Effects of Whirling Disease (*Myxobolus cerebralis*) Exposure on Juvenile Mountain Whitefish (*Prosopium williamsoni*)

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September, 2010

Introduction

Mountain whitefish *(Prospoium williamsoni)* are native to the Yampa and White River drainages in Colorado. Feast (1938) reports that whitefish ranged on the White River from Meeker up to Stillwater, on the North Fork. They were reported to have spawned on the Stillwater in late September. In the Yampa River, the range was reported to be from the lower drainage all the way up to the town of Steamboat Springs, with Elk Creek being one of the most important spawning tributaries. The species was introduced into the Colorado River drainage in the 1940's, either through water diversions or via human transport. Feast (1938) states that "A movement is under way among many of the sportsmen of the Colorado River drainage to secure the transplanting or stocking of whitefish from the White River to the upper waters of the Colorado, mainly the Roaring Fork and Eagle Rivers." Whitefish were intentionally stocked into the Cache la Poudre River drainage in 1956 from a collection of whitefish during the October spawning run in Harrison Creek, a tributary of the Yampa River (Klein 1974).

Mountain whitefish population declines have recently been reported in Colorado in the Yampa River and its tributaries. These reports are primarily anecdotal in nature, as good historical population estimates are relatively sparse. Evidence from other locations in the Western United States suggests that many other previously robust mountain whitefish populations may be in decline. These locations, like the Yampa River, also harbor *Myxobolus cerebralis*, the parasite that causes salmonid whirling disease. Several locations in Montana where reductions in recruitment of young whitefish have been noted include the Blackfoot River drainage and Upper Madison River, including the population in Hebgen Reservoir (Vincent 2008). Mission Creek, a tributary of the Flathead River, is also suspected to be affected, where up to 20% of the out-migrant whitefish exhibited caudal deformities (Vincent 2008), a common clinical sign of whirling disease. Population declines of mountain whitefish have also been observed in the Big Lost River in Idaho, where the parasite was identified as early as 1987 (Elle 1998). Adult mountain whitefish abundance is estimated to be 1.5% of historic levels in this drainage (Idaho Department of Fish and Game 2007). The cause for population declines of mountain whitefish in all of these locations has not been specifically identified, although numerous possibilities exist including habitat degradation, altered flow regimes, drought, migration barriers, competition and predation with other species of fish, and disease.

The apparent onset of population declines in the decade after introduction of *M. cerebralis* in these locations raised the question of the role of whirling disease in these losses. Whirling disease has been implicated as the primary factor in the loss of natural reproduction in rainbow trout in Colorado (Nehring and Walker 1996), but impacts of the parasite on mountain whitefish are largely unknown. A laboratory experiment was conducted by MacConnell and Zale (1999) in which heavy mortality and infection severity was observed among whitefish fry exposed to *M. cerebralis*. Mortality was also high among control groups, so conclusive evidence as to the role of *M. cerebalis* in whitefish losses remained uncertain. This study was initiated to re-evaluate the effects of the parasite on Mountain whitefish in a laboratory setting. Several laboratory experiments were conducted in this study, with varying success. In addition, samples were collected by field biologists throughout Colorado to evaluate prevalence and severity of infection in whitefish observed in the wild.

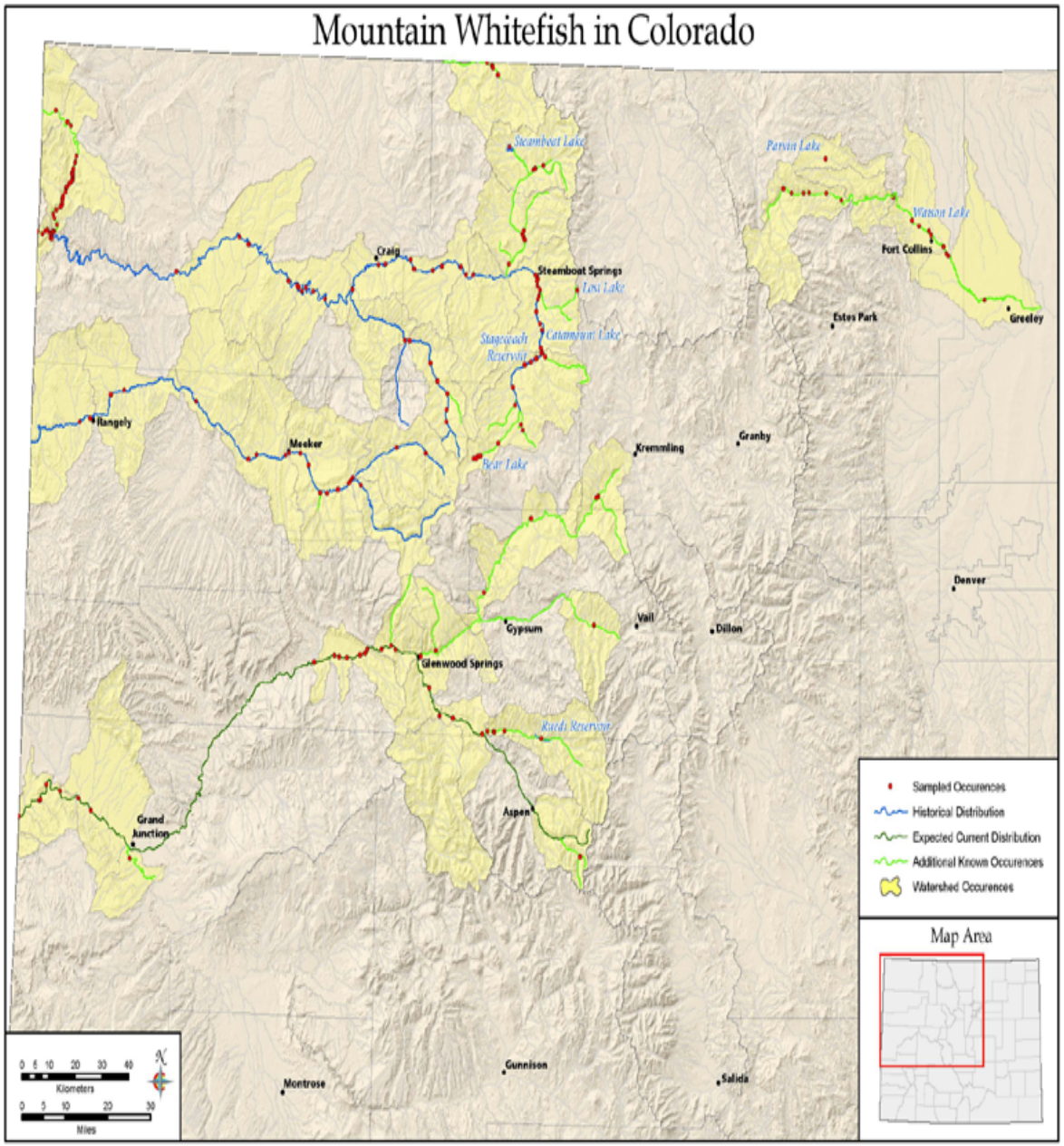

Figure 1. Mountain whitefish sample locations and distribution in Colorado. Map created by Grant Wilcox, Colorado Division of Wildlife GIS specialist.

Hatching and Rearing

Since little was known about the culture and early development of this species at the outset of this project, a considerable amount of trial and error was involved in collecting eggs as well as hatching and rearing the fry. Eggs were first collected in the fall of 2005 from a wild spawning run in the Yampa River, and from Grizzly Creek, a tributary of the Colorado River. In subsequent years, eggs were also collected from the Bear River and Fish Creek (tributaries of the Yampa River), Mad Creek (a tributary of the Elk River) and the White River. The females spawned for this project averaged 347 mm, with the smallest being 282 mm, and the largest 470 mm. While eggs per female were not calculated for this project, they are reported by Brown (1952) to range from 1,426 to 24,143, generally coinciding with length of the fish.

Mountain whitefish eggs are small compared with other salmonids, averaging 3.7 mm after water-hardening (Brown 1952). Other authors have reported eggs as slightly smaller with Sigler (1951) providing a range of 1.94 to 2.12 mm, and Rajagopal (1979) a range of 3.10 to 3.24 mm. The eggs reach eye-up stage quite rapidly, followed by a much longer post-eyed incubation period (Figure 2). Culturists accustomed to hatching other types of salmonid eggs should be made aware of this difference when incubating whitefish eggs, as the expectation that hatching will occur soon after eye-up is not borne out with this species. Rajagopal (1979) provides an excellent description of the development of whitefish embryos at 6 °C. He reports optic lobes developing at 11 days (65.6 degree-days), pigmentation in the eye at 25 days (150 degree-days), chromatophores appearing along the dorsal and lateral surfaces at 31 days (187.8 degree-days), movement of jaws and gills at 70 days (416.7 degree-days) and hatching occurring at 74 days (444.4 degree-days). Photographs of embryo development were taken over the course of our experiments from eggs incubated at 4.5 °C (Figure 3), which illustrate the development over a similar duration. Over the course of these experiments, it was noticed that hatching could be initiated in advanced embryos with sudden increases in temperature or physical disturbance.

Whitefish eggs are very susceptible to *Saprolegnia sp.* infection. Typically, formalin treatments for salmonid eggs of 1,600 to 2,000 ppm for 15 minutes (Piper et al. 1982) are administered periodically until eye-up to prevent infection. Treatments are then ended to prevent damage to the embryo and emerging fry. Since whitefish eggs reach eye-up so early in the incubation cycle, cessation of formalin treatments at eye-up results in the eggs being vulnerable to fungal infection for an extended period of time after eye-up. Some lots of eggs were incubated in Imhoff cones with flow entering the bottom of the cone and exiting a hole near the top. Eggs incubated in these arrangements did not develop severe fungal infections due to the constant motion of the eggs in the water column. Eggs were placed in the cones as early as two weeks post-fertilization without causing mortality. Standard hatching jars or Montana hatching boxes would be effective for incubating large numbers of whitefish eggs.

Incubation temperature proved to be critical for hatch success and health of the fry. As noted by Rajagopal (1979) when some small lots of eggs incubated at temperatures over 10 °C, hatching success was very poor, and higher rates of cripples were observed in the fry that did hatch (Figure 4). A detailed evaluation of effects of incubation temperatures is provided by Brinkman and Vieira (2009).

As with initial attempts at rearing other wild strains of fish, live *Artemia* (brine shrimp) were fed initially to all lots of fry (Figure 5). While the whitefish readily accepted the *Artemia*, later transitions to other diets were problematic. Some success was observed with using freeze-dried bloodworms and *Artemia* (Brinkman and Vieira 2009). The whitefish would reject other standard diets, leading to emaciation and eventual death. Recent feeding trials revealed that soft-moist trout diet, slightly air-dried and sieved through a 500 µm screen, was taken by the fry if provided exclusively soon after hatch. It appears that a certain amount of imprinting occurs on the feed type to which the fry are initially exposed. Early and exclusive exposure to the selected type of feed may be important for rearing large numbers of this species. Further investigations into suitable diets will be necessary if large-scale aquaculture production is a goal.

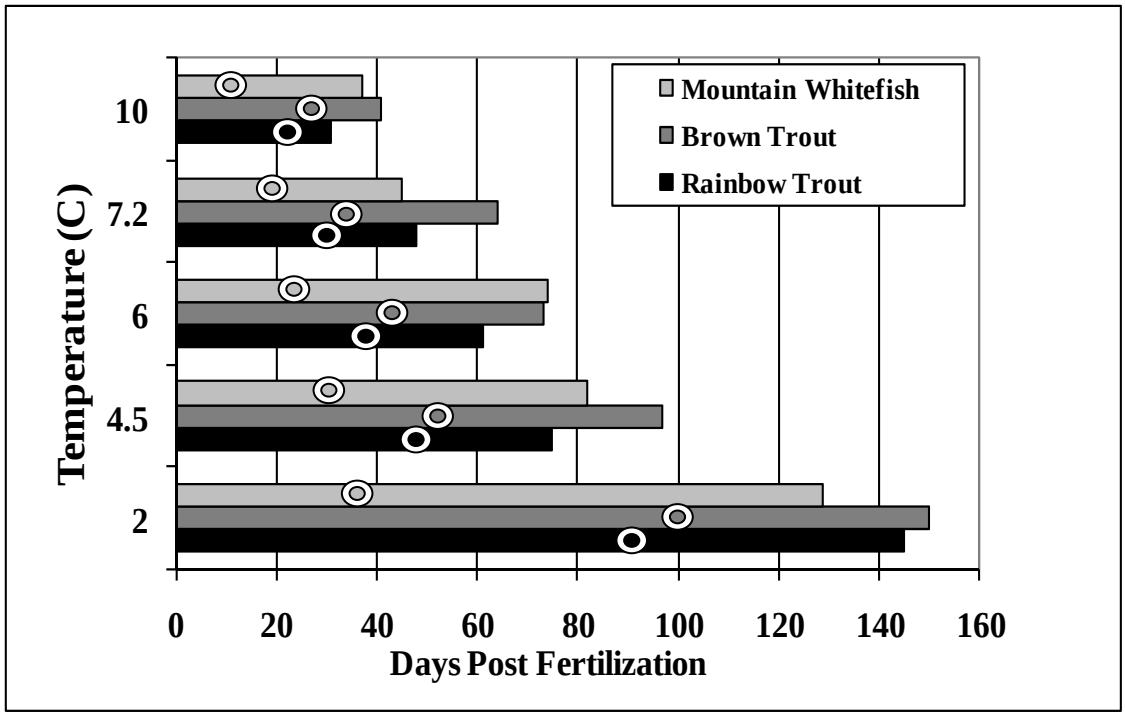

Figure 2. Days to hatch for mountain whitefish, brown trout and rainbow trout. Circles denote time at eye-up.

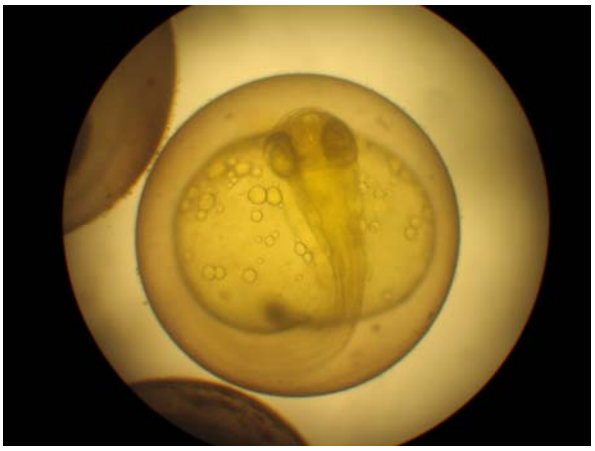

Figure 3a. Mountain whitefish egg at 12 days post-fertilization (54 degree-days C).

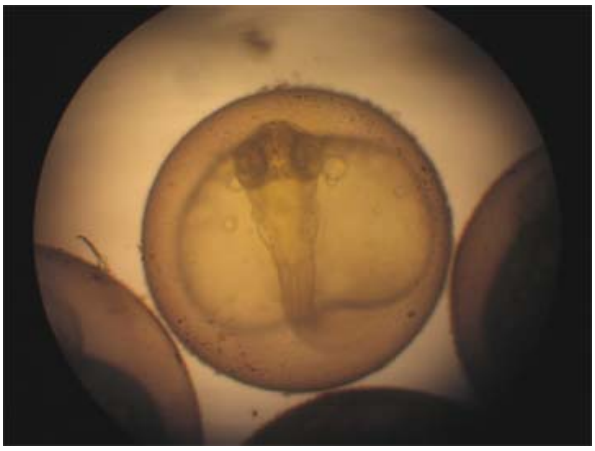

Figure 3b. Mountain whitefish egg at 18 days post-fertilization (81 degree-days C).

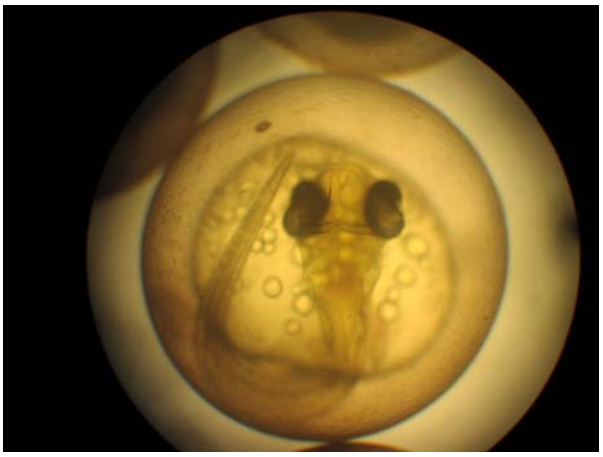

Figure 3c. Mountain whitefish egg at 26 days post-fertilization (117 degree-days C).

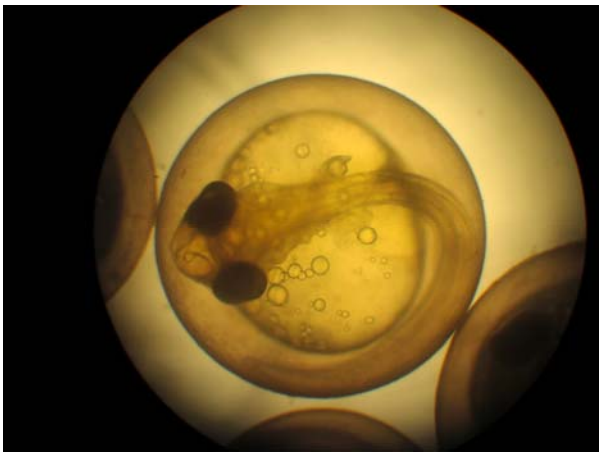

Figure 3d. Mountain whitefish egg at 30 days post-fertilization (135 degree-days C).

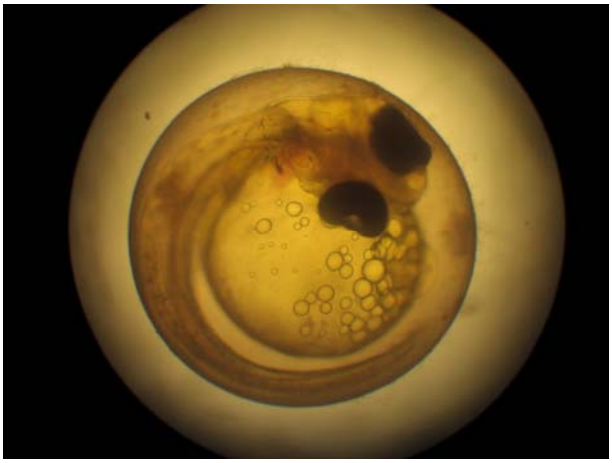

Figure 3e. Mountain whitefish egg at 40 days (180 degree-days C).

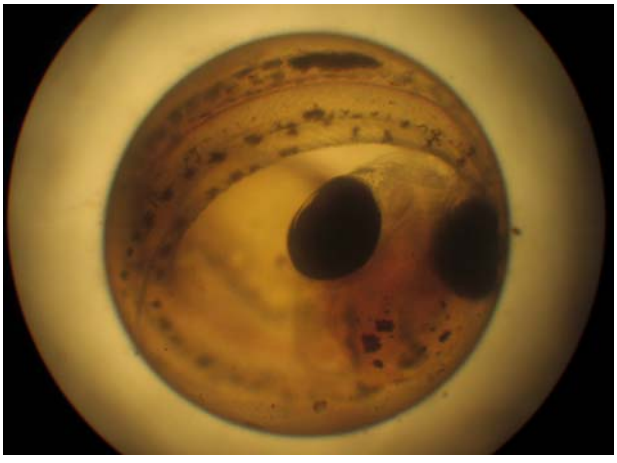

Figure 3f. Mountain whitefish egg at 70 days post-fertilization (315 degree days C).

Figure 4. Healthy mountain whitefish sac fry (left), and whitefish sac fry (right) showing deformities due to high incubation temperatures.

Figure 5. Healthy whitefish fry reared to six months on *Artemia* diet.

Experiment 1

The primary objective of the first experiment was to determine the extent of the infection in whitefish fry with a low exposure to the parasite. The experiment was similar to many laboratory experiments conducted previously with other salmonids, in which test groups of fish are exposed to *M. cerebralis* (Markiw 1992, Schisler et al. 2006). A known dose of triactinomyxons (TAMs), the stage of the parasite infective to fish, are administered to the treatment groups, while the control fish are left unexposed. These experiments are usually initiated when the fish are less than three months old to ensure they are still vulnerable to the effects of the parasite.

Methods

The whitefish fry were reared as described in the earlier text until they were 10 weeks post-hatch and had accumulated a total of 350 degree-days C. The treatment and control groups consisted of five replicates of 20 fish each, for a total of 100 treatment and 100 control fish. The *M. cerebralis* exposure was conducted by introducing triactinomyxons (TAMs) into each treatment tank at a rate of 1,000 TAMs per fish. This rate of exposure is relatively low, but sufficient to cause infections in susceptible species of fish such as rainbow trout. The fish were reared for 130 days post-exposure to evaluate mortality and infection severity. One additional treatment and control group was used for periodic sampling to evaluate the progress of the infection development in the fish.

Each of the replicate groups were reared in individual 76 liter aquariums at ambient laboratory water temperature, ranging from 2.9 °C at the beginning of the experiment to 14.8 °C by the end of the experiment, and averaging 8.8 °C. Each aquarium was equipped with a sponge-style up-flow filter and an air stone. Water changes of 4-20 liters were made every three to four days as aquariums were cleaned. Fish were fed several times daily with a combination of live *Artemia* and micro-encapsulated trout diet. Mortalities were removed daily. Some of the bodies that were not completely degraded were placed into 10% neutral buffered formalin (NBF) for later histological testing. Two fish were collected at 24 hours, 5 days, 10 days, 20 days, 30 days, 60 days, 100 days, and 120 days post exposure from the additional replicate tank and similarly placed into 10% NBF for histological evaluation. By the end of the experiment, very few fish remained in any of the replicate groups. Eight fish from two of the positive groups and one fish from one of the control groups that were still alive at the end of the experiment were evaluated for *M. cerebralis* infection with the enzymatic pepsin-trypsin digest (PTD) method (Markiw and Wolf 1974). The entire body of each fish, rather than just the cranial cartilage, was evaluated for presence of myxospores to increase the chances of finding the parasite.

Results

Mortality progressed at a fairly steady rate over the first 100 days of the experiment in both the treatment and control groups (Figure 6). Higher mortality began to occur at about 110 days post-exposure. Mortality at 130 days post-exposure was 67.0% (SD = 19.2) for the

treatment groups and 58.0% (SD = 13.0) for the control groups, which did not differ significantly ($F_{[1,8]} = 0.7500$; $P = 0.4117$).

Histological evaluation of the whitefish at 24 hours, 5 days, 10 days, 20 days, 30 days and 60 days did not result in positive identification of the parasite in the exposed fish. *M. cerebralis* lesions grading 1+ and 2+ on the MacConnell-Baldwin scale of 0-5 (Andree et al. 2002) were found with histology in exposed fish evaluated at 100 days and 120 days post-exposure (Figures 7-9). Myxopores were also found in exposed fish with histology at 120 days post-exposure. The lesions and spores in the infected fish were concentrated in caudal area of spine. Myxospores were not found in any of the fish tested with PTD.

Discussion

The increase in mortality at 110 days post-exposure was due to the fry selecting only the live *Artemia* as food items, and refusing the artificial diet. *Artemia* are not very nutritious, and are generally used as a starter item for wild fish varieties to encourage them to transition to an artificial diet. As a result, the fish became increasingly weakened as the experiment progressed, and attrition due to what appeared to be malnutrition occurred. Nonetheless, positive identification of the parasite was observed in all of the whitefish fry at 100 and 120 days post-exposure, which is comparable with other salmonids. At this stage the sporogonic stages move out of the nerve bundles and into the cartilage. Some spore development was observed in the histological sectioning, but may have been of insufficient quantity to be found with the PTD testing.

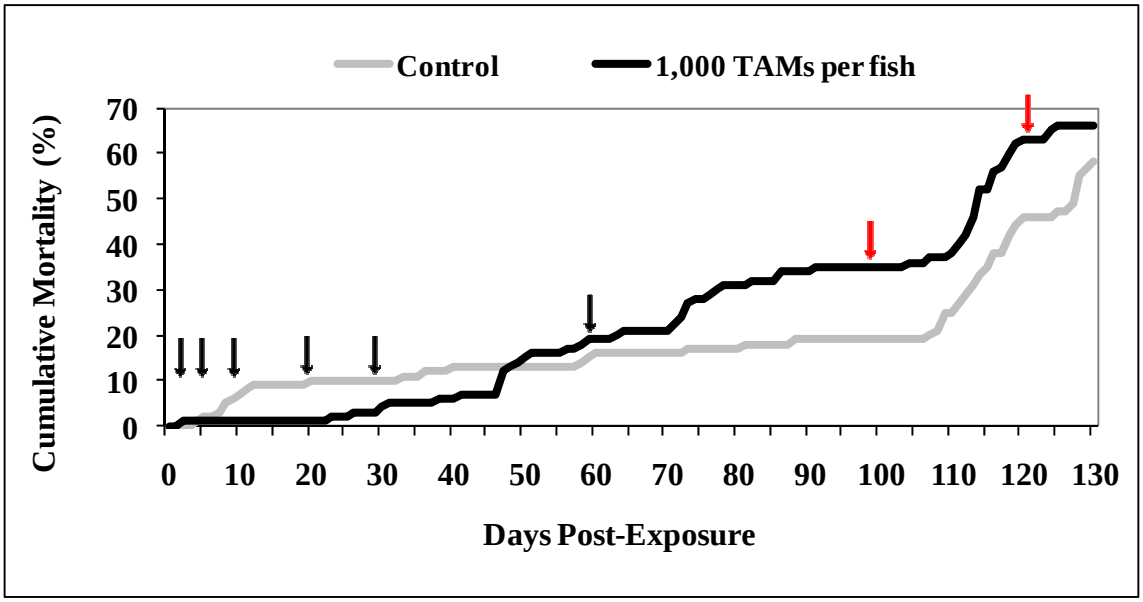

Figure 6. Cumulative morality of whitefish fry exposed to 1,000 TAMs per fish at six weeks of age. Arrows indicate samples collected for histological testing for presence of *M. cerebralis* in the cartilage of the fish collected. Black arrows indicate negative histological results, while red arrows indicate presence of the parasite in the histological sections.

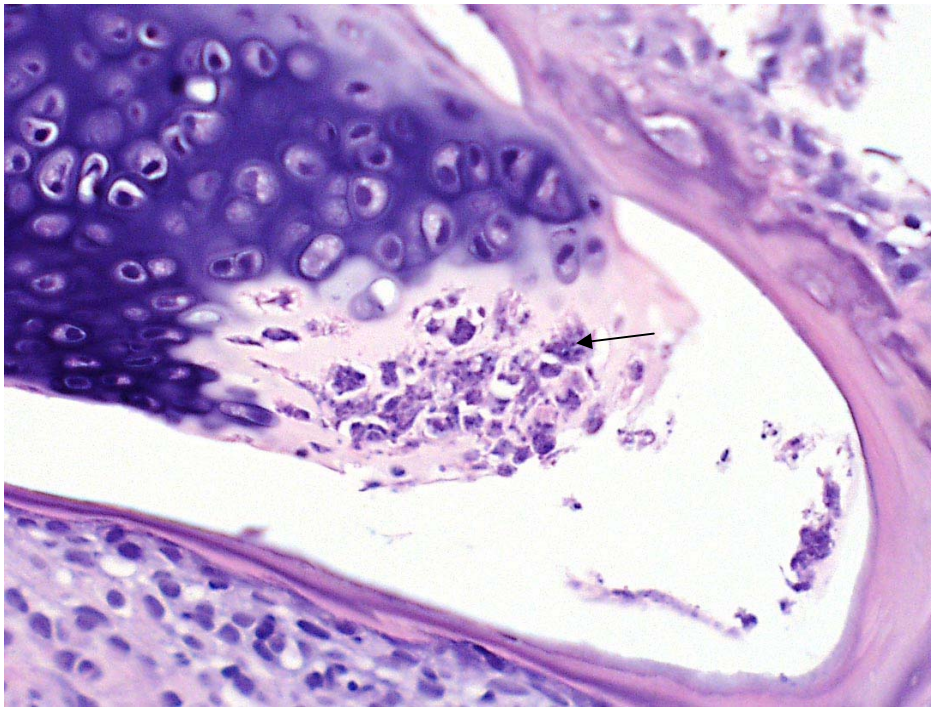

Figure 7. Developmental stages of *M. cerebralis* (area indicated with arrow) in fin ray 100 days post exposure.

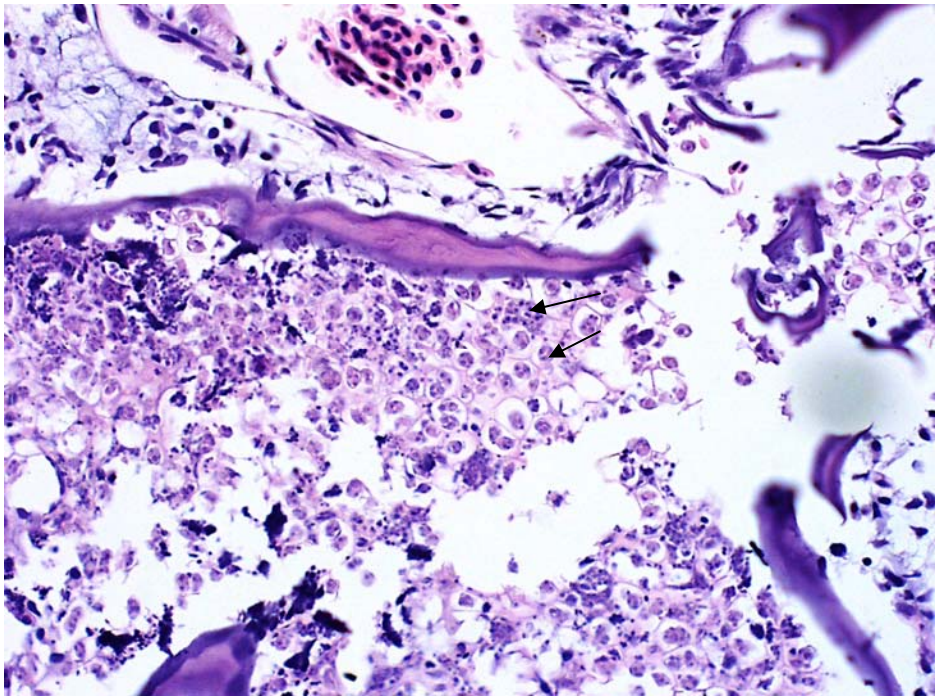

Figure 8. M. cerebralis development in whitefish fry at 120 days post exposure. Sporogonic and myxospore stages are present in cranial cartilage (indicated by arrows). Inflammatory cells are also present.

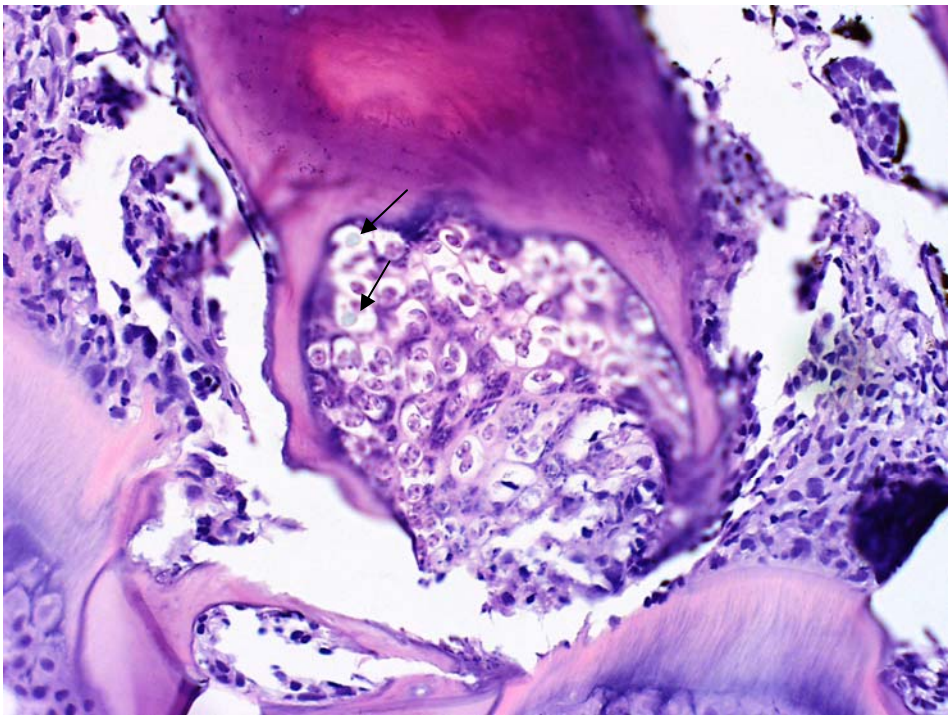

Figure 9. Sporogonic stages of *M. cerebralis*, as well as mature myxospores (indicated by arrows) in the tail plate/vertebrae of a mountain whitefish fry at 120 days post exposure.

Experiment 2

Given the non-significant differences in mortality between the treatment and control groups in the first experiment, a second experiment was initiated to determine if higher exposure levels would influence the whitefish fry differently. Rainbow and brown trout fry were also included in an ancillary experiment to compare infection severity at similar exposure levels.

Methods

One control and two treatment groups consisting of five replicates of 15 fish each were used in this experiment. The treatment groups in this case were exposures to 1,000 and 5,000 TAMs per fish. The fish had been reared fourteen weeks post hatch and approximately 730 degree-days C, at the start of the experiment.

Five control and five treatment replicates of 15 fish each of rainbow and brown trout fry were reared separately. The treatment fish were exposed to *M. cerebralis* at a rate of 1,000 TAMs per fish. In order to age-match the whitefish in degree-days, later exposure dates were used for the brown trout and rainbow trout fingerlings, of seven days and five weeks, respectively. The intent of this ancillary experiment was to obtain comparable myxospore counts for brown and rainbow trout reared under similar conditions with 1,000 TAMs per fish exposure levels. These fish were reared to six months post-exposure.

As with the first experiment, the fish were reared in 76 liter aquariums at ambient laboratory water temperatures. Fish were fed several times daily with a combination of *Artemia* and micro-encapsulated trout diet. Mortalities were removed daily. At the end of the experiment, most of the remaining fish were preserved in 70% ethyl alcohol. Infection severity in these fish was evaluated with a molecular-based technique called TaqMan PCR (Kelley et al. 2004). After the mortalities, histology, and PCR samples were collected, only one fish from each whitefish treatment group remained for testing with PTD.

Results

Heavy early onset of mortality occurred in the treatment group exposed to 5,000 TAMs per fish (Figure 10). These early mortalities stabilized after the first week post-exposure. However, the differences between the groups were significant at 60 days post exposure ($F_{[2,12]} = 7.12$; $P = 0.0091$). At about 65 days post-exposure, an increase in mortality occurred in all of the test groups. Nonetheless, the 5,000 TAMs per fish group still had higher mortality than the control or 1,000 TAMs per fish group after 90 days post exposure and the differences between the groups were significant ($F_{[2,12]} = 10.19$; $P = 0.0026$).

M. cerebralis lesions were found with histology in exposed fish at 90 days post exposure. TaqMan PCR did not identify any infection in the control fish. Three of the ten fish in the low dose group were found to be positive, and four of the nine fish in high dose were found to be positive. As with the whitefish fish evaluated with PTD the first experiment, none were identified as infected with *M. cerebralis.* There were adequate numbers of brown and rainbow trout raised in parallel and exposed to 1,000 TAMs per fish to conduct PTD testing.

Prevalence of infection in brown trout was 17.6% with an average myxospore count of 547 (N = 17, SD = 794). Prevalence of infection was 50.0% in rainbow trout, with an average myxospore count of 11,112 (N = 24, SD = 4,221).

Discussion

The increase in mortality at around 65 days post-exposure occurred when the fish were about six months of age. This is roughly the same age at which the fish in the first experiment began to die due to apparent malnutrition. Despite higher mortalities due to feeding issues late in the experiment, significant differences were still found in the high dose group. This was largely due to the high mortalities early in the experiment.

No myxospores were found with PTD in any of the whitefish in either the first experiment or this experiment. The prevalence of infection in the rainbow and brown trout exposed to 1,000 TAMs per fish in this experiment indicate that rainbow trout are most susceptible, brown trout are less susceptible, and whitefish are least susceptible, as tested with PTD. However, the mortality in the whitefish suggests that individual whitefish that developed more severe infections may be dying soon after exposure, leaving only those with little or no infection in the sample pool.

Verification of infection in treatment groups was found with both histology and TaqMan PCR. It is clear that the initial exposure of the young whitefish fry resulted in a dramatic increase in mortality over the other test groups. If whitefish fry in the wild exposed to heavy doses of the parasite die at a similar rate, early exposure of the fry to *M. cerebralis* may explain population declines

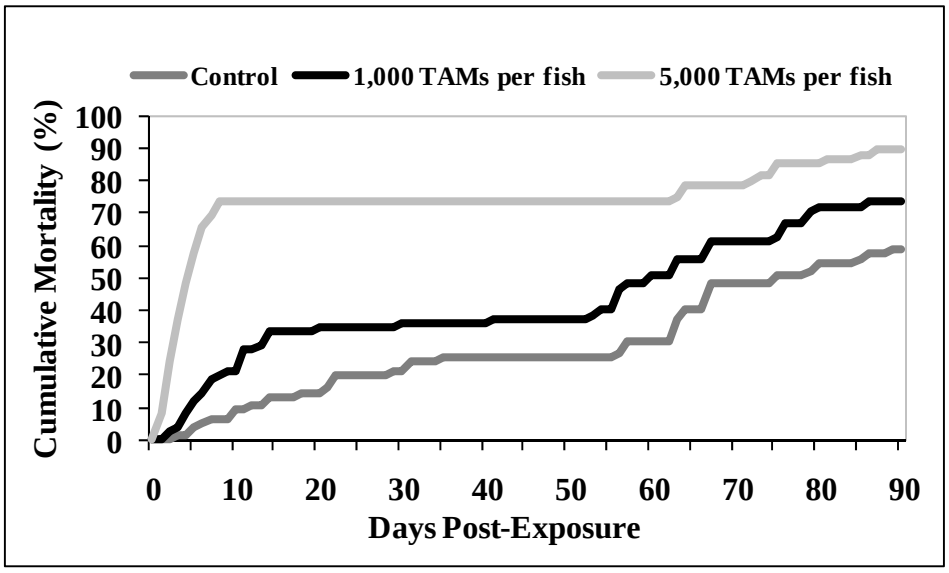

Figure 10. Cumulative mortality in mountain whitefish fry exposed to *M. cerebralis* at 14 weeks post-hatch.

NEGATIVE CONTROL		1,000 TAMS PER FISH			5,000 TAMS PER FISH		
TANK	**HEAD**	**TANK**	**HEAD**	**SPINE**	**TANK**	**HEAD**	**SPINE**
Tank 8	0	Tank 44	4,994	0	Tank 26	217,142	25,492
Tank 8	0	Tank 44	0	0	Tank 26	0	0
Tank 9	0	Tank 45	0	1,568	Tank 26	0	0
Tank 9	0	Tank 45	0	0	Tank 26	629,552	0
Tank 13	0	Tank 46	0	0	Tank 27	0	0
Tank 13	0	Tank 46	0	0	Tank 28A	0	0
Tank 14	0	Tank 47	0	0	Tank 28B	27	0
Tank 14	0	Tank 47	0	0	Tank 28B	0	0
Tank 15	0	Tank 48	1,665,253	109	Tank 29	231,048	56,670
Tank 15	0	Tank 48	0	0			
AVERAGE	**0**	**AVERAGE**	**167,025**	**168**	**AVERAGE**	**119,752**	**9,129**

Table 1. TaqMan PCR results at 90 days post-exposure for mountain whitefish fry exposed to *M. cerebralis* at 14 weeks post-hatch. Values shown are copies of *M. cerebralis* DNA per 10^6 host cells.

Scanning Electron Microscopy

The results of the Experiment 2 clearly demonstrated that mortality among whitefish fry is severe within the first week post-exposure when they are subjected to high doses of TAMs. The relatively low infection prevalence in the test fish and early acute mortality strongly suggested that the mortality was due to the initial exposure rather than from the subsequent infection. This was interesting because in most salmonids, mortality due to *M. cerebralis* typically occurs as the chronic infection gradually weakens and disfigures the fish.

In order to help determine the cause of mortalities in whitefish fry exposed to *M. cerebralis,* we felt it was important to determine exactly what effects the parasite was having on the fish during initial exposure. Scanning electron microscopy (SEM) conducted on whitefish fry preserved during exposure would allow us to observe these effects, similar to work that had been conducted by El-Matbouli et al. (1999) on rainbow trout exposed to the *M. cerebralis*.

Individual whitefish fry were reared to one month post-hatch at 7.5°C. Five fry were randomly allocated to two groups exposed to either 15,000 or 30,000 TAMs per fish. The high doses of TAMs were used to ensure that suitable images could be obtained, given the short duration of time the fish could be exposed before time of fixation. The fish were exposed by pouring the known quantity of parasites into a container holding the fish. Fish were exposed for one minute before being anesthetized with MS-222. The fish were removed from the exposure container using a pair of forceps and placed directly into a fixative solution of 2.5% gluteraldehyde and 1.6% paraformaldehyde. Total time from first exposure to fixation ranged from one minute, fifteen seconds to two minutes, 21 seconds for each fish. The fish were stored in the fixative for 24 hours, and then moved to a 1.5 M Sorenson's phosphate buffer solution until the dehydration phase of the process. Immediately prior to the dehydration phase, the trailing half of the opercules were removed by dissection to allow observation of the gills during the SEM. After the dehydration phase, the fish were mounted on aluminum stubs and coated in preparation for observation in the SEM.

Large numbers of TAMs were observed attached to the whitefish fry at both exposure levels (Figure 11). The TAMs attached to every available surface on the fry, including the skin, eyes, gills, mouth, and fins (Figure 12). Even within the short duration of exposure, some sporoplasms had completely penetrated the epithelium of the fish (Figures 13 - 15). Sites of TAM attachment and sporoplasm penetration exhibited heavy physical damage (Figure 16). Given the fragile nature of whitefish fry, these locations of heavy damage could be playing a role with regard to early mortalities. It is quite obvious from the images that the health of the whitefish fry could be seriously compromised when exposure to large number of TAMs occurs. Osmotic imbalance could occur as plasma leaks from the sites of attachment, and secondary infections could easily be established at these sites as well.

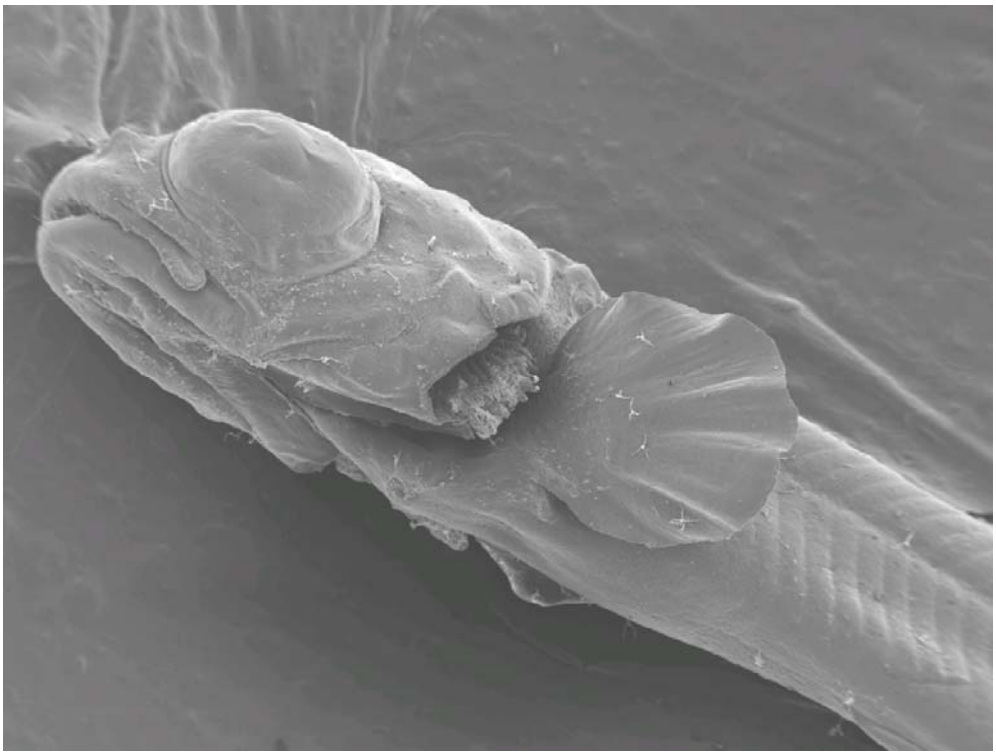

Figure 11. Large numbers of TAMs covering the body of a whitefish fry.

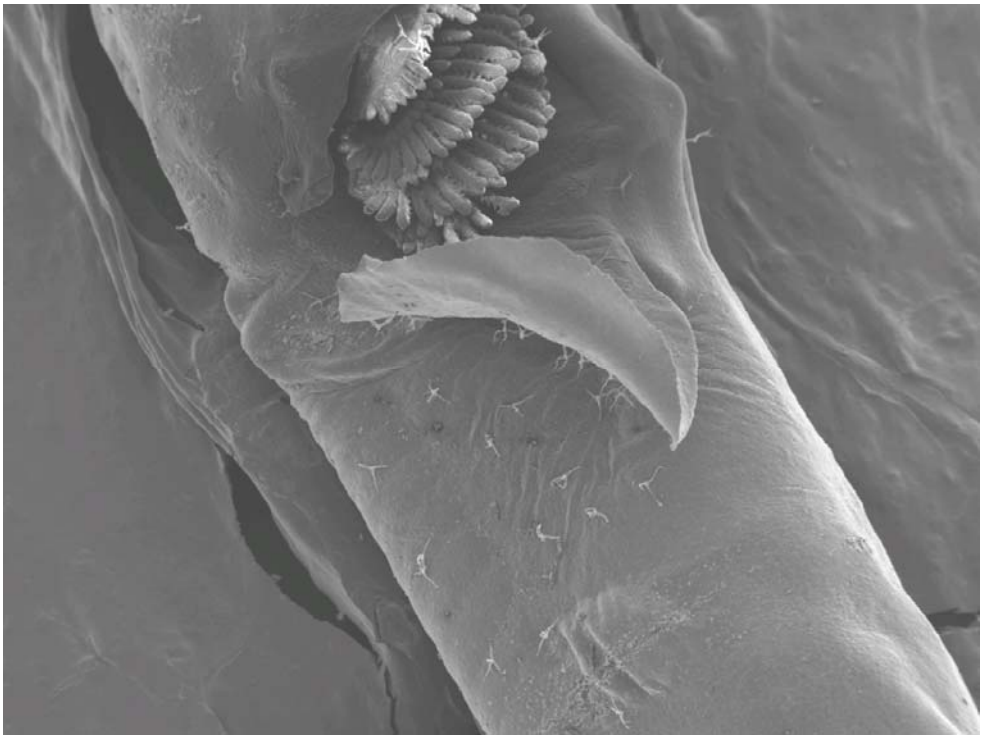

Figure 12. Magnification of the cut-away opercula showing attachment of the TAMs to the gills and skin under the pectoral fin.

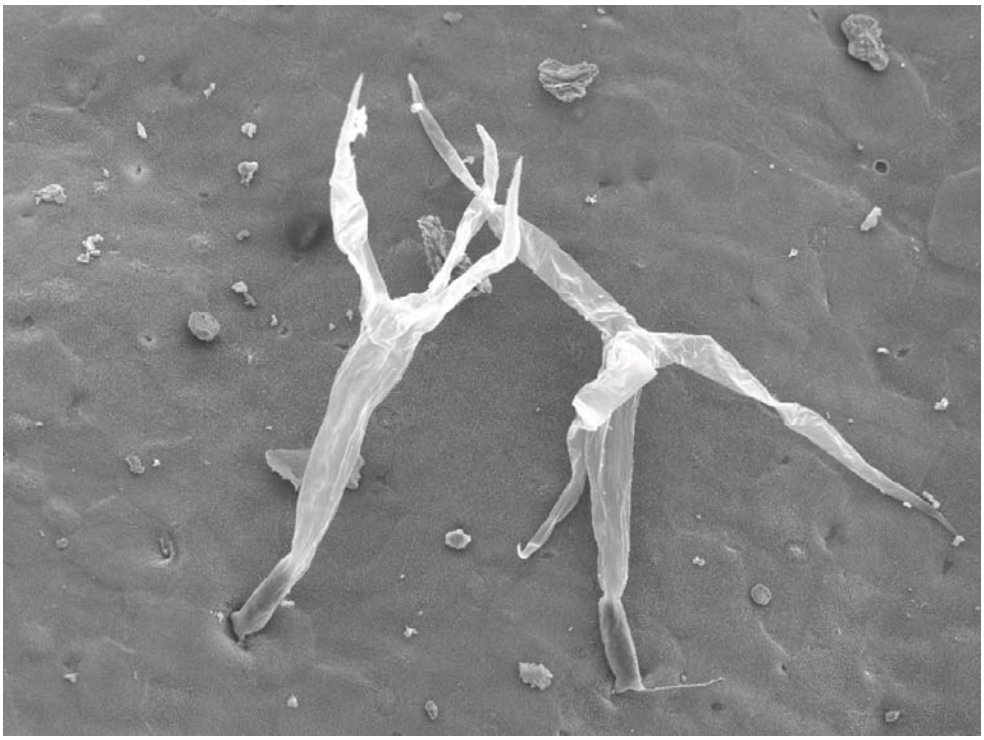

Figure 13. Magnification of two TAMs attached to the epidermis of a whitefish fry.

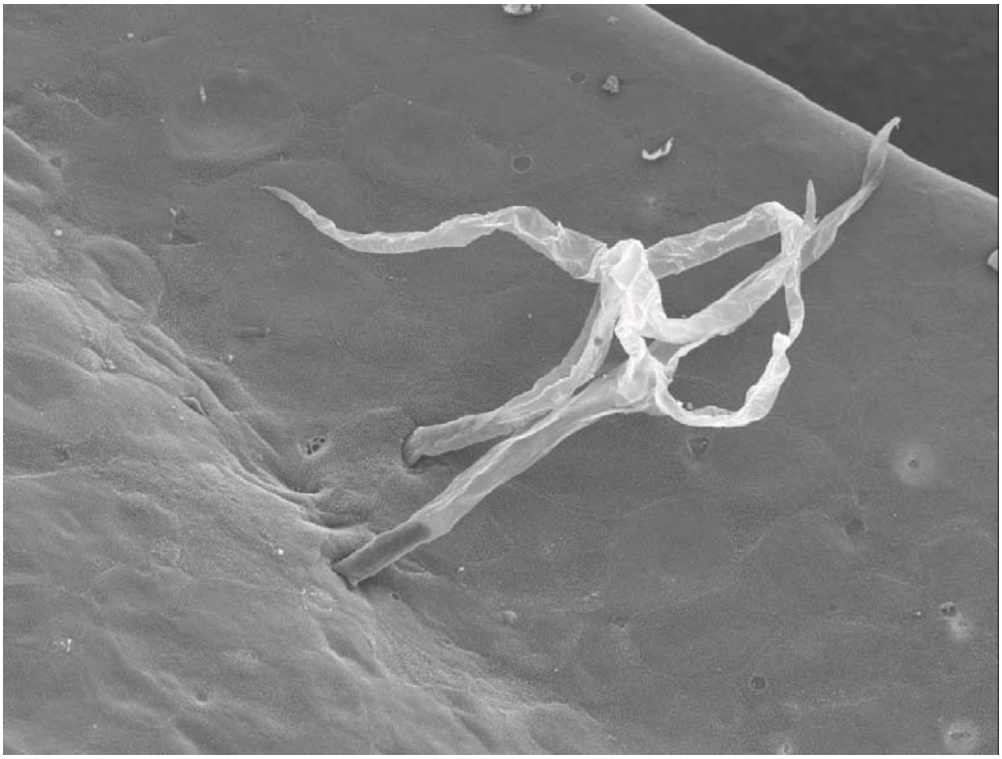

Figure 14. Two TAMs attached to the base of the adipose fin. The sporoplasm of the upper TAM has completely migrated into the epidermis.

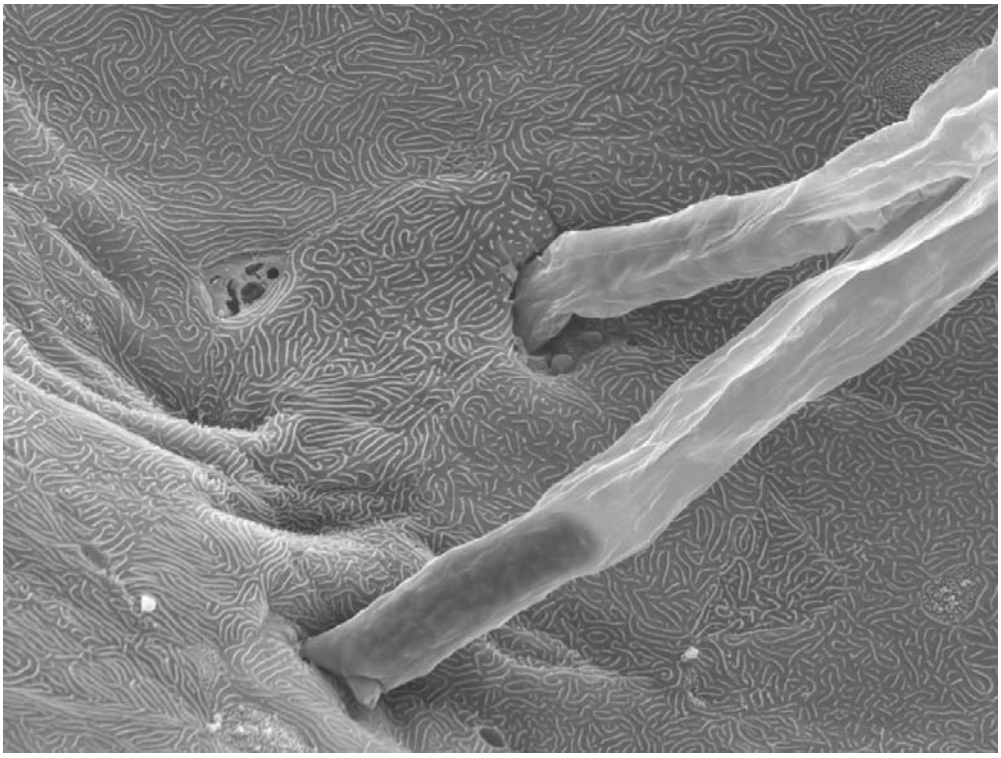

Figure 15. Two TAMs attached to the base of the adipose fin. The sporoplasm of the upper TAM has completely migrated into the epidermis.

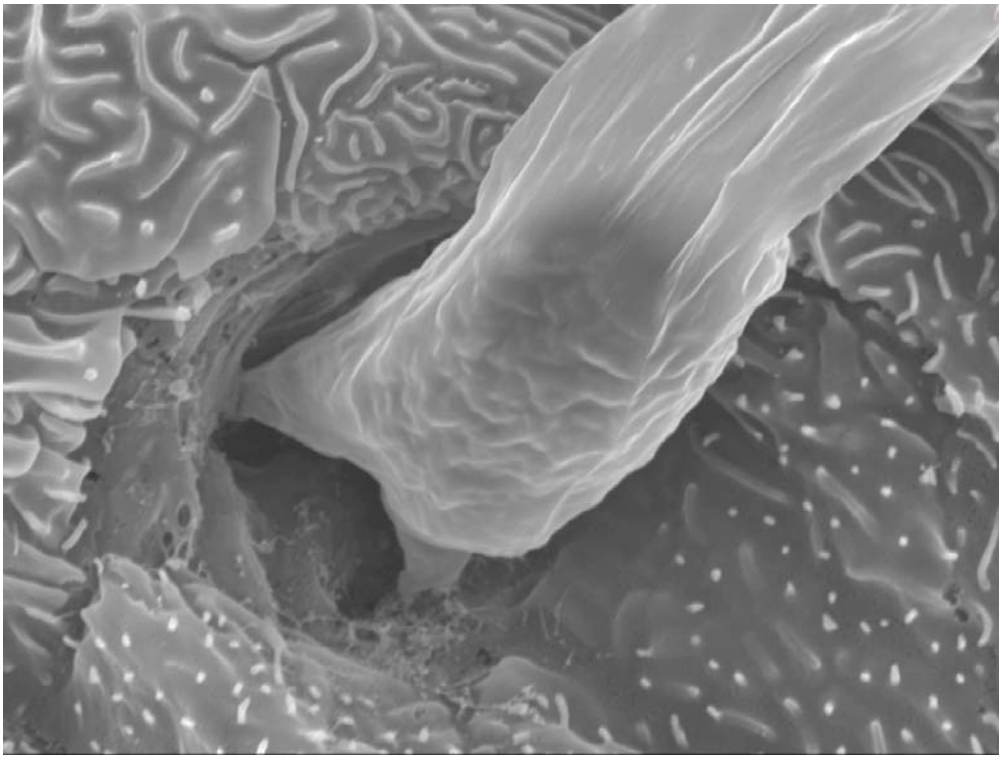

Figure 16. Sporoplasm of a TAM migrating into the epidermis.

Experiment 3

High mortality and obvious physical damage to whitefish fry occurred in the earlier experiments when they were exposed at a very young age and small size. The next question of interest was whether or not older and slightly larger fish would be as susceptible to heavy early mortality when exposed to high numbers of TAMs, as was observed in the previous experiments. In 2008, whitefish fry were trained on a diet of freeze-dried *Artemia*, bloodworms, and TetraFin™ aquarium flake food. This group had survived past the vulnerable fry stage and was well conditioned to the diet, so the fish were good candidates for an experiment to determine if fingerling whitefish had the same reaction to *M. cerebralis* as did the whitefish fry.

Methods

Five month-old fingerling whitefish (Figure 17), which had been reared to approximately 1,500 degree-days C were used in this experiment. The fish were randomly assigned to four groups, which were further randomly assigned to three replicates of 10 fish each. The four groups consisted of a control group and three exposure groups of 1,000, 5,000 and 10,000 TAMs per fish (total of 30 fish per group). The extremely high exposure level of 10,000 TAMs per fish was added to this experiment because of the older age of the whitefish, which would presumably be more resistant to the parasite than younger fish. The fish were reared for 120 days post exposure in 76 L aquariums, similar to the previous two experiments. Water was delivered at a rate of 0.25 L/min in a single-pass flow-through system using well water. Water temperatures ranged from 10.6 °C to 16.2 °C, averaging 14.9 °C over the course of the experiment.

Figure 17. Five month-old whitefish fingerling, reared to 1,500 degree days.

Fish were fed twice daily with the previously-described feed mixture. Mortalities were removed as they occurred from each tank, and preserved in 70% ethyl alcohol for later PCR testing. At the end of the rearing period, the remaining fish were weighed, measured, and processed for *M. cerebralis* testing. Five fish from each aquarium were evaluated for infection with PTD. Remaining fish were preserved in 10% NBF for histological evaluation, of which one fish from each replicate was submitted for testing.

Results

Heavy post-exposure mortalities were not observed in this experiment as were observed in the previous experiments. In fact, very little mortality was observed throughout the experiment. Overall, the control groups sustained 15.0% mortality, while the treatment groups of 1,000, 5,000, and 10,000 TAMs per fish sustained 10.0%, 7.5% and 7.5% mortality, respectively. These results were not significantly different ($F_{[3,12]} = 0.71$, $P = 0.5667$). Average lengths were 71.5 mm, 68.3 mm, 68.2 mm, and 65.9 mm for the control, 1,000, 5,000, and 10,000 TAMs per fish exposure groups, respectively, at the end of the experiment. These results were not significantly different ($F_{[3,12]} = 1.08$, $P = 0.3946$). Average weights were 2.9 g, 2.2 g, 2.2 g, and 2.1 g, respectively, for the control, 1,000, 5,000, and 10,000 TAMs per fish exposure groups, which were also not significantly different ($F_{[3,12]} = 2.27$, $P = 0.1327$). *M. cerebralis* was not found in any of the whitefish tested in this experiment with PTD, histology, or PCR.

Discussion

The complete absence of *M. cerebralis* in all of the whitefish in this experiment, even with PCR testing, was somewhat surprising, particularly given the high dose of TAMs to which some of the fish were exposed. These fish were much older than those used in the previous experiments, and were therefore not nearly as physically fragile as the smaller fish. At three to four months of age, whitefish fry begin to develop noticeable physical changes, such as obvious scale formation. It is possible that this results in protection from exposure-related physical trauma. Immune response may also be occurring with the increase in age and size, as occurs with other salmonids (O'Grodnick 1979, Markiw 1991 and 1992, Ryce et al. 2005). The other, albeit unlikely, possibility is that the TAMs used in the exposures were not viable.

If indeed whitefish become resistant to attacks from the parasite at the fingerling stage, it could explain the differences in observed population trends where the parasite is present. If very young fry avoid exposure to large numbers of TAMs, then later move to areas of heavy infection, *M. cerebralis* may not be deleterious. In other situations, TAM release may not coincide with fry emergence, so temporal separation of the vulnerable fry from the high numbers of TAMS may prevent severe infections from occurring. As a result, only in locations where heavy, early exposures to the parasite are occurring would population-level effects exist.

Experiment 4

The results of the previous three experiments indicated that age at exposure was important with respect to infection severity and survival of juvenile whitefish. In an attempt to bracket the age of susceptibility of mountain whitefish to *M. cerebralis*, a lot of fish was reared specifically to be exposed at one, two , and three months post-hatch. To ensure viability of TAMs and measurable infection in this experiment, rainbow trout were evaluated in parallel with the whitefish at each age at exposure, and exposed with TAMs from the same filtrate.

Methods

Rainbow trout and whitefish fry were reared to one month, two months, and three months of age at 10 °C prior to exposure (roughly 300, 600, and 900 degree-days old). Three replicates of each group, consisting of 20 fish each, were used in the one-month and two-month post-hatch exposure groups. Fifteen fish were used in the replicates of the three-month post-hatch exposure group. The treatment groups were exposed to 2,000 TAMs per fish. Each replicate was reared in individual 76 L aquariums as in the previous experiments, and water was delivered at a rate of 0.25 L/min in a single-pass flow-through system using well water.

The whitefish were fed daily using the same freeze-dried Artemia, bloodworm and flake food mixture as in the previous experiment. The rainbow trout were fed a standard trout food diet. At five months post-exposure, the one-month post-hatch exposure groups of rainbow trout and whitefish were weighed, measured, and preserved in 10% NBF for histological evaluation. Two fish from each tank were submitted for histological evaluation. When the two-month post-hatch and three-month post-hatch exposure groups reached five months post-exposure, a subset of fish were preserved for histological evaluation and a subset were submitted for PTD testing.

Results

In the one-month post-hatch group, a high proportion of the rainbow trout were found to be infected. Five of the six rainbow trout tested with histology were identified as infected with all five being rated as 4.0 on a scale of 0 to 5. Signs of whirling disease were quite prevalent in the exposed rainbow trout, with 83.7% (36/43) exhibiting at least one clinical sign of disease. By comparison, only 1.7% (1/58) of the control rainbow trout exhibited any sort of deformities, which were not necessarily related to whirling disease. *M. cerebralis* could not be found in any of the 10 whitefish remaining at the end of the experiment as tested with histology, and none exhibited signs of whirling disease.

In the two-month post-hatch group, histological tests identified three of the six rainbow trout examined as infected. The average overall score was 1.3, with one of the positive fish being rated as 2.0 and the other two positive fish being rated as 3.0. Prevalence of infection as tested by PTD in the exposed group was 46.7% (14/30), and average myxospore count was 12,085 per fish. Signs of disease occurred in 18.6% (11/59) of the rainbow trout in the exposed group, and 0.0% in the control group (0/58). None of the twelve surviving whitefish were found to be infected by either histology (0/6) or PTD (0/6), and none exhibited any signs of whirling disease.

In the three-month post-hatch group, no infection was found in any of the rainbow trout tested with histology (0/6). Examination of cartilage from the fish in this group showed no definitive signs of *M. cerebralis* infection or parasites. Since these were such large heads it is very possible that small foci of infection could be missed. However, no myxospores were found in any of the exposed rainbow trout (0/30) tested with PTD. No evidence of infection was found in the 16 whitefish surviving at the end of the experiment with either histology (0/6) or PTD (0/10), and none exhibited any signs of whirling disease.

Discussion

The histological results in this experiment suggest that at similar exposure levels, rainbow trout are more likely to be identified as infected by *M. cerebralis* than whitefish. It also appears that whitefish do not develop the high myxospore loads that are observed in other salmonids such as rainbow trout. As with some of the earlier experiments, low survival in the whitefish groups in this experiment makes interpretation of the results somewhat difficult. It is possible that the immune response of mountain whitefish can reduce the effects of the parasite compared to rainbow trout. However, it may also be possible that the very heavily infected mountain whitefish die when exposed to the parasite, and do not survive to the point where infection is observed. The pattern of infection prevalence and severity in the rainbow trout used in this experiment was consistent with past studies, in which high prevalence and severity of infection was observed in fry exposed at younger ages, and decreasing infection and prevalence in the older fingerlings.

Wild Fish Collections

Wild whitefish samples collected for whirling disease testing in Colorado were historically sparse, primarily due to the lack of interest in the species. Some data exist as part of collections made during research and biologist activities in the 1990's and 2000's. These data are summarized in the following section.

Yampa River

Population declines in mountain whitefish in the Yampa River from 1998 to present have been quite dramatic (Figure 18). Population declines in rainbow trout began to occur in 2002. After being identified as negative for *M. cerebralis* in 1995, the river was identified as positive for *M. cerebralis* in 1999. A sample of 10 whitefish and 21 rainbow trout collected in 1995 was found to be free of the parasite. In 1999, thirteen rainbow trout and two brook trout were tested for the parasite with PTD. Only one of the rainbow trout was positive for the parasite, and had a myxospore count of 456. Both brook trout were found to be positive, with an average spore count of 3,530. In 2001, a sample of four whitefish and 13 rainbow trout was collected from the tailwater of Stagecoach Reservoir. Fifty percent (2/4) of the whitefish were positive for *M. cerebralis*, with an average spore count of 4,021. Seventy-seven percent (10/13) of the rainbow trout were positive for the parasite with an average spore count of 21,444. A single whitefish fingerling was collected in 2002, which tested negative for the parasite. Rainbow trout collected from the river and reared in captivity in 2002 had myxospore counts ranging from 105,488 in

fish collected in the Stagecoach Reservoir tailwater to 818,337 in fish collected downstream of the Service Creek confluence. It is possible that the whitefish fry in this drainage are exposed early, resulting in high mortality. It is also possible that other factors are leading to the population declines, one of which is drought conditions causing extremely low flows in the years of 2002 through 2004. It is also possible that migration barriers such as Stagecoach reservoir, which was filled in 1991, could have reduced spawning runs, leading to declines of whitefish in the system. Introduction of *M. cerebralis* and high parasite loads have occurred concurrently with the declines in whitefish. However, until larger numbers of fry can be collected and tested for the parasite, it is difficult to make any definitive statements about the direct influence of *M. cerebralis* on the Yampa River whitefish population.

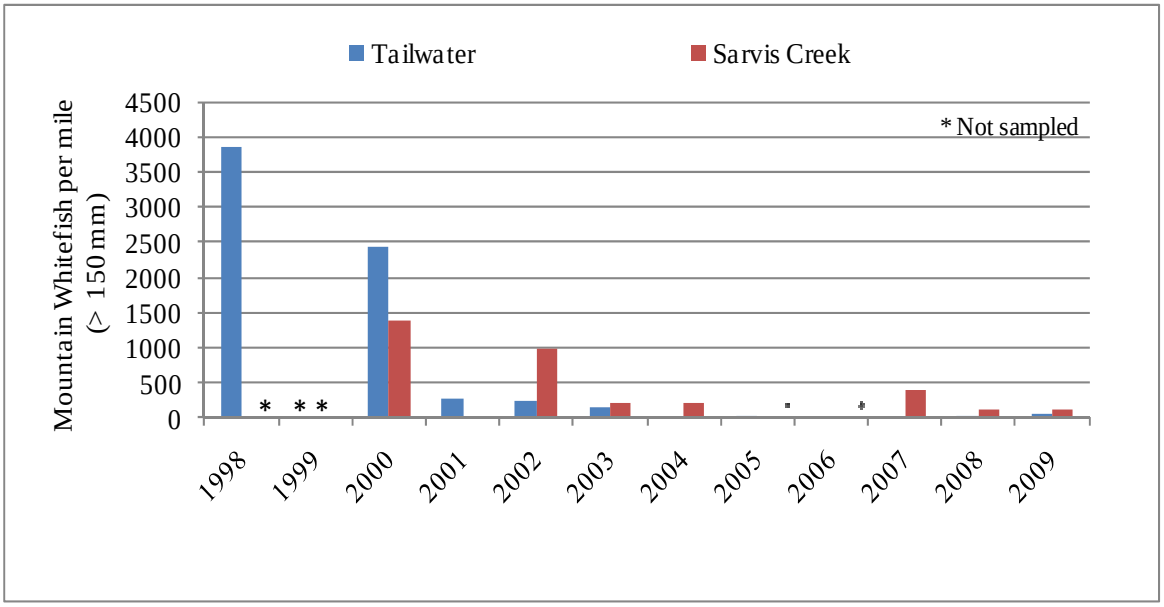

Figure 18. Mountain whitefish in the Yampa River from 1998 through 2009. Data courtesy Billy Atkinson, Colorado Division of Wildlife Northwest Region aquatic biologist.

Little Snake River

The Little Snake River is a tributary of the Yampa River with its confluence near Maybell, Colorado. In August of 2000, a collection of 31 whitefish, 28 rainbow trout, and six brook trout was submitted for PTD testing. Myxospores were found in 29% (8/28) of the rainbow trout, with an average myxospore count of 2,151 per fish. No myxospores were found in any of the whitefish or brook trout. This appears to be a situation where a low ambient level of infection had occurred, but no indication that the resident whitefish were infected. Neither population estimates or whitefish collections for *M. cerebralis* testing have occurred since 2000. This location warrants additional sampling to determine if the parasite has yet appeared in the whitefish population.

White River

The whitefish population in the White River remains robust, consisting of all age classes and sizes of whitefish (Figure 19). The population is a highly regarded recreational fishery, and annual charity fishing events occur specifically for harvest of whitefish. In June 2001, forty-three whitefish were collected from the White River, and all were found to be negative for *M. cerebralis* as tested with PTD. Ten brown trout and one rainbow trout were also collected, and all were found to be negative for *M. cerebralis* as tested with PTD.

Samples were collected again from the White River in 2006. *M. cerebralis* infection was not found in any of the age 3+ and 4+ whitefish (N = 29) collected. However, one of the six age 1+ whitefish collected was identified as positive for the parasite with a mean myxospore burden of 17,778. One of the two age 1+ rainbow trout collected at the same time was identified as infected with a spore count of 34,444. In 2008, 30 whitefish ranging in size from 250 to 395 mm were collected and tested for *M. cerebralis* with PTD. An additional 10 age 1+ fish were submitted for PTD testing, and 25 fry were collected for PCR testing in late May and early June. No myxospores were found in any of the fish tested with PTD. However, PCR testing identified infection in 64% (16/25) of the fry samples, and average infection score for all 25 fish was 1.6 on a scale of 0-3. Average infection score among fish testing positive was 2.5 on a scale of 0-3. This is a relatively high prevalence of infection, and a very high infection severity among those testing positive. These samples were collected in late May, so heavy infections in the young fry would have been from recent exposure. Fry were not found in the same locations when follow-up sampling was conducted only three weeks later. Fifteen whitefish fry were collected in early May of 2009 and submitted for PCR testing. Sixty-seven percent (10/15) were found to be positive for the parasite, with an overall average infection score for all 15 fish of 1.7, and infection score of 2.6 among those testing positive. Again, these results suggest that the whitefish fry testing positive were highly infected.

The lack of parasites in the adult fish in this population is interesting, given the high infection in the fry. It could mean that the infection in the drainage is just starting to become established, or that all of the heavily infected fry are dying before reaching maturity, leaving only the uninfected fish in the population. Additional samples of whitefish and other salmonids will continue in the coming years to monitor any changes in population densities and infection.

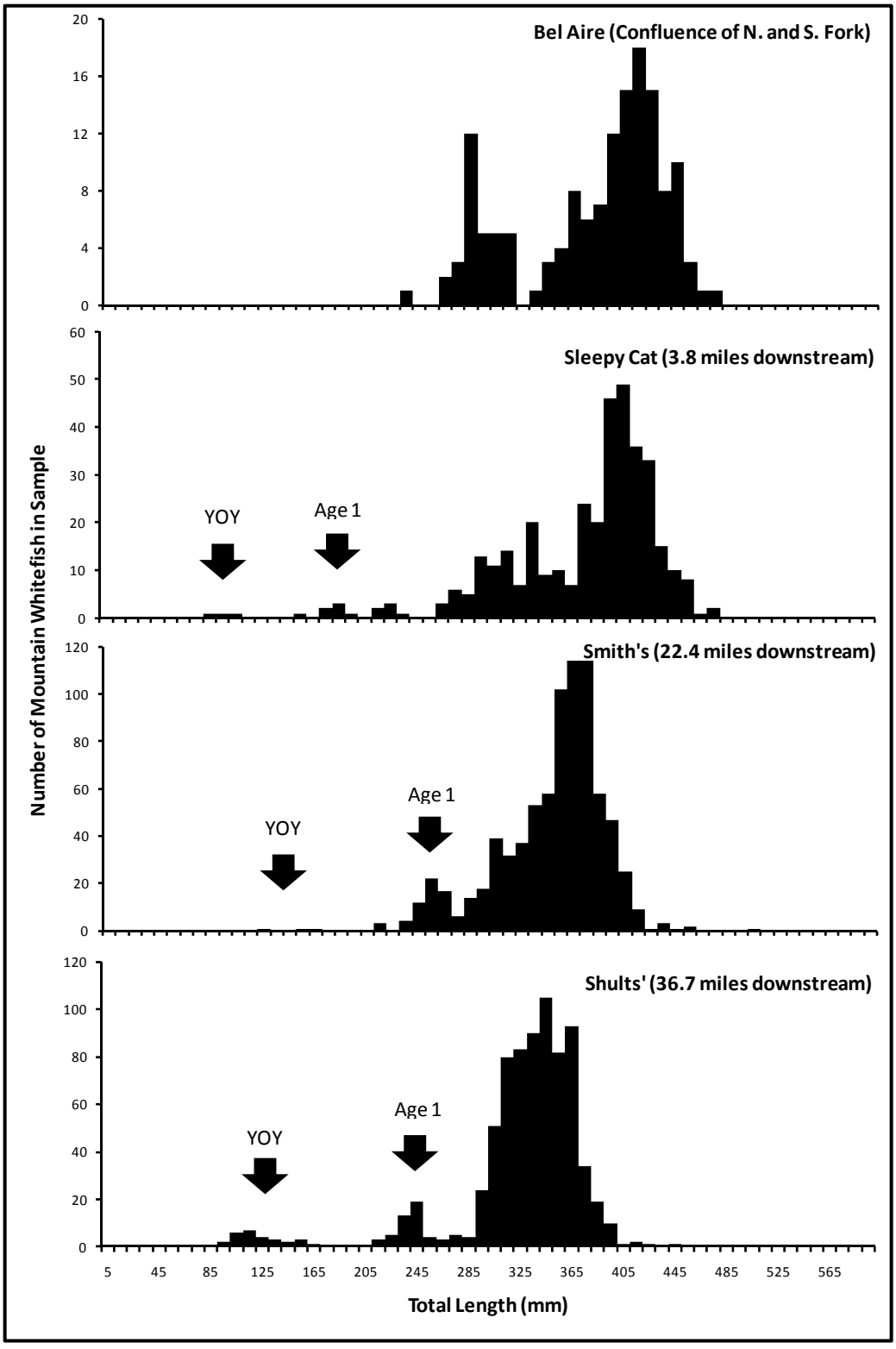

Figure 19. White River mountain whitefish population, 2009. Figure courtesy Boyd Wright, Colorado Division of Wildlife Northwest Region aquatic biologist.

Fish	Location/Date	Score
#01	White River MWF-YOY 5/28/08	+++
#02	White River MWF-YOY 5/28/08	+++
#03	White River MWF-YOY 5/28/08	+++
#04	White River MWF-YOY 5/28/08	-
#05	White River MWF-YOY 5/28/08	-
#06	White River MWF-YOY 5/28/08	-
#07	White River MWF-YOY 5/28/08	-
#08	White River MWF-YOY 5/28/08	-
#09	White River MWF-YOY 5/28/08	+++
#10	White River MWF-YOY 5/28/08	-
#11	White River MWF-YOY 5/28/08	++
#12	White River MWF-YOY 5/28/08	-
#13	White River MWF-YOY 5/28/08	++
#14	White River MWF-YOY 5/28/08	++
#15	White River MWF-YOY 5/28/08	++
#16	White River MWF-YOY 5/28/08	+++
#17	White River MWF-YOY 5/28/08	+
#18	White River MWF-YOY 5/28/08	+++
#19	White River MWF-YOY 5/28/08	+++
#20	White River MWF-YOY 5/28/08	-
#21	White River MWF-YOY 5/28/08	+++
#22	White River MWF-YOY 5/28/08	+++
#23	White River MWF-YOY 5/28/08	-
#24	White River MWF-YOY 5/28/08	+
#01	White River MWF-YOY 6/09/08	+++

Table 2a. PCR results from 25 whitefish fry collected from the White River in 2008

Fish	Location/Date	Score
#01	White River MWF-YOY 5/7/09	++
#02	White River MWF-YOY 5/7/09	+++
#03	White River MWF-YOY 5/7/09	+++
#04	White River MWF-YOY 5/7/09	+++
#05	White River MWF-YOY 5/7/09	-
#06	White River MWF-YOY 5/7/09	+++
#07	White River MWF-YOY 5/7/09	-
#08	White River MWF-YOY 5/7/09	-
#09	White River MWF-YOY 5/7/09	+++
#10	White River MWF-YOY 5/7/09	-
#11	White River MWF-YOY 5/7/09	+++
#12	White River MWF-YOY 5/7/09	++
#13	White River MWF-YOY 5/7/09	-
#14	White River MWF-YOY 5/7/09	++
#15	White River MWF-YOY 5/7/09	++

Table 2b. PCR results from 15 whitefish fry collected from the White River in 2009.

Cache la Poudre River

The Cache la Poudre River has been positive for *M. cerebralis* since the late 1980's, and is known to harbor very high numbers of the parasite. Natural recruitment failures in rainbow trout have occurred there since the early 1990's. Whitefish samples had not been submitted for evaluation in the past primarily due to the scarcity of the species in the standard population estimate samples. The species has never been abundant in the Cache la Poudre River, with estimates of only 175 fish (>152 mm) per mile reported by Klien (1974) in the most populated reaches from 1962 through 1973. Population estimates reveal only sporadic appearance of whitefish of any size in recent years. In 2007, nine individual age-1 whitefish were collected from the river for testing. The samples were split longitudinally. One half was placed in 10% NBF for TaqMan PCR testing, and the other half was placed in a plastic bag and frozen for later

PTD testing. In 2008, only five individuals could be found during the sampling events, and were tested for *M. cerebralis* myxospores with PTD.

The 2007 samples had only one fish identified as positive for *M. cerebralis* with PTD, with an average spore count of 51,472, which is a relatively high infection level. Taqman PCR identified that same fish as positive for the parasite (Figure 3). Three other fish in the 2007 samples were found to contain from 1 to 10 copies of the DNA for the parasite per 10^6 host cells. These would be considered very low infection levels. The remaining five fish had no evidence of presence of the parasite in the tissue by either testing method. The 2008 samples, all fish tested negative for *M. cerebralis* myxospores.

The prevalence and severity of infection in the whitefish sampled is quite low, with one notable exception that was identified as heavily infected by both TaqMan PCR and PTD. In a river such as the Cache la Poudre, high infection severity would be expected in all of the fish sampled, due to the very high ambient level of infection in the drainage. Once again, the results of these samples could lead one to different conclusions. The young fry that do emerge may be getting heavily exposed to the parasite and the very few that don't die and remaining in the population are those without infection. Other factors such as flows and temperature regimes could be limiting the whitefish population in the river.

Fish	Location	Date Collected	Length (mm)	Weight (grams)	Estimated spores per fish	Actual spores observed	Taqman PCR (Head)	Taqman PCR (Tail)
1	Pasquinel's	16-Oct-07	102	10	0	0	1	0
2	Pasquinel's	16-Oct-07	110	14	0	0	0	0
3	Pasquinel's	16-Oct-07	107	11	0	0	0	0
4	Pasquinel's	16-Oct-07	108	13	0	0	0	0
5	Pasquinel's	16-Oct-07	87	7	51,472	5	20,924	124,991
6	Pasquinel's	16-Oct-07	106	15	0	0	0	0
7	Pasquinel's	16-Oct-07	102	13	0	0	0	0
8	Pasquinel's	16-Oct-07	91	8	0	0	2	0
9	Pasquinel's	16-Oct-07	107	12	0	0	10	0
1	Firelane	05-Sep-08	206	100	0	0		
2	Firelane	05-Sep-08	180	79	0	0		
3	Pasquinel's	05-Sep-08	90	6	0	0		
4	Pasquinel's	05-Sep-08	172	48	0	0		
5	Pasquinel's	05-Sep-08	183	67	0	0		

Table 3. Whitefish samples collected from the Cache la Poudre River during 2007 and 2008. Taqman PCR values shown are copies of *M. cerebralis* DNA per 10^6 host cells.

Colorado River

The Colorado River is known to be heavily infected with *M. cerebralis* in its upper reaches near Granby, Colorado. Whitefish are not common in these upper reaches, but a healthy population is found downstream near the town of Radium, consisting of 1,045 fish per km (Figure 20). Despite the apparent strong population existing in this section of the Colorado River, local anglers have reported a decline in numbers compared to previous years (Jon Ewert, Colorado Division of Wildlife, personal communication).

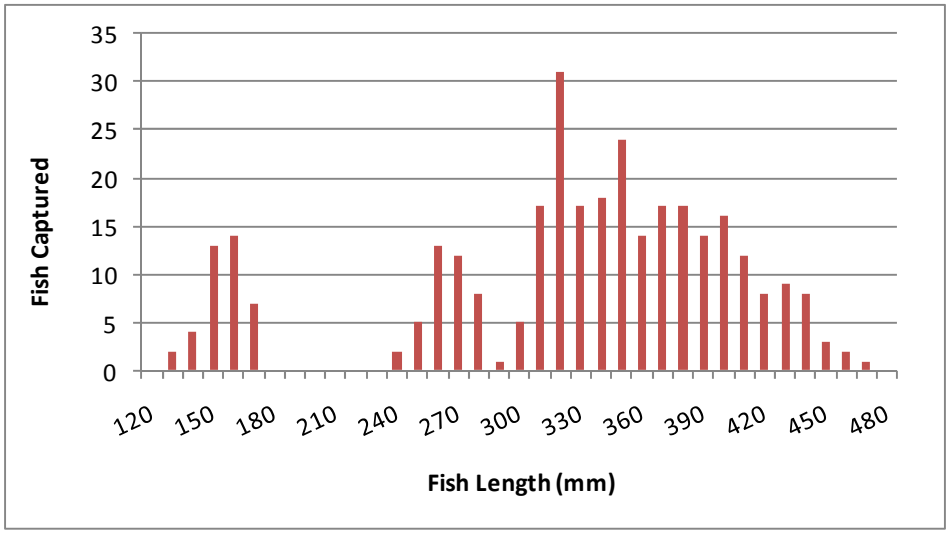

Figure 20. Population structure of mountain whitefish at the Radium site in spring, 2009. Data courtesy Kendall Bakich and Jon Ewert, Colorado Division of Wildlife Northwest Region aquatic biologists.

Whitefish samples were collected in 2008 in the Colorado River at four separate sites further downstream from this highly infected area. One site was near the town of Radium and another upstream of the town of Dostero. Two other sites were near the town of Newcastle, downstream of the confluence with the Eagle River. A total of 68 fish were collected, representative of all size classes from 70 mm up to 470 mm.

Only two fish were found to be infected with *M. cerebralis* among the samples collected and tested with PTD. The first was a 105 mm fish with a myxospore count of 17,133. The second was a 380 mm fish with a myxospore count of 15,672. This apparent lack of *M. cerebralis* infection in the sampled population is similar to other locations where the parasite is known to exist, but is not readily identified with myxospore analysis. Additional samples of young whitefish should be collected in the Colroado River and evaluated using PCR to gain better understanding of exposure and prevalence in immature fish.

Roaring Fork River

The Roaring Fork River is a tributary of the Colorado River, with its confluence near the town of Glenwood Springs, Colorado. Rainbow trout in the Roaring Fork River were identified as positive for *M. cerebralis* as early as 1988. By 1995, the rainbow trout population (278 $\pm$ 145 per kilometer) exhibited a skewed size distribution dominated by fish over 30 cm in length, indicating lack of rainbow trout recruitment. Brown trout were plentiful (600 $\pm$ 240 per kilometer) and the population consisted of large and small fish. Whitefish were the most abundant species present with a robust population (727 $\pm$ 258 per kilometer) of both small and large fish.

The first positive identification of the parasite in mountain whitefish in Colorado was from a single young-of-the-year whitefish collected from the Roaring Fork River on October 15, 1996. In this case the fish was identified as positive with histology. A subsequent test in 1997 identified the parasite in a high proportion of the samples collected. One lot of 10 age 2+ and 3+ fish was tested individually, and a prevalence of 80% and an average of 6,100 parasites per fish was observed. A second lot of 50 fish was evaluated as pooled sampled, so prevalence could not be determined, but the average myxospore count was 10,060 per fish. In 2003, a relatively large sample of rainbow trout, brown trout, and whitefish were collected. Rainbow trout had an average myxospore count of 101,218 with a prevalence of 83.3%. Brown trout had an average myxospore count of 12,370 with a prevalence of 57.9%. Whitefish had an average myxospore count of 4,660 with a prevalence of only 25.0%. These results appear to be consistent with the laboratory studies that suggested whitefish were less likely to be identified as positive for *M. cerebralis* infection as tested with the PTD method. Whitefish remain abundant in the Roaring Fork River. Given the high infection rate in rainbow trout in this river, one would presume that if *M. cerebralis* were a limiting factor for mountain whitefish, this would be a location where population declines would occur. It is possible that the very young fry are not being exposed to TAMs in the spawning areas, but do become exposed later in the rearing locations. However, these sites are not well defined, and specific details of the life history of whitefish in the drainage need to be determined to make any conclusive statements about this population.

Other States

Information from other States regarding *M. cerebralis* infection in wild whitefish is lacking. One exception is work conducted by Burkhardt (2002) in Wyoming, in which PCR and histology were used to test for presence of the parasite in whitefish, rainbow and brown trout. In 23 sites he tested that had brown trout and whitefish together, brown trout were identified as infected with PCR testing at all of the sites, whereas only 65% (15/23) of those same sites had whitefish identified as infected. Overall, 66.4% of all brown trout were found to be infected as tested by PCR at those sites compared with only 22% of the whitefish. In those same 23 sites, 70% (16/23) had positive brown trout histology samples, where only 17% (4/23) of the sites had positive whitefish histology samples. Hubert at al. (2002) theorized that high mortality among very young whitefish exposed to *M. cerebralis* may have been a reason for failure to find evidence of whirling disease among wild mountain whitefish in Wyoming. These results once again suggest that whitefish are less likely to be identified as infected by the parasite than rainbow or brown trout when exposed to similar levels of infection. Whether this is due to early

mortality of susceptible whitefish fry, later exposure of more resistant whitefish fingerlings, or a combination of both is unknown.

Conclusions

Laboratory experiments have shown that young whitefish fry can undoubtedly be killed very quickly with high exposure levels to *M. cerebralis*. Infection can be found in fish exposed at less than three months of age with PCR, histology, and to a lesser extent, PTD. Mortality from initial exposure appears to be more detrimental than subsequent infection. On the other hand, lesion severity and parasite development appears to be somewhat reduced compared with rainbow and brown trout. Advanced fingerling mountain whitefish actually appear to be completely non-susceptible to *M. cerebralis*. The specific mechanism for this is unknown, but may be related to the improved immune response or physical development of the fish at three to four months of age.

The effects of whirling disease on mountain whitefish populations are still not completely understood. The fragile nature of mountain whitefish fry can lead to high mortality from virtually any source of stress or physical injury. This makes identifying *M. cerebralis* as the sole cause of the population declines tenuous at best. *M. cerebralis* is not readily found in wild whitefish populations, particularly with PTD testing. Myxospore loads in whitefish, as tested with PTD, are lower than those found in rainbow and brown trout in every location where concurrent samples were collected. This could be due to early die-offs of young susceptible whitefish, leaving only healthy, non-infected individuals in the populations. It could also be due to a spatial or temporal disconnect between fry emergence and TAM releases, leading to lower or later exposures to the parasites.

The information compiled here does not appear to explain why mountain whitefish populations have declined, but hopefully initiates more discussion as to the possible causes of the losses. Detailed life-history information for the species is lacking in every location where they exist in Colorado. Additional investigations to identify spawning, rearing, and overwintering sites are needed to produce a better understanding of not only the effects of whirling disease, but of other factors that may limit the species.

Acknowledgements

Dr. Ron Hedrick, University of California-Davis, provided histological and Taqman PCR evaluations, as well as the histological images in this report, and was instrumental in conducting these evaluations. Kevin Rogers, Aquatic Wildlife Researcher, collected the whitefish eggs for the laboratory experiments conducted in this project. Steve Brinkman, Aquatic Wildlife Researcher, hatched eggs and provided fry for several of the experiments, along with providing substantial insight for this project. Barry Nehring, Aquatic Wildlife Researcher, provided the TAMs for the laboratory experiments. Mikael Catanese, Eric Fetherman, and Elise Titze assisted in rearing the whitefish fry. Beth McConnell, Headwaters Fish Pathology, provided many of the histological evaluations. John Wood, Pisces Molecular, provided some of the PCR testing. Kim Davidson at Colorado State University provided the SEM images. Joanne Tarman at the Colorado Division of Wildlife Fish Health Lab conducted the PTD testing. Colorado Division of

Wildlife aquatic biologists Billy Atkinson, Jon Ewert, Kendall Bakich, Boyd Wright, and Kurt Davies provided field data and wild fish collection samples.

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