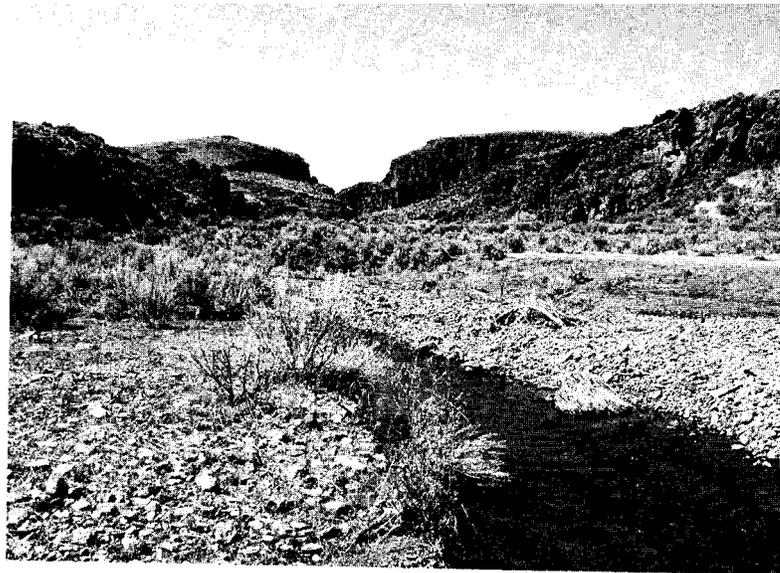


FISHERY RESEARCH



IDAHO NEUROTROPIC *MYXOBOLUS* INFECTIVITY TRIALS
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ABSTRACT

While screening salmonids for *Myxobolus cerebralis*, the causative agent of whirling disease, the Eagle Fish Health Laboratory detected spores of a neurotropic *Myxobolus* sp. that were morphologically similar to the *M. cerebralis* spore but found in brain and spinal cord. Having previously concentrated our research on the development of a polymerase chain reaction (PCR) based diagnostic test for the neurotropic *Myxobolus* (to help distinguish it from *M. cerebralis*, Hogge et al. 2004) and the characterization of its spore stage, our objectives for this study were: 1) to demonstrate that Duncan Creek water (Owyhee County, Idaho) carries the infective stage for the parasite, 2) to determine when parasite DNA and mature spores can be detected in the anterior spinal cord, and 3) to determine how the prevalence and intensity of the infection change over time. Three exposure trials of sentinel rainbow trout *Oncorhynchus mykiss* were performed. Exposure I revealed fish exposed in Duncan Creek were positive for the neurotropic *Myxobolus* in brain tissue by PCR at 104 CTU (74 days PE) and by pepsin/trypsin digest (PTD) and histology at 1885 CTU (145 days PE). After observing mature spores in the spinal cord of a specimen from Exposure I (histology), a test was designed to look at the entire spinal cord of native redband trout from Duncan Cr. This revealed mature spores in all four quarters of the spinal cord. With this information, the second trial was designed. Exposure II revealed fish positive for the parasite in the anterior spinal cord at 247 CTU (19 days PE) by PCR and 2106 CTU (162 days PE) by PTD. Exposure III revealed the parasite can be detected in the anterior spinal cord by PCR at 468 CTU (36 days PE) following a 7 day trial, at 234 CTU (18 days PE) following a 14 day trial, and at 104 CTU (8 days PE) following a 21 day trial. Since these exposure trials we have published a characterization of the parasite naming it *Myxobolus neurotropus*, including a new diagnostic PCR test, 18S rDNA sequence information, and phylogenetic analysis (Hogge et al., 2008).

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INTRODUCTION

The Eagle Fish Health Laboratory of the Idaho Department of Fish and Game first confirmed *Myxobolus cerebralis* in Idaho waters in 1987 (IDFG, 1987). Since that time we have sampled both cultured and wild salmonid populations from all river systems statewide using Blue Book Protocols (Fish Health Section of the American Fisheries Society, MacConnell, 2003). During these investigations we observed *Myxobolus* spores having similar morphology to *M. cerebralis* that could not be confirmed as *M. cerebralis*. Histology demonstrated *Myxobolus* spores in brain and other nerve tissues. We began calling this *Myxobolus* sp. the Idaho neurotropic *Myxobolus*. We developed a discriminatory polymerase chain reaction (PCR) based diagnostic technique to differentiate the two species of *Myxobolus*, sequenced the neurotropic *Myxobolus* and performed phylogenetic analysis to characterize its relatedness to other known *Myxobolus* species (Hogge et al., 2004)

Duncan Creek, Owyhee County, Idaho was chosen as our study area for the neurotropic *Myxobolus* infectivity trials (Figure 1). Duncan Creek is an isolated, sparsely populated area within the mid Snake River Basin. The creek is intermittent, connecting with Big Jacks Creek only during high water years. Review of the fish stocking history for the area reveals Jack's Creek was stocked once in 1946 with rainbow trout *Oncorhynchus mykiss* from Hagerman hatchery. That date is prior to the introduction of *M. cerebralis* into the United States (Bartholomew & Reno, 2002). Our routine testing of the indigenous redband rainbow trout in Duncan Creek has been positive for the Idaho neurotropic *Myxobolus* and negative for *M. cerebralis*.

Having previously concentrated our research on the development of a diagnostic test for the neurotropic *Myxobolus* and the characterization of its spore stage, our objectives for this study were: 1) to demonstrate that Duncan Creek water carries the infective stage for the parasite (Exposure I), 2) to determine when parasite DNA as well as when mature spores can first be detected in the anterior spinal cord (Exposure II), and 3) to determine how the prevalence and intensity of the infection change over time (Exposure III).

METHODS

Study site

Duncan Creek is located in Owyhee County, Idaho in the southwestern part of the state (Figure 1). Two exposure sites were selected: Site I at Pyramid rock (579946E 4711179N mapping datum WGS84) and Site II at Big rock (580334E 4711749N).

Experimental animals and exposure methods

For each exposure trial triploid rainbow trout *Oncorhynchus mykiss* (RBT) eggs were obtained from Hayspur Hatchery and reared in well water at the Eagle Fish Health Laboratory (EFHL) wet lab. They were placed in a cylindrical aluminum live-box that measured 47 cm in

length x 30.3 cm in diameter. Fish were transported to and from the exposure site in plastic bags with water and oxygen. Each live-box was equipped with a STOWAWAY XTI temperature logger to monitor water temperatures at 30 minute intervals. An YSI meter (model 556 MPS) was used to determine pH, temperature, dissolved oxygen, percent dissolved oxygen, and conductivity (Appendix A). A group of control (unexposed) fish (n=10) were selected and retained in the wet lab until sampled.

Exposure I

On May 5, 2005 one live-box was placed in Duncan Creek at Site I (Pyramid Rock, 579946E 4711179N) and a second box at Site II (Big Rock, 580334E 4711749N). Each live-box contained 50 RBT averaging 1.4 grams per fish. The exposure duration was 21 days. On May 26, 2005 the live boxes were retrieved and the fish placed into two rearing tanks at the EFHL wet lab in 13°C well water. The fish were sampled at 962 accumulated Celsius temperature units (CTU's), 1430 CTU, 1885 CTU, and 2340 CTU. On the sample date the total number of fish were counted and one fifth of the total number were randomly selected for sampling. The fish to be sampled were euthanized with tricaine methane sulphonate (MS222) and weights and lengths recorded. We removed the heads and split each longitudinally in half fixing one half-head in 10% neutral buffered formalin (NBF) and freezing the second. We cut the remaining fish body into three pieces horizontally, fixed them in 10% NBF, and archived them.

For each sampling date we tested 18 fish, performing PCR (Hogge et al., 2004) and histology on eight (half-heads from the same fish) using standard techniques. Pepsin-trypsin digest (PTD, Markiw & Wolf 1974) was performed on the remaining 10 fish.

Exposure II

On May 9, 2006 one live-box was placed at Site I and one at Site II (see Exposure I above), each containing 100 Hayspur strain RBT averaging 1.6g per fish. The exposure duration was 22 days. On May 31, 2006 the live boxes were retrieved and the fish from Site II placed into one rearing tank at the EFHL wet lab in 13°C well water. The live box placed at Site I contained no fish. Fish were prepared for sampling as in Exposure I above. The anterior spinal cord was removed at 247 CTU, 468 CTU, 702 CTU, 975 CTU for PCR testing of parasite DNA from the vegetative stage, and at 1651 CTU, 1885 CTU, 2106 CTU, 2340 CTU and 2574 CTU for PTD testing (protocols as in Exposure I) to visualize myxospores.

Exposure III

On September 21, 2006 two live boxes were placed at Site II (see Exposure I above) each containing 75 Hayspur strain RBT averaging 1.2g per fish. On Sept. 28th 25 fish were removed from each live box and pooled (7 day exposure A). On October 5th 25 fish were removed from each live box and pooled (14 day exposure B). On October 12th, the remaining fish were removed and pooled (21 day exposure C). Each exposure was placed in a rearing tank at the EFHL wet lab in 13°C well water. Each of the three exposures (A, B, & C) was then sampled at 104 CTU, 234 CTU, 351 CTU, and 468 CTU. The anterior spinal cord was tested for the parasite by PCR.

RESULTS

Exposure I

Brain tissue was positive for the neurotropic *Myxobolus* by PCR at 962 CTU. PTD and histology examinations were positive for spores at 1885 CTU (Table 1). No vegetative stages were observed by histology. Spores were seen lying freely in the brain tissue with no host response. In one sample that contained some spinal cord, mature spores were observed in the spinal cord as well as the brain. All control (unexposed) fish (n=10) were negative by PCR.

Exposure II

The anterior spinal cord was positive for the neurotropic *Myxobolus* at 247 CTU by PCR and at 2106 CTU by PTD (Table 2). All control (unexposed) fish (n=10) were negative by PCR.

Exposure III

The anterior spinal cord was positive for the neurotropic *Myxobolus* by PCR at 468 CTU following a 7 day trial, at 234 CTU following a 14 day trial, and at 104 CTU following a 21 day trial (Table 3). All control (unexposed) fish (n=10) were negative by PCR.

DISCUSSION

Duncan Creek water carries the neurotropic *Myxobolus* infective stage. In Exposure I the brain of exposed fish tested positive by PCR in our first sampling at 962 CTU. We did not see mature spores by PTD or histology until 1885 CTU. This is much later than what we have observed for *M. cerebralis* when sentinel RBT fry were handled similarly. Typically we can detect *M. cerebralis* spores in the cranial cartilage by PTD in challenged fish at 900 CTU.

We changed our tissue sample from brain to spinal cord in exposure II. While performing the histological test in Exposure I we observed mature spores not only in the brain but also in the spinal cord. We sampled a group of indigenous redband trout from Duncan Creek, dissecting the entire spinal cord and, dividing it into quarters, then performed PTD's on each quarter. We demonstrated mature spores in all four quarters of the spinal cord.

Exposure II revealed that we could detect the neurotropic *Myxobolus* in the spinal cord at 247 CTU, much earlier than we could detect it in the brain (exposure I). We conclude that the parasite moves from the spinal cord into the brain. We did not detect mature spores in the spinal cord until 2106 CTU's, the next sampling interval after we were able to detect them in Exposure I.

Exposure III revealed that whereas a 7 day period in Duncan Creek water was sufficient to achieve exposure to the parasite, it required 36 days post exposure to demonstrate it in the spinal cord. Following a 21 day period in Duncan Creek only 8 days were required to demonstrate the parasite in the spinal cord.

Low mortality was observed in all exposures while the fish were held at EFHL. This is likely due to the low pathogenicity of the parasite. Histology of the brain and spinal cord revealed no pathology or host response to the parasite. With the exception of *M. cerebralis*, *Myxobolus* species do tend to be fairly benign.

The importance of this parasite lies in the fact that we, in Idaho, commonly encounter it while testing heads for *M. cerebralis* and that it is morphologically similar to *M. cerebralis*. The Duncan Creek area was chosen for our study site due to its remote location and the lack of fish stocking in the area. Therefore the neurotropic *Myxobolus* appears to be indigenous to Idaho. Since the pathogenicity of the two *Myxobolus* species are very different we need to be diligent in confirmation of all *Myxobolus* spores that we see.

In addition to the type host, rainbow trout, we have documented the neurotropic *Myxobolus* in cutthroat trout (both Yellowstone and westslope subspecies), bull trout, Chinook salmon, and kokanee and sockeye salmon in Idaho.

Since undertaking this series of infectivity trials we have published the characterization of this new species naming it *M. neurotropus* after it's affinity for nervous tissue (Hogge et al., 2008). Therein we look at its relatedness to other *Myxobolus* species found in salmonids with 18S rDNA sequence information and phylogenetic analysis. Our diagnostic PCR is given to help identify the species.

The distribution of *M. neurotropus* appears to be widespread. We have confirmed the species in rainbow trout from Washington and in Bonneville cutthroat trout from Utah. It may also be present in Oregon (Lorz et al., 1989) and California (Hedrick et al., 1991).

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Table 1. Duncan Creek sentinel exposure I prevalence obtained in May 2005 expressed as number of RBT positive / total RBT examined.

Sample	PTD	PCR	Hist
962 CTU	0/10	8/8	0/8
74 days PE			
1430 CTU	0/10	8/8	0/8
110 days PE			
1885 CTU	2/10	7/8	1/8
145 days PE			
2340 CTU	7/10	8/8	8/8
180 days PE			

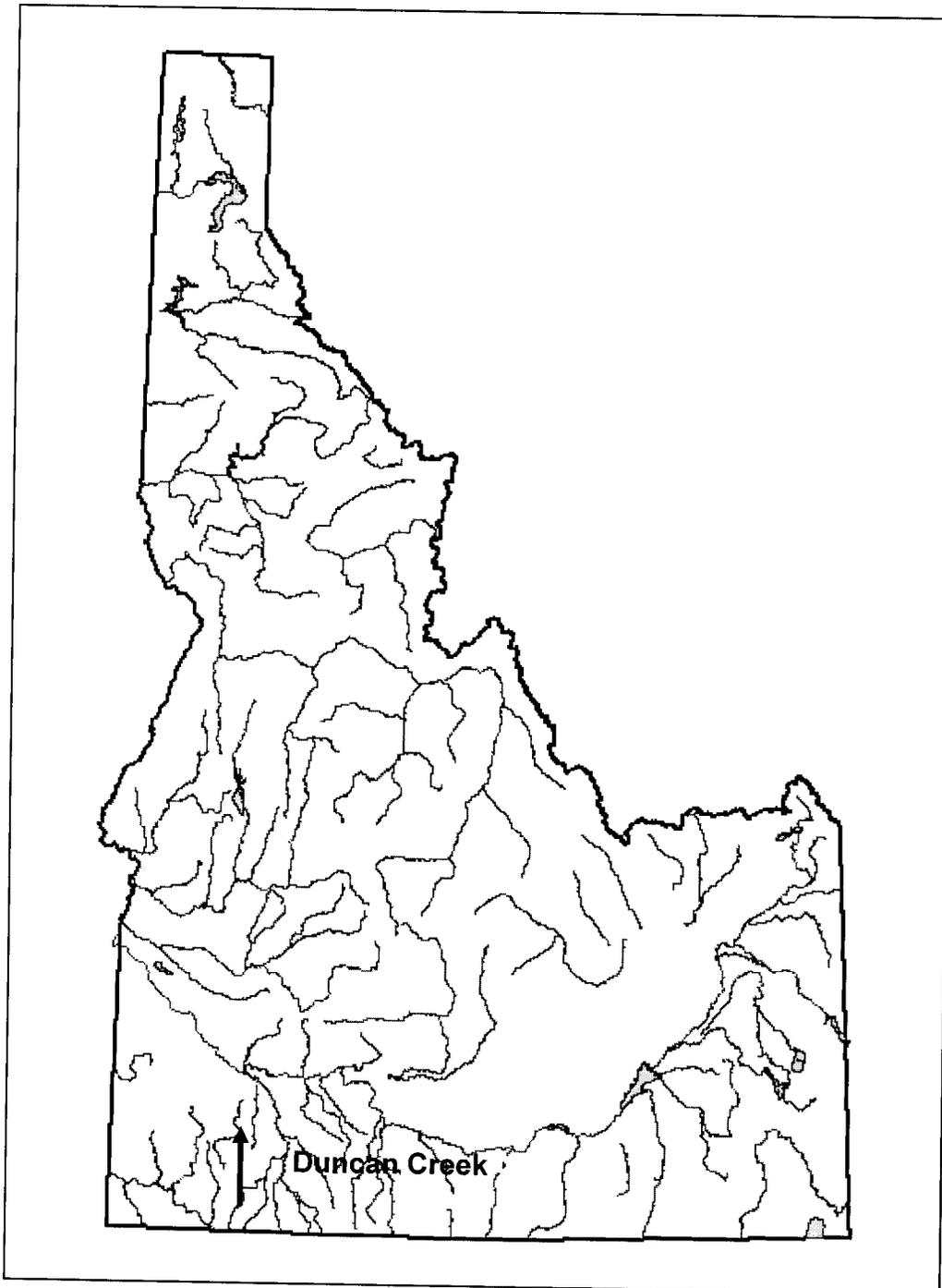
Table 2. Duncan Creek sentinel exposure II prevalence obtained in May 2006 using spinal cord tissues (expressed as number of RBT positive / number of RBT examined).

Sample interval		PCR	PTD
CTU	Days PE		
247	19	7/10	
468	36	4/10	
702	54	7/10	
975	75	6/10	
1651	127		0/10
1885	145		0/10
2106	162		2/10
2340	180		5/10
2574	198		7/10

Table 3. Duncan Creek sentinel exposure III prevalence obtained in Sept.-Oct. 2006 using PCR test with anterior spinal cord tissues (expressed as number of RBT positive / number of RBT examined).

Exposure	Sample I 104 CTU 8 days PE	Sample II 234 CTU 18 days PE	Sample III 351 CTU 27 days PE	Sample IV 468 CTU 36 days PE
A (7 days)	0/10	0/10	0/10	1/10
B (14 days)	0/10	1/10	3/10	1/9
C (21 days)	2/10	2/6	3/6	4/7

Figure 1. Sentinel exposure study area is Duncan Creek, Owyhee County, Idaho.



APPENDICES

Appendix A. Duncan Creek water chemistry parameters during three exposure trials

Trial	Site	Date	Time	Temp. °C	pH	DO	Turbidity	
				mean (std)			mg/l	FTU
I	I	5/5/2005	11:05	9.24 (1.39)	7.54	8	5.15	0.116
	11	5/5/2005	13:30	9.24 (1.39)	7.38	7.44	5.81	0.118
II	II	5/9/2006	12:05	11.72 (1.90)	7.96	7.89	2.21	0.133
	I	5/31/2006	11:34	12.28 (2.64)	8.1	7.8	2.76	0.131
III A	II	9/21/2006	12:05	12.87 (1.39)	7.76	7.66	4.34	0.126
III B	II	9/28/2006	12:30	12.83 (1.29)	8	8.83	2.8	0.125
III C	II	10/5/2006	11:15	12.50 (1.32)	7.95	7.79	3.18	0.125

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A handwritten signature in black ink, appearing to read "Edward B. Schriever", written over a horizontal line.

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