerated using standard techniques (Thoeson 1994). The plankton centrifuge method was used on half heads sampled at 120, 180 and 240 d pe. Five half heads were evaluated at each sampling time. In addition, at 240 d pe five whole heads were processed individually and all remaining fish processed as whole head pooled samples. Heads were ground with tap water in a Waring Blender covered with parafilm for ~ 1 min or until the head was adequately blended. The blended sample was then strained through a disinfected urinary calculi filter. The filtered material was added to a separatory funnel located directly above the plankton centrifuge. The plankton centrifuge was then turned on and the filtered material was allowed to flow at a slow and steady pace into the plankton centrifuge cup. The separatory funnel was rinsed three times with tap water (~ 20 ml) after each sample run to flush down any remaining sample material into the plankton centrifuge cup. The plankton centrifuge cup was scraped with a rubber spatula to liberate the spores from the wall of the cup and both the spatula and the cup were rinsed with 5 to 20 ml of water. The sample was pipetted out into a labeled specimen cup and the final volume of the

collected sample was recorded. The plankton centrifuge, blender and calculi filter were disinfected between samples for 10 min in 10% bleach (5.25% sodium hypochloride).

The pepsin trypsin digest method was used on fish sampled at 150 d pe. Boiling water was added to each half head sample in a 50 ml vial. The half heads were left in the water until the eye turned white. Each half head was then strained in a calculi filter and defleshed. The bones and cartilage were ground in a Waring blender with 0.5 % pepsin solution and allowed to digest in a 50 ml vial in a 34 °C shaking water bath until the digested material became a milky white. The samples were then centrifuged for 10 min at 1000 X g. The supernatant was poured off, 2 mls of 0.5% Trypsin/Rinaldi's (pH color indicator solution) was added and the pH was adjusted with 1 N NaOH to 7-7.4. Samples were then placed in a 24 °C shaker water bath for 30 min. The samples were strained through a calculi filter, poured into a 15 ml vial with 1 ml 10% bovine albumin and centrifuged for 10 min at 1200 X g. The supernatant was poured off and 1 ml of 1% bovine albumin/0.5% Hank's Buffered Salt Solution added to each vial using a transfer pipet. The samples were layered using a transfer pipet on 2 ml of glucose in a 15 ml vial and centrifuged for 30 min at 1200 X g. The supernatant was poured off and 2 ml of 70% ethanol was added to the pelleted sample (Markiw and Wolf 1974; Thoesen 1994). The blender and filter were disinfected with bleach between each sample, and new 15 ml and 50 ml vials were used for each sample.

#### Spore Enumeration and Calculation

Samples were evaluated using light microscopy for the absence or presence of spores. Observed spores were also evaluated for normalcy or deformity. Mansour El-

Matbouli supplied photos of transmission electron micrographs of normal and deformed spores as a evaluation guide. The micrographs were examples of normal spore ultrastructure and deformities to the polar capsules resulting from fumagillin treatment. A hemacytometer was used to enumerate the spores. Spores were counted in all 18 grids of the hemacytometer (Benjamin 1961). The spore counts were recorded and the total number of spores/half head calculated as follows:

$$\frac{\text{\# of spores} \times 10^4 \times \text{total volume of the sample}}{18}$$

### PCR

Polymerase chain reaction (PCR) genetically confirms the presence or absence of a specific sequence of DNA. Specifically for *Mc*, a product of 413 base pairs (bp) is the sequence of interest. Frozen heads were thawed, a 6 mm punched sample was collected from each head and a Qiagen DNA extraction kit used to obtain *Mc* DNA from the sample. The nested procedure developed by Andree et al. (1997) was used to confirm the presence of *Mc* infection at day 45 pe. Extracted samples were amplified using Sigma brand Tr 5-16 and Tr 3-16 primers for the first round on a Perk and Elmer thermocycler. The second round amplification of *Mc* DNA was conducted using Sigma brand Tr 5-17 and Tr 3-17 primers. A 1.5% agarose gel consisting of 24 wells was loaded with the amplified samples. An electric current was passed through the gel to allow the DNA fragments to migrate down the gel and separate into bands. The gel was stained with ethidium bromide, destained in water and the results were documented on a UV gel documentation system.

### Operculum Blots

Operculum blots were collected immediately following anesthesia, and before any invasive/disruptive procedures were performed on the fish samples. Operculums were removed with a scalpel, and the cut edge of each sample was blotted on a microscope slide four times. Blots were air dried and stained with 55 ul of a 0.083% concentration of methylene blue (Hoffman and Markiw 1977). A cover slip was mounted on the slide and the samples were read for the presence of spores immediately after staining.

### Pathology

#### Histology

Histology samples were collected from the half head from which the operculum had been removed for the operculum blot. Histology samples were preserved in Davidson's fixative for 48 h, transferred to 70% ethanol and then processed by standard histological procedures (Sheehan and Hrapchak 1980). Two slides were prepared from each half head sample. Each slide contained a midline sagittal and a lateral tissue section. One slide was stained with Hemotoxylin and Eosin and the other with Giemsa. Each slide was examined by light microscopy to determine the severity of *Mc* infection according to the MacConnell/Baldwin Scale, which describes the presence and level of infection of the parasite and the inflammatory response. The MacConnell/Baldwin Scale ratings are as follows: Grade 0: no abnormalities noted. *Myxobolus cerebralis* in not observed; Grade 1: small, discrete, focus or foci of cartilage degeneration. No or few associated leukocytes; Grade 2: single, locally extensive focus or several small foci of

cartilage degeneration and necrosis. Inflammation is localized; few to moderate numbers of leukocytes infiltrate or border lytic cartilage; Grade 3: multiple foci of cartilage degeneration and necrosis. Moderate number of leukocytes are associated with lytic cartilage. Inflammatory cells extend minimally into surrounding tissues; Grade 4: multifocal to coalescing areas of cartilage necrosis. Moderate to large numbers of leukocytes border and/or infiltrate lytic cartilage. Locally extensive leukocyte infiltrates extend into surrounding tissues; Grade 5: multifocal to coalescing areas of cartilage necrosis. Moderate to large numbers of leukocytes border and/or infiltrate necrotic cartilage. The inflammatory response is extensive and leukocytes infiltrate deeply into surrounding tissues. This classification is characterized by loss of normal architecture and is reserved for the most severely affected fish (MacConnell, personal communication).

#### Blood Parameters

Blood Smear. The caudal peduncle was excised at a 45° angle. Blood was collected from the caudal vein into a 25 ul capillary tube by holding the fish at a 45° angle to the tube. Tubes were filled ~2/3 full. A small drop of blood was dabbed on a clean labeled microscope slide and spread in a monolayer across the slide for blood cell analysis. The remaining blood in the capillary tube was plugged with creosil and set aside for hematocrits. The blood smear was air dried, fixed for 1 min in methanol and stained with Leishman Giemsa 24 to 48 h later (Yasutake and Wales 1983). The slides were allowed to air dry after staining and then coverslipped. The starting point for cell counts was 1.5 inch from the leading edge of the blood smear and 0.5 inch from the top

edge of the slide. Five hundred erythrocytes were counted and the stage of cellular development (i.e. blast cell, immature cell, mature cell, degenerative cell or smudge cell) for each erythrocyte determined. In addition, the number of lymphocytes in each smear was noted.

Hematocrit. The capillary tubes plugged with creosil were centrifuged for 10 min at 10,000 rpm in an International Capillary centrifuge, which separated the buffy coat, plasma, and erythrocytes into three distinct layers. The capillary tubes were removed from the centrifuge and placed on an International Micro-Capillary reader. Total hematocrit was determined by measuring the percent packed erythrocyte volume.

### Microscopy Evaluations

#### Light Microscopy

Operculum blots, spore counts, histology samples, and hematological samples were evaluated on a Nikon Labphot – 2 light microscope. Optical lenses of 4, 10, 40 and 100X were used to evaluate these samples. Results were recorded.

#### Electron Microscopy

Samples for transmission and scanning electron microscopy (TEM and SEM) were immediately fixed in modified Karnovsky's fixative (2.5% glutaraldehyde /4% paraformaldehyde in 0.1 M cacodylate buffer), stored at 4 °C overnight and then overnighed to Germany. Final processing and evaluation of all EM samples was conducted by Mansour El-Matbouli at the University of Munich. The TEM samples were

embedded in Epoxy resin for thin and ultra-thin sectioning to obtain micrographs of spores. The SEM samples were processed by a standard protocol (El-Matbouli et al. 1999) to obtain micrographs. These micrographs were evaluated to determine potential treatment affects.

#### Statistical Analysis

Statistical tests performed were a two-sample t-test, and a parametric or non parametric analysis of variance (ANOVA). These tests were used to detect effect of fumagillin treatments on : (1) number of *Mc* spores in fish heads; (2) histological rating of *Mc* infection; (3) mortality; (4) fish weights; and (5) clinical signs of *Mc* infection. A Tukey's pair-wise multiple comparison test was performed when the normality and equal variance tests passed. A Dunn's pair-wise multiple comparison test was conducted when either or both the normality and equal variance tests failed. Where differences are stated to be significant, a level of  $P \leq 0.05$  is implied. All study data from the termination date of each trial (150, 180 or 240 d pe) were analyzed by SigmaStat Version 2.03 (SPSS Inc. 1992).

## RESULTS

Six trials (2 laboratory and 4 field trials) were conducted in which fumagillin or TNP-470 medicated feed was fed to juvenile rainbow trout that had been exposed to *Mc* triactinomyxons. Fish were fed medicated feed at various dosages, at various times with respect to the time of exposure, and for varying lengths of time. Fumagillin was either top-coated on feed or incorporated in feed. Two different formulations of fumagillin were evaluated. TNP-470 was incorporated in feed. Presence and level of *Mc* infection was determined by polymerase chain reaction (Figure 4), operculum blots, histology, spore counts, and electron microscopy evaluation conducted 45 to 240 d pe. Hematology samples were also collected to evaluate potential toxic effects of treatment. Data indicated that fumagillin top-coated treatment was not efficacious in preventing or reducing *Mc* infection. Although incorporated fumagillin and TNP-470 administered for 10 or 26 d in the WTRL 2000 trial did result in a reduction in level of *Mc* infection, results were not significantly different from positive controls. No treatment in any of the 6 trials was effective in preventing or controlling *Mc* infection. Although fumagillin fed for 10 or 14 d and TNP-470 fed for 10 d did not appear to negatively impact fish performance, toxicity was observed in fish fed TNP-470 for 26 d. These fish became lethargic 30 d pe, and blood samples revealed low hematocrits, severely decreased lymphocytes, and reduced numbers of blast cells. Although, electron microscopy revealed spore deformation resulting from fumagillin and TNP-470 treatment, treatment did not impact the level of infection. In general, results were very inconsistent both within and between studies. Results of these 6 trials indicated that fumagillin and TNP-

470 are not effective therapeutants for use in the prevention or control of *Mc* infection in rainbow trout.



**Figure 4.**-Agrose gel showing the nested polymerase chain reaction (PCR) products from two samples from the Pahsimeroi SFH, ID; Chalk Cliffs SFH, CO; Eagle SFH, ID; Willow Creek, MT; and the Wild Trout Research Laboratory (1998), MT trials. Lanes 1 and 19 were DNA ladders, lanes 2 and 20 had no samples, lane 3 and 4 were negative controls, lane 5 was WTRL 3.5 mg/kg BW/day at 0 d pe, lane 6 was WTRL negative control, lane 7 was Willow Creek negative control, lane 8 was Willow Creek 7.5 mg/kg BW/day at 5 d prior to exposure and 3 d pe, lane 9 was Eagle 7.5 mg/kg BW/day at 10 d pe, lane 10 was Eagle positive control, lane 11 was Pahsimeroi steelhead fed at 7.5 mg/kg BW/day at 14 d pe, lane 12 was Pahsimeroi steelhead fed at 7.5 mg/kg BW/day at 14 d pe, lane 13 was Pahsimeroi chinook salmon positive control, lane 14 was Pahsimeroi chinook salmon positive control, lane 15 was Chalk Cliffs 7.5 mg/kg BW/day at 0 d pe, lane 16 was Chalk Cliffs positive control and lane 17 and 18 were positive controls. A band of 413 base pairs indicates *Myxobolus cerebralis* infection detected.

### Results by Individual Trial

#### Pahsimeroi State Fish Hatchery

Fumagillin treatments of SHT and SCS exposed to naturally occurring TAMs in Pahsimeroi SFH source water (Pahsimeroi River) were not efficacious in the prevention or control of whirling disease. *Myxobolus cerebralis* infection was confirmed by PCR in the positive control of SCS and the 7.5 mg/kg BW/d treatment group of SHT 45 d pe.

Mean spore count for the SHT positive control group 180 d pe was 39,055, compared to mean spore counts for the SHT treated groups of 82,900 (3.75 mg fumagillin/kg BW/day initiated 14 d pe) and 112,804 (7.5 mg fumagillin/kg BW/day initiated 14 d pe).

Although spore counts were higher for treated groups, the difference was not significant (Table 4).

**Table 4.**-Spore count data from steelhead trout half-heads 180 d pe to TAMs at the Pahsimeroi SFH, Ellis, ID 1998 trial. (Note: fish were exposed in hatchery source water from the Pahsimeroi River).

Treatment <sup>1</sup>	Spore count <sup>2</sup> (mean value)	Standard deviation	95% C.I	Coefficient of variation
Positive Control N <sup>4</sup> =2	39,055	4,478	40,234	11.5%
3.75 mg Fumagillin/kg BW @ 14 d <sup>3</sup> N=1	82,900	----	----	----
7.5 mg Fumagillin/kg BW @ 14 d <sup>3</sup> N=3	112,804	50,321	12500	44.6%

<sup>1</sup> All controls and treatments conducted in triplicate

<sup>2</sup> Mean of five fish sampled per replicate.

<sup>3</sup> Fumagillin treatment initiated 14 d pe, fumagillin fed for 10 d

<sup>4</sup> N = number of replicate tanks per treatment sampled

Histological evaluation of these fish revealed the presence of three other *Myxobolus spp.*, *Flavobacterium spp.* and presence of a gill fluke, which may have influenced spore count data. These multiple parasites could also have impacted the fish's immune system and treatment effectiveness.

Mean spore count for the SCS positive control group 180 d pe was 4,656, compared to mean spore count for the SCS treated groups of 11,626 (3.75 mg fumagillin/kg BW/day initiated 14 d pe) and 8,359 (7.5 mg fumagillin/kg BW/day initiated 14 d pe) (Table 5).

**Table 5.-** Spore counts from chinook salmon half-heads 180 d pe to TAMs at the Pahsimeroi SFH, Ellis, ID 1998 trial. (Note: fish were exposed in hatchery source water from the Pahsimeroi River).

Treatment <sup>1</sup>	Spore count <sup>2</sup> (mean value)	Standard deviation	95% C.I	Coefficient of variation
Positive Control N <sup>4</sup> =3	4,656	4,790	11,899	102.9%
3.75 mg Fumagillin/kg BW @ 14 d <sup>3</sup> N=3	11,626	4,382	10,886	37.7%
7.5 mg Fumagillin/kg BW @ 14 d <sup>3</sup> N=3	8,359	8,989	22,331	107.5%

<sup>1</sup> All controls and treatments conducted in triplicate

<sup>2</sup> Mean of five fish sampled per replicate.

<sup>3</sup> Fumagillin treatment initiated 14 d pe, fumagillin fed for 10 d

<sup>4</sup> N = number of replicate tanks per treatment sampled

Chinook salmon spore counts were significantly lower than SHT spore counts.

Histological evaluation of SCS also revealed the presence of a considerable number of spores of other *Myxobolus spp.* and other parasitic infections. Because of the

complicating disease conditions, MacConnell/Balwin histological scores were not determined for either species in this trial. Spore count data with respect to both species indicated there was no apparent difference between fumagillin dosages of 3.75 or 7.5 mg/kg BW/d.

Mean total mortality was not reported for either species as a result of the mitigating effects of the presence of other parasites, a severe outbreak of *Ichthyophthirius multifiliis* ("Ich") that occurred during the study period, and water turbidity problems. Although, mean weight of SHT was somewhat lower in both treated groups compared to the positive control, the mitigating factors described above could have influenced mean weight (Table 6). Clinical signs of whirling disease were observed in steelhead trout in treated groups and in the positive control group. Mean fish weight of chinook salmon was similar in treated groups and the positive control. Chinook salmon in this trial had less variation in size than fish in any other trial. Furthermore, no clinical signs of whirling disease were observed in SCS in either the positive control or treated groups (Table 7).

#### Chalk Cliffs State Fish Hatchery

Fumagillin treatment of Erwin rainbow trout exposed to a naturally occurring TAM population in Chalk Cliffs SFH source water (Chalk Creek), was not efficacious in the prevention or control of whirling disease. *Myxobolus cerebralis* infection was confirmed by PCR in the positive control 45 d pe. Mean spore count for the positive control group 240 d pe was 88,598, compared to 245,917 in the treated group (7.5 mg fumagillin/kg BW/d initiated at the time of exposure) (Table 8). Although the spore

**Table 6.**-Mean total mortality, mean fish weight, and mean incidence of clinical signs of whirling disease<sup>1</sup> in steelhead trout 180 d pe to TAMs at the Pahsimeroi SFH, Ellis, ID 1998 trial. (Note: fish were exposed in hatchery source water).

Treatment <sup>2</sup>	Mean percent total mortality	Mean fish weight (g)	Percent mean incidence of clinical signs of <i>Mc</i> <sup>3</sup>
Positive Control <i>N</i> <sup>7</sup> =2	---- <sup>5</sup>	15.1	30
3.75 mg Fumagillin/kg BW @ Day 14 <sup>4</sup> <i>N</i> =1	----	10.7 <sup>6</sup>	60 <sup>6</sup>
7.5 mg Fumagillin/kg BW @ Day 14 <sup>4</sup> <i>N</i> =3	----	12.0	33

<sup>1</sup> Primary clinical signs of whirling disease evaluated included whirling behavior, cranial deformities, opercular deformities, and skeletal deformities.

<sup>2</sup> All controls and treatments conducted in triplicate

<sup>3</sup> Determination based on evaluation of all fish remaining in each tank at study termination

<sup>4</sup> Fumagillin treatment initiated 14 d pe, fumagillin fed for 10 d

<sup>5</sup> Mortality data not reported as a result of unrelated diseases.

<sup>6</sup> Single replicate tank only

<sup>7</sup> *N* = number of replicate tanks per treatment sampled

count was nearly three times higher in the treated group than in the positive control, the

difference was not significant. Mean histology score 240 d pe was 3.6 for the positive

control, compared to 3.7 for the treated group (Table 9), indicating no treatment effect.

Mean total mortality was higher in the treated group (49.3%) than in the positive control

(25.3%), suggesting possible toxicity of the fumagillin treatment (Table 10). However,

**Table 7.**-Mean total mortality, mean fish weight, and mean incidence of clinical signs of whirling disease<sup>1</sup> in chinook salmon 180 d pe to TAMs at the Pahsimeroi SFH, Ellis, ID 1998 trial. (Note: fish were exposed in hatchery source water).

Treatment <sup>2</sup>	Mean percent total mortality	Mean fish size (g)	Percent mean incidence of clinical signs of <i>Mc</i> <sup>3</sup>
Positive Control <i>N</i> <sup>6</sup> =3	---- <sup>5</sup>	10.3	0
3.75 mg Fumagillin/kg BW @ Day 14 <sup>4</sup> <i>N</i> =3	----	10.9	0
7.5mg Fumagillin/kg BW @ Day 14 <sup>4</sup> <i>N</i> =3	----	10.1	0

<sup>1</sup> Primary clinical signs of whirling disease evaluated included whirling behavior, cranial deformities, opercular deformities, and skeletal deformities.

<sup>2</sup> All controls and treatments conducted in triplicate

<sup>3</sup> Determination based on evaluation of all fish remaining in each tank at study termination

<sup>4</sup> Fumagillin treatment initiated 14 d pe, fumagillin fed for 10 d

<sup>5</sup> Mortality data not reported as a result of unrelated diseases.

<sup>6</sup> *N* = number of replicate tanks per treatment sampled

an outbreak of coldwater disease occurred during the study period which may have affected the significance of mortality data. Clinical signs of whirling disease were observed in both the positive control and the treated group (Table 10). Mean fish weight was greater in the treated group (60.1 g) than in the positive control group (45.8 g) (Table 10). It is likely that the larger size of treated fish was an artifact of decreased rearing density that resulted from increased mortality in this group. The flow indexes and

**Table 8.-**Spore count data from rainbow trout half-heads 240 d pe to TAMs at the Chalk Cliffs SFH, Nathrop, CO 1998 trial. (Note: fish were exposed in hatchery source water).

Treatment <sup>1</sup>	Spore count <sup>2</sup> (mean value)	Standard deviation	95% C.I	Coefficient of variation
Positive Control <i>N</i> <sup>4</sup> =3	88,598	45,791	113,751	51.7%
7.5 mg Fumagillin/kg BW @ 0 d <sup>3</sup> <i>N</i> =3	245,917	30,414	75,553	12.4%

<sup>1</sup> All controls and treatments conducted in triplicate

<sup>2</sup> Mean of five fish sampled per replicate.

<sup>3</sup> Fumagillin treatment initiated at 0 d (day of exposure), fumagillin fed for 10 d

<sup>4</sup> *N* = number of replicate tanks per treatment sampled

**Table 9.-**Histology score data from rainbow trout half-heads 240 d pe to TAMs at the Chalk Cliffs SFH, Nathrop, CO 1998 trial. (Note: fish were exposed in hatchery source water).

Treatment <sup>1</sup>	Histology score <sup>2,3</sup> (mean value)	Standard deviation	95% C.I	Coefficient of variation
Positive Control <i>N</i> <sup>5</sup> =3	3.6	0.7	3.9	19.4%
7.5 mg Fumagillin/kg BW @ 0 d <sup>4</sup> <i>N</i> =3	3.7	1.2	6.9	32.4%

<sup>1</sup> All controls and treatments conducted in triplicate

<sup>2</sup> Mean from five fish sampled per replicate

<sup>3</sup> Histological score based on the MacConnell/Baldwin Scale

<sup>4</sup> Fumagillin treatment initiated at 0 d (day of exposure), fumagillin fed for 10 d

<sup>5</sup> *N* = number of replicates per treatment

density indexes for all treatment groups were within the suggested parameters of < 1.5 for flow index and < 0.5 for density index (Piper et al. 1992).

**Table 10.**-Mean total mortality, mean fish weight, and mean incidence of clinical signs of whirling disease<sup>1</sup> in rainbow trout 240 d pe to TAMs at the Chalk Cliffs SFH, Nathrop, CO 1998 trial. (Note: fish were exposed in hatchery source water).

Treatment <sup>2</sup>	Mean percent total mortality	Mean fish weight (g)	Percent mean incidence of clinical signs of <i>Mc</i> <sup>3</sup>
Positive Control N <sup>6</sup> =3	25.3 <sup>5</sup>	47.0	73
7.5 mg Fumagillin/kg BW @ 0 d <sup>4</sup> N=3	49.3 <sup>5</sup>	60.0	67

<sup>1</sup> Primary clinical signs of whirling disease evaluated included whirling behavior, cranial deformities, opercular deformities, and skeletal deformities.

<sup>2</sup> All controls and treatments conducted in triplicate

<sup>3</sup> Determination based on evaluation of all fish remaining in each tank at study termination

<sup>4</sup> Fumagillin treatment initiated at 0 d (day of exposure), fumagillin fed for 10 d

<sup>5</sup> Mortality data impacted by an outbreak of coldwater disease

<sup>6</sup> N = number of replicate tanks per treatment sampled

#### Eagle State Fish Hatchery

Fumagillin treatment of Kamloop strain rainbow trout exposed to naturally occurring TAMs in the South Fork of the Boise River for 10 d prior to transfer to the Eagle SFH, was not efficacious in the prevention or control of whirling disease.

*Myxobolus cerebralis* infection was confirmed by PCR in the positive control and the treated group 45 d pe. No significant difference was found between mean spore counts

for the positive control group 240 d pe (19,767) compared to spore counts for the treated group (46,448) fed 7.5 mg fumagillin/kg BW/day initiated 10 d pe (Table 11).

**Table 11.**-Spore count data from rainbow trout half-heads 240 d pe to TAMs at the Eagle SFH, Eagle, ID 1998 trial. (Note: fish were exposed in the South Fork of the Boise River and then transported to the Eagle SFH).

Treatment <sup>1</sup>	Spore count <sup>2</sup> (mean)	Standard deviation	95% C.I	Coefficient of variation
Positive Control N <sup>4</sup> =3	19,767	27,085	67,284	137.0%
7.5 mg Fumagillin/kg BW @ Day 7 <sup>3</sup> N=3	46,448	68,186	169,383	146.8%

<sup>1</sup> All controls and treatments conducted in triplicate

<sup>2</sup> Mean of five fish sampled per replicate

<sup>3</sup> Fumagillin treatment initiated 10 d pe, fumagillin fed for 14 d

<sup>4</sup> N = number of replicate tanks per treatment sampled

Mean histology score 240 d pe was 0.9 for the positive control, compared to 0.7 for the treated group (Table 12). Mean total mortality was higher in the treated group (16.3%) than in the positive control (12.1%), suggesting possible toxicity of the fumagillin treatment. Clinical signs of whirling disease were observed in both the positive control and the treated group. There was no difference in mean fish weight between the positive control (11.1 g) and the treated group (11.7 g) (Table 13).

**Table 12.**-Histology score data from rainbow trout half-heads 240 d pe to TAMs at the Eagle SFH, Eagle, ID 1998 trial. (Note: fish were exposed in the South Fork of the Boise River and then transported to the Eagle SFH).

Treatment <sup>1</sup>	Histology score <sup>2,3</sup> (mean value)	Standard deviation	95% C.I	Coefficient of variation
Positive Control <i>N</i> <sup>5</sup> =3	0.7	0.1	0.7	14.3%
7.5 mg Fumagillin/kg BW @ 7 d <sup>4</sup> <i>N</i> =3	0.9	0.6	3.5	66.7%

<sup>1</sup> All controls and treatments conducted in triplicate

<sup>2</sup> Mean of five fish sampled per replicate

<sup>3</sup> Histological Score based on the MacConnell/Baldwin Scale

<sup>4</sup> Fumagillin treatment initiated 10 d pe, fumagillin fed for 14 d

<sup>5</sup> *N* = number of replicate tanks per treatment sampled

#### Montana (Willow Creek)

Fumagillin treatment administered prophylactically and/or pe to Erwin rainbow trout exposed to naturally occurring TAMs in Willow Creek, MT for a period of 3 or 7 d prior to transfer to the WTRL was not efficacious in the prevention or control of whirling disease. *Myxobolus cerebralis* infection was confirmed by PCR in the 7.5 mg/kg BW/d treatment group 45 d pe. Mean spore counts for the 3 and 7 d pe positive control groups 240 d pe were 145,389 and 344,656, respectively, compared to a range of 43,500-152,495 for treated groups (Table 14). Although spore counts were lower for treated groups except for the 7.5 mg/kg BW/day administered at 7d pe, this difference was not significant. Mean histology score 240 d pe was 4.0 for the positive controls as compared

**Table 13.**-Mean total mortality, mean fish weight, and mean incidence of clinical signs of whirling disease<sup>1</sup> in rainbow trout 240 d pe to TAMs at the Eagle SFH, Eagle, ID 1998 trial. (Note: fish were exposed in the Boise River and then transported to the Eagle SFH).

Treatment <sup>2</sup>	Mean percent total mortality	Mean fish weight (g)	Percent mean incidence of clinical signs of <i>Mc</i> <sup>3</sup>
Positive Control <i>N</i> <sup>5</sup> =3	12.1	11.7	33
7.5 mg Fumagillin/kg BW @ 7 d <sup>4</sup> <i>N</i> =3	16.3	11.1	20

<sup>1</sup> Primary clinical signs of whirling disease evaluated included whirling behavior, cranial deformities, opercular deformities, and skeletal deformities.

<sup>2</sup> All controls and treatments were in triplicate

<sup>3</sup> Determination based on evaluation of all fish remaining in each tank at study termination

<sup>4</sup> Fumagillin treatment initiated 10 d pe, fumagillin fed for 14 d

<sup>5</sup> *N* = number of replicate tanks per treatment sampled

to a range of 3.2 – 4.0 for the treated groups (Table 15). Spore count and histological score data also indicated no difference between time of treatment relative to time of exposure (5 d prophylactically, 3 or 7 d pe) with respect to treatment efficacy. Mean total mortality was higher in both positive controls (33.3% and 35.8%) and all treated groups (range 40.0 - 54.2%) than in the negative control (6.7%), indicating *Mc* infection was probably responsible for at least a portion of increased mortality. Slightly elevated mortality in treated groups as compared to the positive controls suggested a possible toxic effect of fumagillin treatment. Clinical signs of whirling disease were observed in both the positive controls and all treated groups (Table 16).

**Table 14.**-Spore count data from rainbow trout half-heads 240 d pe to TAMs in Montana 1998 trial. (Note: fish were exposed in Willow Creek at Harrison, MT and then transported to the WTRL, Bozeman, MT).

Treatment <sup>1</sup>	Spore count <sup>2</sup> (mean)	Standard deviation	95% C.I	Coefficient of variation
Negative Control <i>N</i> <sup>9</sup> =2	0	0	0	----
Positive Control (3 d) <sup>3</sup> <i>N</i> =2	145,389	55,673	500,205	38.3%
Positive Control (7 d) <sup>4</sup> <i>N</i> =2	344,656	435,170	3,909,845	126.3%
7.5 mg Fumagillin/kg BW Prophylactic and @ 3 d <sup>5</sup> <i>N</i> =2	100,756	31,852	286,176	31.6%
7.5 mg Fumagillin/kg BW Prophylactic and @ 7 d <sup>6</sup> <i>N</i> =2	78,795	84,907	762,862	107.8%
7.5 mg Fumagillin/kg BW @ 3 d <sup>7</sup> <i>N</i> =2	43,500	10,370	93,175	23.8%
7.5 mg Fumagillin/kg BW @ 7 d <sup>8</sup> <i>N</i> =2	152,495	111,134	998,498	72.9%

<sup>1</sup> All controls and treatments groups conducted in duplicate

<sup>2</sup> Mean of five fish sampled per replicate

<sup>3</sup> Fish exposed to TAMs in Willow Creek for 3 d, no treatment

<sup>4</sup> Fish exposed to TAMs in Willow Creek for 7 d, no treatment

<sup>5</sup> Fish fed fumagillin prophylactically for 5 d; fumagillin treatment initiated again 3 d pe for 10 d

<sup>6</sup> Fish fed fumagillin prophylactically for 5 d; fumagillin treatment initiated again 7 d pe for 10 d

<sup>7</sup> Fumagillin treatment initiated 3 d pe, fumagillin fed for 10 d

<sup>8</sup> Fumagillin treatment initiated 7 d pe, fumagillin fed for 10 d

<sup>9</sup> *N* = number of replicate tanks per treatment sampled

**Table 15.**-Histology scores from rainbow trout half-heads 240 d pe to TAMs in Montana 1998 trial. (Note: fish were exposed in Willow Creek at Harrison, MT and then transported to the WTRL, Bozeman, MT).

Treatment <sup>1</sup>	Histology score <sup>2,3</sup> (mean)	Standard deviation	95% C.I	Coefficient of variation
Negative Control <i>N</i> <sup>10</sup> =2	0.0	0.0	0.0	----
Positive Control (3 d) <sup>4</sup> <i>N</i> =2	3.8	0.4	15.9	10.5%
Positive Control (7 d) <sup>5</sup> <i>N</i> =2	4.2	0.3	12.7	7.1%
7.5 mg Fumagillin/kg BW Prophylactic and @ 3 d <sup>6</sup> <i>N</i> =2	3.6	0.6	25.5	16.7%
7.5 mg Fumagillin/kg BW Prophylactic and @ 7 d <sup>7</sup> <i>N</i> =1	3.2	1.7	76.4	53.1
7.5 mg Fumagillin/kg BW @ 3 d <sup>8</sup> <i>N</i> =2	3.9	0.1	6.4	2.7%
7.5 mg Fumagillin/kg BW @ 7 d <sup>9</sup> <i>N</i> =2	4.0	0.5	23.2	12.5%

<sup>1</sup> All controls and treatments conducted in duplicate

<sup>2</sup> Mean of five fish sampled per replicate

<sup>3</sup> Histological Score based on the MacConnell/Baldwin Scale

<sup>4</sup> Fish exposed to TAMs in Willow Creek for 3 d, no treatment

<sup>5</sup> Fish exposed to TAMs in Willow Creek for 7 d, no treatment

<sup>6</sup> Fish fed fumagillin prophylactically for 5 d; fumagillin treatment initiated again 3 d pe for 10 d

<sup>7</sup> Fish fed fumagillin prophylactically for 5 d; fumagillin treatment initiated again 7 d pe for 10 d

<sup>8</sup> Fumagillin treatment initiated 3 d pe, fumagillin fed for 10 d

<sup>9</sup> Fumagillin treatment initiated 7 d pe, fumagillin fed for 10 d

<sup>10</sup> *N* = number of replicate tanks per treatment sampled

**Table 16.**-Mean total mortality, mean fish weight, and mean incidence of clinical signs of whirling disease<sup>1</sup> in rainbow trout 240 d pe to TAMs in Montana 1998 trial. (Note: fish were exposed in Willow Creek at Harrison, MT and then transported to the WTRL, Bozeman, MT).

Treatment <sup>2</sup>	Mean percent total mortality	Mean fish weight (g)	Percent mean incidence of clinical signs of <i>Mc</i> <sup>3</sup>
Negative Control <i>N</i> <sup>10</sup> =2	6.7	8.6	0
Positive Control (3 d) <sup>4</sup> <i>N</i> =2	33.3	16.9	60
Positive Control (7 d) <sup>5</sup> <i>N</i> =2	35.8	13.3	90
7.5 mg Fumagillin/kg BW Prophylactic and @ 3 d <sup>6</sup> <i>N</i> =2	41.6	14.9	80
7.5 mg Fumagillin/kg BW Prophylactic and @ 7 d <sup>7</sup> <i>N</i> =2	54.2	21.7	100
7.5 mg Fumagillin/kg BW @ 3 d <sup>8</sup> <i>N</i> =2	40.0	18.8	40
7.5 mg Fumagillin/kg BW @ 7 d <sup>9</sup> <i>N</i> =2	40.8	16.8	90

<sup>1</sup> Primary clinical signs of whirling disease evaluated included whirling behavior, cranial deformities, opercular deformities, and skeletal deformities.

<sup>2</sup> All controls and treatments conducted in duplicate

<sup>3</sup> Determination based on evaluation of all fish remaining in each tank at study termination

<sup>4</sup> Fish exposed to TAMs in Willow Creek for 3 d, no treatment

<sup>5</sup> Fish exposed to TAMs in Willow Creek for 7 d, no treatment

<sup>6</sup> Fish fed fumagillin prophylactically for 5 d; fumagillin treatment initiated again 3 d pe for 10 d

<sup>7</sup> Fish fed fumagillin prophylactically for 5 d; fumagillin treatment initiated again 7 d pe for 10 d

<sup>8</sup> Fumagillin treatment initiated 3 d pe, fumagillin fed for 10 d

<sup>9</sup> Fumagillin treatment initiated 7 d pe, fumagillin fed for 10 d

<sup>10</sup> *N* = number of replicate tanks per treatment sampled

Wild Trout Research Laboratory 1998

Fumagillin treatment of Erwin rainbow trout exposed to a known concentration of TAMs (1900/fish for 2 h) was not efficacious in the prevention or control of whirling disease. *Myxobolus cerebralis* infection was confirmed by PCR in 3.5 mg/kg BW/d treatment group 45 d pe. Mean spore count for the positive control group 240 d pe was 603,111, compared to mean spore counts for the treated groups that ranged from 122,337 – 294,940 (Table 17). Although spore counts were lower for treated groups, differences were not significant due to the large variation among replicates. Mean histology score 240 d pe was 3.2 for the positive control, compared to 3.4 for the treated groups (Table 18). Spore counts and histological scores did not indicate a difference between either treatment dose (3.75 or 7.5 mg fumagillin/kg BW/d) or time of treatment relative to time of exposure (0 or 14 d pe). Mean total mortality was higher in the positive control (11.7%) and all treated groups (range 15.0 - 27.2%) than in the negative control (3.8%), indicating *Mc* infection was responsible for at least a portion of increased mortality (Table 19). Elevated mortality in treated groups as compared to the positive control suggested a possible toxic effect of fumagillin treatment. Clinical signs of whirling disease were observed in the positive control and all treated groups (Table 19).

Wild Trout Research Laboratory 2000

Fumagillin and TNP-470 treatments of Arlee strain rainbow trout exposed to a known concentration of TAMs (1000/fish for 2 h) were not efficacious in the prevention or control of whirling disease. *Myxobolus cerebralis* infection was confirmed in positive control and both fumagillin and TNP-470 treated groups by spore counts and histological

**Table 17.**-Spore count data from rainbow trout half-heads 240 d pe to TAMs at the Wild Trout Research Lab, Bozeman, MT 1998 trial. (Note: fish were exposed to 1,900 TAMs per fish for 2 h).

Treatment <sup>1</sup>	Spore count <sup>2</sup> (mean)	Standard deviation	95% C.I	Coefficient of variation
Negative Control <i>N</i> <sup>5</sup> =3	556	962	2390	173.0%
Positive Control <i>N</i> =2	603,111	561,930	5,048,738	93.2%
3.75 mg Fumagillin/kg BW @ 0 d <sup>3</sup> <i>N</i> =3	294,940	176,191	437,684	59.7%
3.75 mg Fumagillin/kg BW @ 14 d <sup>4</sup> <i>N</i> =3	122,337	81,651	202833	66.7%
7.5 mg Fumagillin/kg BW @ 0 d <sup>3</sup> <i>N</i> =3	205,333	91,681	227,749	44.6%
7.5 mg Fumagillin/kg BW @ 14 d <sup>4</sup> <i>N</i> =3	185,641	89,055	221,224	48.0%

<sup>1</sup> All controls and treatments groups conducted in triplicate

<sup>2</sup> Mean of five fish sampled per replicate

<sup>3</sup> Fumagillin treatment initiated at 0 d (day of exposure), fumagillin fed for 10 d

<sup>4</sup> Fumagillin treatment initiated 14 d pe, fumagillin fed for 10 d

<sup>5</sup> *N* = number of replicate tanks per treatment sampled

evaluation. Mean spore count for the positive control group 150 d pe was 29,516, compared to mean spore counts in the treatment groups that ranged from 8,066 to 13,533 (Table 20). Although spore counts were lower for the treated groups, the differences were not significant. Mean histology score 150 d pe was 3.4 for the positive control, and

**Table 18.**-Histology score data from rainbow trout half-heads 240 d pe to TAMs at the Wild Trout Research Lab, Bozeman, MT 1998 trial. (Note: fish were exposed to 1,900 TAMs per fish for 2 h).

Treatment <sup>1</sup>	Histology score <sup>2,3</sup> (mean)	Standard deviation	95% C.I	Coefficient of variation
Negative Control N <sup>6</sup> =3	0.0	0.0	0.0	----
Positive Control N=2	3.2	0.0	0.0	0.0%
3.75 mg Fumagillin/kg BW @ 0 d <sup>4</sup> N=3	3.1	0.7	4.0	22.6%
3.75 mg Fumagillin/kg BW @ 14 d <sup>5</sup> N=3	3.3	0.4	2.2	12.1%
7.5 mg Fumagillin/kg BW @ 0 d <sup>4</sup> N=3	3.9	0.2	1.4	5.0%
7.5 mg Fumagillin/kg BW @ 14 d <sup>5</sup> N=3	3.2	0.5	3.0	15.6%

<sup>1</sup> All controls and treatments groups conducted in triplicate

<sup>2</sup> Mean of five fish sampled per replicate

<sup>3</sup> Histological Score based on the MacConnell/Baldwin Scale

<sup>4</sup> Fumagillin treatment initiated at 0 d (day of exposure), fumagillin fed for 10 d

<sup>5</sup> Fumagillin treatment initiated 14 d pe, fumagillin fed for 10 d

<sup>6</sup> N = number of replicate tanks per treatment sampled

**Table 19.-** Mean total mortality, mean fish weight, and mean incidence of clinical signs of whirling disease<sup>1</sup> in rainbow trout 240 d pe to TAMs at the Wild Trout Research Laboratory, Bozeman, MT 1998 trial. (Note: fish were exposed to 1,900 TAMs per fish for 2 h).

Treatment <sup>2</sup>	Mean percent total mortality	Mean fish weight (g)	Percent mean incidence of clinical signs of <i>Mc</i> <sup>3</sup>
Negative Control <i>N</i> <sup>6</sup> =3	3.8	21.9	0
Positive Control <i>N</i> =2	11.7	18.8	10
3.75 mg Fumagillin/kg BW @ 0 d <sup>4</sup> <i>N</i> =3	27.2	21.7	33
3.75 mg Fumagillin/kg BW @ 14 d <sup>5</sup> <i>N</i> =3	15.0	29.1	27
7.5 mg Fumagillin/kg BW @ 0 d <i>N</i> =3	26.1	18.4	27
7.5 mg Fumagillin/kg BW @ 14 d <i>N</i> =3	25.0	24.2	27

<sup>1</sup> Primary clinical signs of whirling disease evaluated included whirling behavior, cranial deformities, opercular deformities, exophthalmia, and skeletal deformities.

<sup>2</sup> All controls and treatments conducted in triplicate

<sup>3</sup> Determination based on evaluation of all fish remaining in each tank at study termination

<sup>4</sup> Fumagillin treatment initiated at 0 d (day of exposure), fumagillin fed for 10 d

<sup>5</sup> Fumagillin treatment initiated 14 d pe, fumagillin fed for 10 d

<sup>6</sup> *N* = number of replicate tanks per treatment sampled

ranged from 3.0 to 3.4 for the fumagillin and TNP-470 treated groups (Table 21).

Histological evaluation identified a moderate level of *Mc* infection in both positive

**Table 20.**-Spore count data from rainbow trout half-heads 150 d pe to TAMs at the Wild Trout Research Laboratory, Bozeman, MT 2000 trial. (Note: fish were exposed to 1,000 TAMs per fish for 2 h).

Treatment <sup>1</sup>	Spore count <sup>2</sup> (mean)	Standard deviation	95% C.I	Coefficient of variation
Negative Control <i>N</i> <sup>5</sup> =5	0.0	0.0	0.0	----
Positive Control <i>N</i> =5	29516	15922	19770	54
7.5 mg Sanofi Incorporated/kg BW/d <sup>3</sup> <i>N</i> =5	8467	3512	4361	41
7.5 mg Sanofi Top- coat/kg BW/d <sup>3</sup> <i>N</i> =5	13533	4179	5189	31
7.5 mg Chinoin Incorporated/kg BW/d <sup>3</sup> <i>N</i> =5	11000	2163	2686	20
7.5 mg TNP-470 Incorporated/kg BW/d <sup>3</sup> <i>N</i> =5	10622	5510	6842	52
7.5 mg TNP-470 Incorporated/kg BW/d <sup>4</sup> <i>N</i> =5	8066	5500	6829	68

<sup>1</sup> All controls and treatments conducted in quintuplet

<sup>2</sup> Mean of 10 fish sampled per replicate

<sup>3</sup> Treatment initiated at 1 d (day after exposure) and fed for 10 d

<sup>4</sup> Treatment initiated at 1 d (day after exposure) and fed for 26 d

<sup>5</sup> *N* = number of replicate tanks per treatment sampled

control and treated groups. Differences between histological scores were not significant ( $p > 0.05$ ). TNP-470 10 d treatment had very comparable results with TNP-470 26 d treatment with respect to spore counts and histology scores. Clinical signs of whirling

disease were observed in all treated (6 to 22 %) and positive control (32%) groups (Table 22).

**Table 21.**-Histology score data from rainbow trout half-heads 150 d pe to TAMs at the Wild Trout Research Lab, Bozeman, MT 2000 trial. (Note: fish were exposed to 1,000 TAMs per fish for 2 h).

Treatment <sup>1</sup>	Histology score <sup>2,3</sup> (mean)	Standard deviation	95% C.I	Coefficient of variation
Negative Control <i>N</i> <sup>6</sup> =5	0.0	0.0	0.0	----
Positive Control <i>N</i> =5	3.4	0.4	0.7	11.8%
7.5 mg Sanofi Incorporated/kg BW/d <sup>3</sup> <i>N</i> =5	3.1	0.7	1.4	22.6%
7.5 mg Sanofi Top- coat/kg BW/d <sup>3</sup> <i>N</i> =5	3.4	0.2	0.5	5.9%
7.5 mg Chinoin Incorporated/kg BW/d <sup>3</sup> <i>N</i> =5	3.2	0.2	0.5	6.3%
7.5 mg TNP-470 Incorporated/kg BW/d <sup>3</sup> <i>N</i> =5	3.3	0.5	0.9	15.2%
7.5 mg TNP-470 Incorporated/kg BW/d <sup>4</sup> <i>N</i> =5	3.0	0.4	0.8	13.3%

<sup>1</sup> All controls and treatments conducted in quintuplet

<sup>2</sup> Mean of 10 fish sampled per replicate

<sup>3</sup> Histological Score based on the MacConnell/Baldwin Scale

<sup>4</sup> Treatment initiated at 1 d (day after exposure) and fed for 10 d

<sup>5</sup> Treatment initiated at 1 d (day after exposure) and fed for 26 d

<sup>6</sup> *N* = number of replicate tanks per treatment sampled

**Table 22.**-Mean total mortality, mean fish weight, and mean incidence of clinical signs of whirling disease<sup>1</sup> in rainbow trout 150 d pe to TAMs at the Wild Trout Research Lab, Bozeman, MT 2000 trial. (Note: fish were exposed to 1,000 TAMs per fish for 2 h).

Treatment <sup>2</sup>	Mean percent total mortality	Mean fish weight (g)	Percent mean incidence of clinical signs of <i>Mc</i> <sup>3</sup>
Negative Control <i>N</i> <sup>6</sup> =5	1.58	23.7	0
Positive Control <i>N</i> =5	1.58	21.4	32
7.5 mg Sanofi Incorporated/kg BW/d <sup>3</sup> <i>N</i> =5	3.68	21.3	22
7.5 mg Sanofi Top-Coat/kg BW/d <sup>3</sup> <i>N</i> =5	2.63	19.7	12
7.5 mg Chinoin Incorporated/kg BW/d <sup>3</sup> <i>N</i> =5	3.16	21.6	8
7.5 mg TNP-470 Incorporated/kg BW/d <sup>3</sup> <i>N</i> =5	0.00	21.7	6
7.5 mg TNP-470 Incorporated/kg BW/d <sup>4</sup> <i>N</i> =5	11.05	22.6	12

<sup>1</sup> Primary clinical signs of whirling disease evaluated included whirling behavior, cranial deformities, opercular deformities, exophthalmia, and skeletal deformities.

<sup>2</sup> All controls and treatments conducted in quintuplet

<sup>3</sup> Determination based on evaluation of all fish remaining in each tank at study termination

<sup>4</sup> Treatment initiated at 1 d (day after exposure) and fed for 10 d

<sup>5</sup> Treatment initiated at 1 d (day after exposure) and fed for 26 d

<sup>6</sup> *N* = number of replicate tanks per treatment sampled

Mean mortality was significantly higher at 11% in the TNP-470 26 d pe treatment group, compared to a range of 0.00 to 3.68% in all other treatment and control groups (Table 22). However, there were no other significant differences in mortality rate

between the treated and the control groups. Elevated mortality rate in the TNP-470 26 d pe treatment group indicated a toxic effect of this treatment regimen. Lethargy and a suppression of appetite were observed in all five replicates of this treatment group 30 d pe. Hematocrit readings at 30 d pe were significantly lower in TNP-470 26 d treated fish (24.4) than in the negative controls (41.3). By 61 d pe however, hematocrit readings for the treated fish were comparable to the controls (Table 23). Blood smears at 30 d pe revealed no blast cells or immature erythrocytes in the TNP-470 26 d treatment group compared to mean numbers of 14.5 and 34.0, respectively, in the negative control. An increase in erythrocyte smudge cells and degenerate cells was also observed in the TNP-470 26 d treatment group at 30 d pe. At 61 d pe however, all erythrocytic cell types that were suppressed at 30 d pe had begun to proliferate. At 150 d pe all erythrocyte counts were comparable to the negative control. Mean lymphocyte number in the TNP-470 26 d group 30 d pe was 0.5 compared to 36.0 in the negative control group. Although the mean lymphocyte number gradually began to increase following completion of treatment, it had not recovered fully at 150 d pe compared to the negative controls (Table 24).

Histological evaluation 30 d pe revealed abnormal cytology in the kidney and the thymus of the TNP-470 26 d treated fish. Compared to normal hematopoietic kidney tissue which is predominately basophilic in composition, the hematopoietic tissue in TNP-470 26 d treated fish was very eosinophilic. Although kidney tubules remained intact, hematopoietic tissue in the kidney of TNP-470 26 d treated fish was replaced with connective tissue (Figure 5 a,b). The basophilic thymocytes in the thymus of these fish

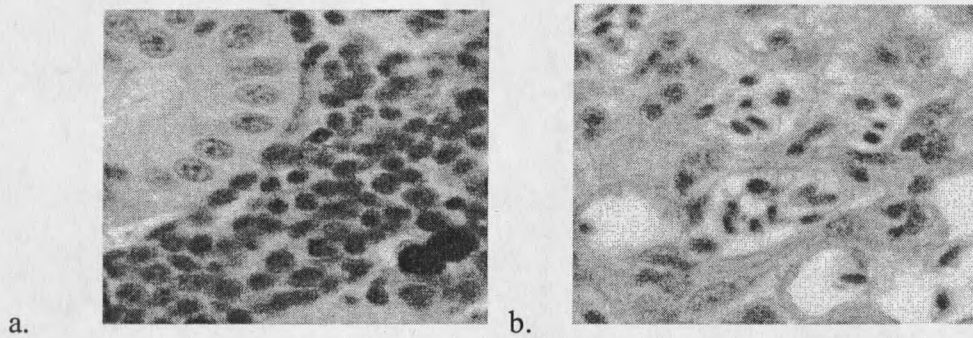
**Table 23.**-Hematocrit values for negative control and TNP-470 26 d treatment at 30 and 61d pe in the Wild Trout Research Laboratory, Bozeman, MT 2000 trial.

Treatment	30 d pe				61 d pe			
	Hematocrit (mean value)	Standard Deviation	95% C.I.	Coefficient of Variation	Hematocrit (mean value)	Standard Deviation	95% C.I.	Coefficient of Variation
Negative Control	41.3	2.1	3.3	5.1	44	2.8	25.4	6.4
TNP-470 Incorporated 26 day feed	24.4	4.7	3.6	19.3	41.2	1.7	3.9	4.1

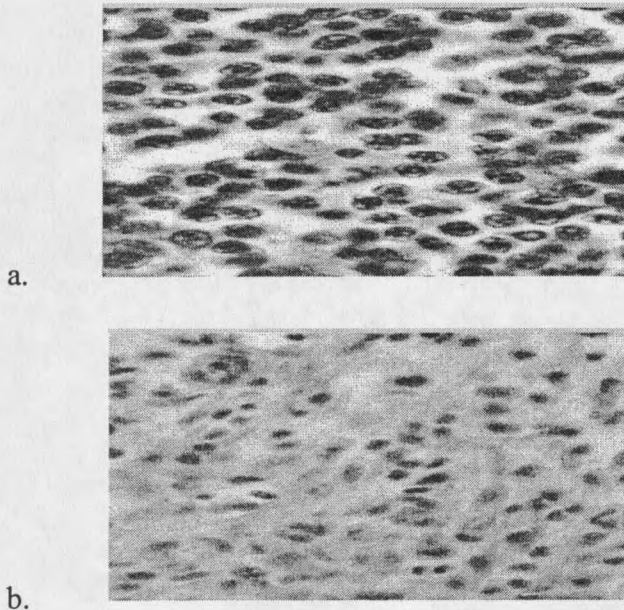
**Table 24.**-Mean number of blood cell types in negative controls and TNP-470 26 d treatment blood smears collected 30, 61 and 150 d pe to TAMs in the Wild Trout Research Laboratory, Bozeman, MT 2000 trial (Note: Fish were exposed to 1,000 TAMs per fish for 2 h).

Cell Type	30 d pe				61 d pe				150 d pe			
	Total cells (mean)	Standard deviation	95 % C. I.	Coefficient of variation	Total cells (mean)	Standard deviation	95 % C. I.	Coefficient of variation	Total cells (mean)	Standard deviation	95 % C. I.	Coefficient of variation
<b>ERYTHROCYTES</b>												
<b>Blast Cells</b>												
Negative Control	14.5	5.0	44.5	34.5	13.0	4.2	38.1	32.3	17.5	14.8	133.4	84.6
TNP-470 26 day treatment	0.0	0.0	0.0	--	25.5	27.2	19.5	106.7	17.7	14.3	10.2	80.8
<b>Immature</b>												
Negative Control	34.0	9.9	89.0	29.1	61.5	3.5	31.8	5.7	35.5	6.4	57.2	18.0
TNP-470 26 day treatment	0.0	0.0	0.0	--	87.2	75.2	53.8	86.2	34.4	34.9	25.0	101.5
<b>Mature</b>												
Negative Control	415.0	18.4	165.2	4.4	394.5	16.3	146.1	4.1	431.5	9.2	82.6	2.1
TNP-470 26 day treatment	446.0	32.9	27.5	7.4	321.1	86.7	62.0	27.0	419.6	75.5	54.0	18.0
<b>Degenerative</b>												
Negative Control	6.0	4.2	38.1	70	8.0	11.3	101.7	141.2	12.5	12.0	108.0	96.0
TNP-470 26 day treatment	15.6	10.9	9.1	69.9	18.6	23.6	16.9	126.9	19.4	30.8	22.1	158.8
<b>Smudge</b>												
Negative Control	30.5	0.7	6.4	2.3	23.0	12.7	114.4	55.2	3.0	0.0	0.0	0.0
TNP-470 26 day treatment	38.4	27.6	23.1	71.9	47.6	34.0	24.3	71.4	8.9	8.7	6.2	97.8
<b>LYMPHOCYTES</b>												
Negative Control	36.0	11.3	101.7	31.4	31.5	27.6	247.8	87.6	25.5	1.4	12.7	23.3
TNP-470 26 day treatment	0.5	0.76	0.6	152	11.3	11.4	8.2	100.9	15.0	12.5	9.0	83.3

were destroyed, leaving only eosinophilic connective tissue and cellular support cells (Figures 6 a,b).

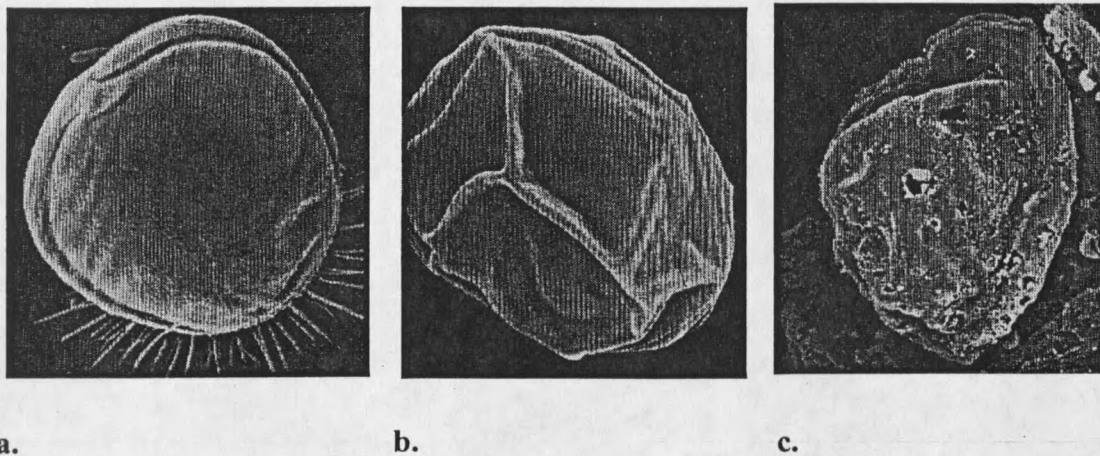


**Figure 5.**-Hemotoxylin and eosin stained kidney tissue. a. Normal kidney tissue appearing basophilic in color. b. TNP-470 treated kidney tissue which appears eosinophilic in color with blood sinuses and connective tissue visible.



**Figure 6.**-Hemotoxylin and eosin stained thymus tissue. a. Normal thymus tissue appearing basophilic in color. b. TNP-470 treated thymus tissue which appears eosinophilic in color with the thymocytes replaced with connective tissue and support cells.

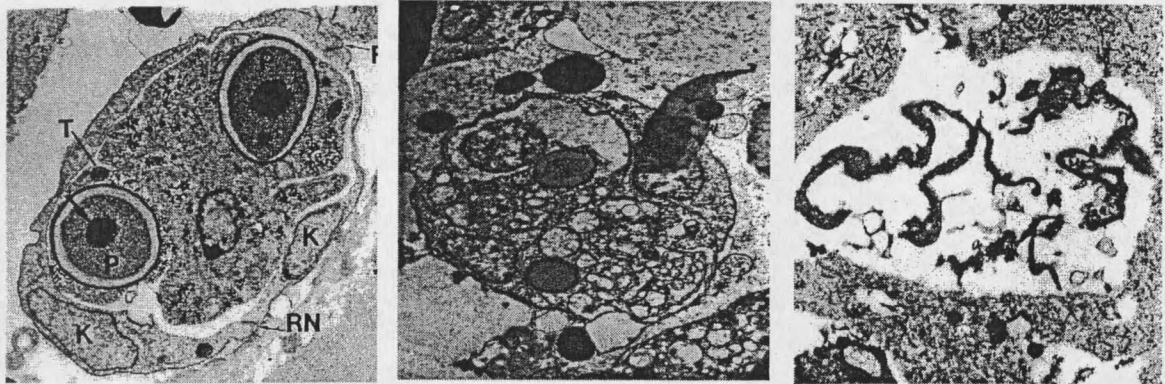
Scanning and transmission electron micrographs from positive controls (Figure 7 a), fumagillin treated groups, and TNP-470 treated groups revealed a treatment effect with respect to spore ultrastructure. Scanning micrographs revealed that TNP-470 treatment resulted in a disruptive effect on the valvogenic cells, causing a collapse of the spore shell (Figure 7 b). The scanning micrographs also showed that both TNP-470 treatments regimens of 10 or 26 d resulted in perforation of the shell valve, and over time, total destruction of the spore ultrastructure (Figure 7 c).



**Figure 7.-** Scanning electron micrographs of *Myxobolus cerebralis* spores. a. A positive control spore with smooth outer spore valve shell. b. Spore from TNP-470 treatment showing deflation of the spore valve shell ultrastructure. c. Spore from TNP-470 treatment showing perforations of the spore valve shell. Pictures compliments of Mansour El-Matbouli.

Transmission micrographs revealed that compared to the control, fumagillin and TNP-470 treatment both affected sporogenesis, but that treatment effect was different for each compound (Figure 8 a). Fumagillin treatment resulted in the vacuolization of

intracellular spore constituents and deformation of polar capsules, without affecting the spore valve shells (Figure 8 b). TNP-470 treatment appeared to disrupt the formation of the spore valve shells, and perhaps the formation of the polar capsules (none were observed in the micrographs analyzed) (Figure 8 c).



a.

b.

c.

**Figure 8.-** Transmission electron micrographs of *Myxobolus cerebralis* spores. a. A spore from a positive control: (P) polar capsules, (K) nuclei, (RN) spore shell junction and (T) electron dense body. b. A spore from fumagillin treatment showing numerous vacuoles and polar capsule deformity. c. A spore from TNP-470 treatment showing necrosis of the spore valve shell and only remnants of intracellular components. Pictures compliments of Mansour El-Matbouli.

## DISCUSSION

El-Matbouli and Hoffman (1991) reported that fumagillin fed orally was able to control whirling disease by decreasing overall spore numbers and clinical signs. Based on their findings, I expected fumagillin and TNP-470 treatment to result in at least some degree of efficacy with respect to level of *Mc* infection. Hedrick et al. (1988), Wishkovsky, et al. (1990), Higgins and Kent (1996), and le Gouvello et al. (1998) have reported some success in the use of fumagillin, and Higgins and Kent (1998) in the use of TNP-470, to control proliferative kidney disease (PKD) in various salmonid species. Since both PKD and *Mc* are caused by myxosporean parasites, I hypothesized that fumagillin would also be efficacious in the control of *Mc*. However, my data do not support this hypothesis.

There were several differences between my study and El-Matbouli and Hoffman's (1991) study, including fumagillin source and dosage, spore extraction and counting procedure, rainbow trout strains, exposure levels, age of fish and time of treatment. Their spore counts and infection rates in German strain rainbow trout were based solely on a head blot to a slide (i.e. no spores were extracted). Also, their treatment dosage was 2.2 mg/kg BW/d incorporated Chinoin fumagillin compared to 3.75 and 7.5 mg/kg BW/d Sanofi and/or Chinoin fumagillin in my trials. They administered medicated feed at 14 or 30 d pe and fish were fed for 50 or 130 d with no toxic side effects. Their reports of no side effects resulting from treatment may be attributed to fish age, fish strain, and the lower dosage of drug used in their study. Of particular note, the German strain of rainbow trout used in their study is reported to be more resistant to *Mc* infection than rainbow

trout strains in the United States (Mansour El-Matbouli, personal communication). If the German strain of rainbow trout used by El-Matbouli and Hoffman (1991) was more resistant to *Mc* infection, the synergistic effects of natural resistance and fumagillin treatment may have combined to result in decreased infection level.

Discussions with Mansour El-Matbouli prior to the initiation of my trials led to the conclusion that treating *Mc* exposed fish earlier than 14 d pe would be more efficacious than treating fish after 14 d or longer pe. This conclusion was based on the hypothesis that if fumagillin was present in fish at (or soon after) the time of exposure, fish would be better able to fend off initial infection. However, based on the non-efficacious results of the 6 trials conducted, this conclusion may have been in error. Upon penetration of the epidermis, *Mc* rapidly migrates into nervous tissue, whereby it gradually migrates to cartilaginous tissue.

The migration of *Mc* to cartilaginous tissue requires approximately 21 d at 15°C (Hedrick et al. 1999). During this period of time in nervous tissue, *Mc* may have been inaccessible to the effects of fumagillin and TNP-470 treatment. A more logical approach may have been to treat fish later as *Mc* was leaving nervous tissue and entering cartilaginous tissue.

Although I have no clear explanation for the observed lack of efficacy of fumagillin or TNP-470 treatment to control or prevent whirling disease, I originally hypothesized that exposing fish to relatively high levels of *Mc* may have "overwhelmed" the ability of fumagillin to control infection level. However, this was not supported by the lack of fumagillin treatment efficacy observed in the Eagle SFH trial where

histological evaluation confirmed a relatively low infection level (0.9, MacConnell/Baldwin Scale). An alternate hypothesis was that a delay in time of treatment relative to time of exposure may have contributed to a lack of observed efficacy (e.g. Pahsimeroi trial treatment at 14 d pe). However, this was not supported by the results of the Willow Creek trial that involved prophylactic treatment, or the Chalk Cliffs, WTRL 1998, and the WTRL 2000 trials where fish were treated at the time of exposure (0 d).

I also hypothesized that fumagillin formulation and/or method of incorporation of fumagillin into feed may have influenced treatment effectiveness. The first five trials all involved top-coated Sanofi fumagillin. Hence, in the WTRL 2000 study I evaluated Sanofi fumagillin top-coated verses incorporated, Chinion fumagillin incorporated feed (the formulation and delivery method reported to be efficacious by El-Matbouli and Hoffman (1991), as well as, TNP-470 incorporated. However, no formulation or delivery method tested proved to be efficacious. Elevated mean total mortality in treated groups in five of the six trials suggested possible therapeutant toxicity. Although previous studies have shown that high dosages of fumagillin (0.5 - 1.0 g/kg feed) fed for an extended period (4-8 weeks) can result in pathological changes in kidney and spleen tissue (Wishkovsky et al. 1990; Hedrick et al. 1988), I did not anticipate that treatment doses and duration used in my trials (3.75 and 7.5 mg/kg BW/d of fumagillin for 10 day) would result in adverse affects because of the relatively low dosages and short treatment duration. Although no side effects were observed in fish fed TNP-470 fed at 0.75 mg/kg BW/d for 10 d, fish fed TNP-470 for 26 d were lethargic, exhibited increased mortality

and decreased appetite 30 d pe. Also, at this time hematocrits were almost 50% below normal (based on Goede 1990), blast cells and immature erythrocytes were not observed, and lymphocytes were almost non-detectable. A diagnosis of hypoplastic anemia was concluded and further supported by histological analysis. Histology showed a destruction of thymocytes in the thymus and hematopoietic cells in the kidney. At 61 d pe, histology of the TNP-470 treated for 26 d fish revealed regeneration and recovery of the thymocytes in the thymus and the hematopoietic cellular constituents in the kidney. The fish appeared to be completely recovered from the toxicity by 150 d pe.

A number of recent studies have reported that the risk or severity of *Mc* infection may be related to stream water temperature (El-Matbouli et al. 1999; Hedrick et al. 1999). These studies report that optimal TAM production occurs at approximately 10-14°C. At temperatures above or below this range, the potential for infection decreases. High levels of infection were observed in my first two trials (Pahsimeroi SFH trial and WTRL 1998 trial), which were conducted at water temperatures ranging from 12 to >15°C. However, purposefully delaying the Willow Creek trial until early October when mean water temperature was 8°C (in an effort to moderate *Mc* exposure level) resulted in the highest mean infection (3.8; McConnell/Baldwin Scale) of all trials conducted. These data strongly indicate that water temperature alone can not be used to predict potential level of *Mc* infection. The pattern of water temperature and the parasite load for a given site needs to be taken into account. From the data, Willow Creek has a high infectivity rate.

One of the potentially more significant findings of my study was that both the plankton centrifuge method and pepsin trypsin digest method resulted in extremely inconsistent and highly variable spore counts within treatments, between treatments, and with respect to time of evaluation post exposure to TAMs (i.e. spore count data at 120 vs. 180 vs. 240 d pe). Other researchers attempting to use spore counts as a quantitative measure of the level of infection have reported similar results (Beth MacConnell, personnel communication). The inconsistency may not be due to the extraction method, but rather to the hemacytometer used to count spores. This technique involves flooding two nine grid sections of the hemacytometer with the sample and covering with a weighted coverslip. Numerous times when only a few spores were observed in the grids, many were observed outside the counting area, biasing the true spore count of that sample. Therefore, counting two or three sub samples from the same fish sample and averaging the counts would be a truer reflection of the total spore count. However, I do not believe averaging would have resulted in statistical significance of spore counts in my trials.

Based upon clinical signs, mortality and severity of infection, no treatment worked in any combination. Histological evaluation resulted in more consistent results within and between treatment replicates. Although histological evaluation is more time consuming and labor intensive, it appears to be a far superior quantitative assessment of level of *Mc* infection compared to spore counts.

Under the environmental conditions in my study, sampling at 90 and 120 d pe did not allow enough time for *Mc* spores to mature and complete sporogenesis. One of the

most important parts of sampling is to allow sufficient degree-days for sporogenesis of *Mc* to occur in order to get an accurate spore count and histological rating of infection.

Polymerase chain reaction of selected tanks from each 1998 trial site resulted in a positive *Mc* DNA confirmation. Since there are over 1200 confirmed Myxosporea and 450 *Myxobolus spp.* in the world, it is imperative to confirm with DNA analysis a true *Mc* infection in the wild (Lom et al. 1995).

Stained operculum blots did not consistently assess *Mc* infection in the fish sampled. Rarely, at 120 d pe were spores detected in operculum blots. When spores were observed, distinguishing between maturation stages of the spore, and normality or deformity, was difficult. Methylene blue stain was used to determine viability in the spores, but not detect deformities (Hoffman and Markiw 1977). The operculum blot procedure lacked the capability to accurately detect infection and should not be relied on to determine if therapeutant treatment results in spore deformities. However, the operculum blots and staining were useful in diagnosing *Flavobacterium sp* infection in the Pahsimeroi fish.

Data on mean fish weight at trial termination indicated some differences between negative control, positive control, and treated groups in 4 trials. Flow indexes and density indexes were calculated for each study to evaluate any differences in treatments and controls (Piper et al. 1992). The flow indexes and density indexes were within recommended values (Piper et al. 1992) suggesting that growth was not adversely affected. Weight and length differences in these trials attributed to lower densities due to mortalities and feeding regime.

Another potentially significant finding from my study was that SEM and TEM revealed definitive *Mc* spore deformities resulting from fumagillin and TNP-470 treatment. Scanning electron microscopy revealed that TNP-470 treatment resulted in a disruptive effect on the valvogenic cells, causing a collapse of the spore valve shell. Also, SEM showed that TNP-470 treatment resulted in perforation of the spore valve shell, and over time, total destruction of the shell. Transmission electron microscopy revealed that although fumagillin and TNP-470 affected sporogenesis, the effects of each compound were different. Fumagillin treatment resulted in vacuolization of intracellular spore constituents and deformation of polar capsules, without affecting the formation of spore valves. TNP-470 treatment appeared to disrupt the formation of the spore valve shells. Similar effects of TNP-470 treatment were also noted during the early developmental stages of sporogenesis. Although these two drugs appear to have different modes of action with respect to the formation of mature spores such as targeting different cell types, treatment with either may result in a major disruption of spore ultrastructure. The disruption in the maturation of polar capsules and spore valve shells negatively effects normal spore function resulting in nonviable spores. Although spores were observed in the fish, the number of viable spores released into the environment after death could potentially decrease over time and eventually lower overall infection level. However, because of the lack of apparent efficacy and the cost of treatment of both fumagillin and TNP-470, alternative treatments to control or prevent whirling disease need to be explored.

Further research will be required to fully understand the mechanism of action and therapeutic potential of fumagillin and/or TNP-470. Timing of administration, duration of treatment and dose of fumagillin and TNP-470 should be evaluated at various times during sporogenesis. Furthermore, if efficacy is achieved due to a different experimental design, an in-depth study should be performed to analyze the parasite/drug interaction by identifying the adhesion molecules involved in binding. If the mode of action of fumagillin and TNP-470 could be understood relative to the deformation of the parasite, other analogs could possibly be synthesized to increase therapeutic value.

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