AN ABSTRACT OF THE THESIS OF

Tamsen M. Polley for the degree of Honors Baccalaurate of Science in Microbiology presented on May 31, 2012. Title: Supplemental Description of *Myxobolus squamalis* (Myxozoa) & Epidemiology of *M. squamalis* at Two Oregon Hatcheries.

Abstract Approved: _____
<div align="center">Jerri Bartholomew</div>

Myxobolus squamalis is a Myxozoan parasite of salmonids, which contributes to lowered fitness of hatchery and wild fish in the Pacific Northwest of North America. The only GenBank DNA sequence of *M. squamalis* is from *Oncorhynchus tschawytscha* (Chinook)*,* but is not linked to a published morphological description. There is a range of salmonid Myxozoans with similar morphology to *M. squamalis* and we suspect that some "*M. squamalis*" observations from different host species might be invalid. We provide a clear re-description of *M. squamalis* from its type host, *O. mykiss* (rainbow trout and steelhead). Our results confirm the morphological and morphometric data of the original description, which we supplement with a SSU rRNA gene sequence. Based on this sequence, we developed a PCR assay for specific detection of *M. squamalis* DNA in environmental water samples. We used the assay to compare parasite levels in the water influent and effluent from two hatcheries that rear different salmon and trout species. Our approach allows for early *M. squamalis* detection, infection level estimates, and estimates for environmental impacts based upon spore release. Early detection of Myxozoan infections is crucial to hatchery and wild fish management, as there is no documented treatment for infected fish.

Key words: *Myxobolus squamalis*, salmon, trout, salmonid, parasite, hatchery, epidemiology, sequence.

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Supplemental Description of *Myxobolus squamalis* (Myxozoa) & Epidemiology at Two

Oregon Hatcheries

by Tamsen M. Polley

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Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

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Tamsen M. Polley, Author

ACKNOWLEDGEMENT

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INTRODUCTION

Myxobolus squamalis, originally described by Iversen in 1954, is a Myxozoan parasite of trout and salmon that contributes to lowered fitness of hatchery and wild fish stocks in the Pacific Northwest of North America. *M. squamalis* creates cysts in the scale pockets that can raise the scales to give fish a wart-like, inflamed appearance commonly described as "salmon pox" (Figure 1). Iversen (1954) reports the hosts of *M. squamalis* as: *Oncorhynchus mykiss* (rainbow trout or steelhead), *O. kisutch* (coho), and *O. keta* (chum). Subsequently, the parasite has been reported from *O. mykiss* (Lom and Noble 1984); *O. kisutch*, *O. keta* (Hoskins et al. 1976); O. *kisutch* and *O. tshawytscha* (Chinook) (Olson 1978). This broad host range is atypical for a Myxozoan parasite (Lom & Dykova 2006), and given that there is a range of salmonid Myxozoans with similar morphology to *M. squamalis* (e.g. *M. cerebralis* Hofer 1904, *M. kisutchi* Yasutake & Wood 1957, *M. neurotropus* Hogge *et al.* 2004), we suspect that some of the observations or identifications of "*M. squamalis*" from different host species may be invalid.

DNA sequence data are a powerful tool for description and discrimination of Myxozoan species, especially morphologically ambiguous *Myxobolus* spp. (e.g. *M. insidiosus - M. fryeri* Ferguson et al. 2008). The only DNA sequence of *M. squamalis* in GenBank (U96495; Andree 1997) is from *O. tschawytscha* and is not linked to a published morphological description.

Accordingly, we sought to provide a clear re-description of *Myxobolus squamalis* from its type host, *O. mykiss*. We characterized multiple parasite isolates using morphology, morphometric, and molecular (small subunit ribosomal RNA gene sequence, SSU) data (Table 1). We sourced the parasite from fish raised in a hatchery with a long history of presence of the parasite, first documented in 1984 (Amandi, Oregon Department of Fish and Wildlife, pers. comm.), and tested the possible host range of the parasite by examining juvenile *O. tschawytscha* raised in the same river and hatchery. Our results confirm the morphological and morphometric data of the original description (Iverson, 1954) and supplement this with a robust SSU sequence.

Figure 1. Brood stock rainbow trout at Leaburg state fish hatchery in 2010 exhibiting clinical signs of *M. squamalis* infection: raised scales and white patches free of scales with epithelial damage due to secondary infections.

Photo courtesy of Edson Adriano.

Table 1. *Myxobolus squamalis* records showing host, sample locality, spore measurements and DNA data.
SFH = state fish hatchery; nd = not determined

Record Fish/sample Code	host species, type, life stage	Locality & Date	*n*	spore body (µm)			polar capsules (µm)		polar filaments	DNA sequence length, GenBank accession #
				length	width	thick-ness	length	width	# turns	
Iversen 1954	*O. mykiss* – rainbow trout juveniles *O. kisutch* – coho salmon adults *O. keta* – chum salmon adults	SFHs Seattle & Olympia, WA 1951	110	9.0 8.1-9.9	8.6 7.7-9.9	6.7 5.6-7.7	4.4 3.9-5.1	3.1 2.6-3.9	nd	nd
GenBank Andree *et al.* 1997	*O. tschawytscha* Chinook salmon spawning adult	Nimbus SFH Sacramento R, CA 1999	nd	nd	nd	nd	nd	nd	nd	1932 nt U96495
present study LEP337110	*O. mykiss* rainbow trout adult broodstock	Leaburg SFH McKenzie R, OR March 2011	19	9.2 7.8-10.	8.6 7.8-9.3	nd	4.4 3.9-5.0	3.0 2.6-3.3	4	1633nt
present study 72T09	*O. mykiss* rainbow trout juvenile	Leaburg SFH McKenzie R, OR December 2011	nd	nd	nd	nd	nd	nd	nd	1905nt
present study 71T10 3 fish pool	*O. mykiss* rainbow trout juveniles	Leaburg SFH McKenzie R, OR January 2011	17	8.9 7.8-10.	7.5 6.1-8.2	5.9 5.2-6.4	4.2 3.5-4.8	2.7 2.3-3.4	4	1550nt
present study 2410 StS 4 fish pool	*O. mykiss* summer steelhead trout juveniles	Leaburg SFH McKenzie R, OR January 2011	14	8.9 8.5-9.4	8.1 7.7-8.7	nd	4.5 4.2-4.8	2.9 2.7-3.7	4	1755nt
present study McK	*O. tschawytscha* fall Chinook salmon spawning adult	McKenzie SFH McKenzie R, OR December 2010	nd	nd	nd	nd	nd	nd	nd	*M. insidiosus*
present study RockCkStW	*O. mykiss* winter steelhead trout spawning adult	Rock Creek SFH Umpqua R, OR Date Unknown	22	9.5 8.9-10.	8.8 8.3-9.6	6.7 6.2-7.2	4.5 4.0-4.9	3.1 2.6-3.5	4	1580nt
present study 71B08	*O. mykiss* rainbow trout adult broodstock	Leaburg SFH McKenzie R, OR December 2010	21	8.4 7.3-9.2	7.8 7.3-8.6	nd	3.9 3.5-4.3	2.7 2.1-3.1	4	1600nt
		January 2011	18	8.4 7.4-9.4	7.9 7.1-9.0	6.2 6.2-6.3	4.1 3.3-4.7	2.9 2.5-3.3	4	

MATERIALS & METHODS

Host fish samples and localities

Samples of infected fish were obtained in close coordination with Oregon Department of Fish and Wildlife (ODFW) fish pathologists Jerry Jones and Dr. Tony Amandi. The ODFW regularly monitors health of fish at more than 30 state fish hatcheries (SFH). For this study, we analyzed samples of infected fish from Leaburg SFH (44.136°N 122.610°W) and McKenzie SFH (44.118°N 122.638°W), which lie within 4 km of each other and are supplied with water from the McKenzie River, Oregon, USA. Leaburg Hatchery raises primarily rainbow and steelhead trout, with some Chinook salmon, and has a long history of episodic *M. squamalis*-infections in both juvenile and adult rainbow and steelhead trout. McKenzie Hatchery rears Chinook salmon, in which *M. squamalis* has never been detected. We characterized the parasite also from adult steelhead trout from the Umpqua River basin, at Rock Creek SFH (43.336°N 123.003°W), to assess inter-regional genetic variation. A single adult Chinook salmon from McKenzie SFH was examined.

M. squamalis myxospore samples from fish

Mortalities and fish with clinical signs of infection were collected by hand net directly from hatchery raceways, and were killed or sedated by MS222. Table 1 shows a list of sampled fish. A scalpel or razor blade was scraped gently across the fish skin to obtain mucous and the contents of scale pockets. Wet-mount tissue squashes were examined with a light microscope at 400× to confirm the presence of round, *M.*

squamalis-like myxospores. Higher magnification bright field and Nomarski differential contrast digital images were taken of 10-20 representative spores from each fish. Spores were measured from these images using Macnification (version number 2.0, Orbicule, 2006-2011), following the guidelines of Lom & Arthur (1989) (Figure 2). Spore samples were both air-dried onto glass microscope slides and frozen for later DNA extraction.

Figure 2. Myxospore standard morphometrics by Lom & Arthur (1989), illustrated by Tamsen Polley (2012).

DNA extraction

Total DNA was extracted from frozen fish tissue samples using a DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA) following the Animal Tissue protocol, and eluted with 2 x 60 μL buffer AE.

DNA amplification and sequencing

Myxozoan SSU rDNA was amplified from fish tissue samples in two overlapping fragments: 5' with universal SSU forward primer ERIB1 (ACC TGG TTG ATC CTG CCA G; Barta et al. 1997) and Myxozoan-specific reverse primer ACT1R (AAT TTC ACC TCT CGC TGC CA; Hallett & Diamant 2001), and 3' with Myxozoan-specific forward primer MyxGen4f (GTG CCT TGA ATA AAT CAG AG, Kent et al., 2000) and universal reverse primer ERIB10 (CTT CCG CAG GTT CAC CTA CGG; Barta et al. 1997) (Table 2). PCRs were in 20 μL volumes: 1 μL DNA, 0.5 μL each primer (10 μM), 0.25 μL GoTaq Flexi polymerase (5U/ μL), 0.4 μL dNTPs (10 mM each), 0.5 μL bovine serum albumin (10 mg/mL), 1.0 μL Rediload dye stock, 1.2 μL $MgCl_2$ (25 mM), 4.0 μL Go Taq Flexi clear buffer and 10.65 μL water. PCRs were carried out on a MJ Research PTC-200 thermocycler using cycling conditions: primary denaturation 180s at 95°C; followed by 35 cycles of 94°C for 20s, 55°C for 30s, and 72°C for 90s; then 72°C for 600s for terminal elongation. PCR products were electrophoresed on 1% TAE gels stained with SYBRsafe (Life Technologies, Carlsbad, CA) to verify amplification. Amplicons were purified by incubation with ExoSAP-it (USB Products; Santa Clara, CA) according to the manufacturer's protocol. Amplicons were directly sequenced at the Center for Genome Research and Biocomputing (CGRB) at Oregon State University

(OSU). Sequences were aligned manually in BioEdit (Hall 1997-2011), with reference to sequence chromatograms to clarify any ambiguous bases.

Table 2. Primers.

Primer	Direction	Sequence	Citation
ERIB1	F	ACCTGGTTGATCCTGCCAG	Barta *et al*. 1997
ACT1R	R	AATTTCACCTCTCGCTGCCA	Hallett & Diamant 2001
MYXGEN4F	F	GTGCCTTGAATAAATCAGAG	Kent *et al*. 2000
ERIB10	R	CTTCCGCAGGTTCACCTACGG	Barta *et al*. 1997
MS193	F	CCAACTACCGGCGTAACG	Present Study
MS684	R	CTATTTGATGTTGAAGCAGTGTG	Present Study
MS205	F	GTAACGGCTTGCTGTTGC	Present Study
MS655	R	GGTACTACATCTGTTTCAACGTT	Present Study

Syntype reference material

Air-dried slides of myxospores were fixed in methanol and stained by Diff-Quik (Dade Behring Inc., Newark, DE). Slides were air-dried and cover slipped, and those that contained the most representative spores were deposited in the Parasitology Collection at the Queensland Museum, Brisbane, Australia, along with spores in 100% ethanol (Accession numbers pending).

RESULTS

Variation between hosts & localities

There was no significant intra-specific variation between spores of differing localities, sampling dates, or source stocks (all samples originated from *Oncorhynchus mykiss*). *M. squamalis* was not found in any Chinook salmon samples taken from either hatchery.

Spore morphology

Myxospore morphology (Figures 3) was equivalent to the previously described and illustrated description (Iversen, 1954) (Figure 4). Similarities included the illustrated (Figure 4) and photographed sutural ridge (Figure 3) and four turns of the polar filament (Figure 3 & 4).

Figure 3. Morphology of *Myxobolus squamalis* myxospores: A) Leaburg hatchery rainbow trout (RbT), 2010; B & C) winter steelhead in Rock Creek; C); D) spring-run steelhead, 2011; E) Leaburg hatchery RbT, 2010; F&G) Leaburg hatchery RbT , 2011; H&I) Leaburg, RbT, 2011.

Figure 4. *M. squamalis* myxospore illustrations by Iversen (1954).

PLATE II

5 μ

A   B   C   D   E

Spore morphometry

Our spore measurement data was equivalent to the type description (Iversen 1954; Table 1). The difference between Iversen's average values and the calculated values ranged from 0 to 0.47 microns and fall within accepted standard deviations.

SSU Sequence

We submitted consensus sequences of *M. squamalis* from rainbow trout and steelhead trout to GenBank (accession number pending, respectively). No polymorphisms were observed within any sample that we sequenced and no variations were seen between any samples. A BLAST search revealed the closest relatives of *M. squamalis* to be the morphologically similar *M. neurobius* (95% similarity; 1532/1613 nt) and *M. cerebralis* (93% similarity; 1670/1796 nt). Our *M. squamalis* sequence was only 77% similar over 1785nt to GenBank *M. squamalis* sequence U96495 of Andree, 1997.

DISCUSSION

Our results fully supported the original taxonomic description of *Myxobolus squamalis* Iversen, 1954 with respect to host, tissue, spore morphometry and morphometrics.

Iversen's original published illustrations display an ovoid spore with a sutural ridge and 4 turns of the polar filament within the polar capsules. The sutural ridge is similarly shown in the side profile of all *O. mykiss* myxospore isolates. Four turns of the polar filament is similarly shown in all myxospore images of the isolates used for the supplemental description. Myxospore isolates were collected at various localities and times of the year from rainbow and steelhead hosts, and exhibited little to no morphometric variation between isolates and Iversen's description (Table 1).

There is a range of salmonid Myxozoans with similar morphology to *M. squamalis* (e.g. *M. cerebralis* Hofer 1904, *M. kisutchi* Yasutake & Wood 1957, *M. neurotropus* Hogge *et al.* 2004) and may be misidentified as "*M. squamalis*" from different host species. *M. cerebralis* is a Myxozoan that destroys the cartilage of the head and vertebral column resulting in "whirling disease," and can be visually confused with *M. squamalis.* Due to *M. cerebralis* infecting cartilaginous tissue it is unlikely they would be isolated together. If contamination occurred *M. cerebralis* would be easily recognized as a misidentification because it is well described and sequenced (U96492). *M. kisutchi* (EF431919) and *M. neurotropus* (DQ846661) are also listed on GenBank and exhibit differing tissue tropism than *M. squamalis*. We are confident in the accuracy of the new description of *M. squamalis* because of the isolates originating from varying localities,

sampling dates, and hosts, and the spore measurements being analogous to Iversen's original description.

The current *M. squamalis* GenBank entry by Andree *et al.* was BLAST searched for sequence similarity resulting in high identity with *M. insidiosus* (97%), *M. fryeri* (97%), and other *Myxobolus* species. The GenBank sequence appears inherently erroneous, being possibly chimeric. We supplemented the description with SSU rRNA gene sequence data. We found that the *M. squamalis* SSU rRNA sequence was identical from multiple McKenzie River rainbow and steelhead trout and from a steelhead trout from the geographically distant Umpqua River basin. We consider our sequence data to be a robust, accurate identifier of *M. squamalis* that could be used for future molecular identification of the parasite and recommend that the GenBank entry U96495 for *M. squamalis* be renamed as an unknown species (*Myxobolus* spp.) or removed, to prevent future confusion. These data are not linked with any morphological description of the source material, which renders critical comparison impossible.

Rainbow and steelhead trout exhibit mild infections but no visibly severe skin damage, heavy infections are required for visible lesions to form. In contrast to documented coho infections that result in raised bumps and sores along the sides and belly of the fish ("salmon pox"). We speculate that skin damage caused by *M. squamalis* may lead to secondary infections in heavily infected, immunodepressed, or spawning fish (Figure 5). While it remains an open question whether Chinook salmon (*O. tschawytscha*) are a permissive host of *M. squamalis*, our data suggest that it is not.

We surveyed historical ODFW fish pathology data for juvenile Chinook from McKenzie hatchery, and could find no record of *M. squamalis* infection, whereas there is

a long history of infection in rainbow trout from Leaburg hatchery, which lies some 4 km upstream on the same water system. The single Chinook salmon from McKenzie SFH that we analyzed molecularly, did not yield an amplicon of *M. squamalis* instead we identified *M. insidiosus*. Rainbow trout held previously at McKenzie hatchery became infected with *M. squamalis*, which demonstrated that the hatchery water supply, at least episodically, contains infectious *M. squamalis* stages. It is possible that there are different genotypes of *M. squamalis* in other river basins with Chinook salmon host specificity. However, we concluded that Chinook salmon in the McKenzie River are not susceptible to parasitism by *M. squamalis*. To determine the source and environmental effect of the parasite we have initiated a project to assess water samples above and below both hatcheries.

Figure 5. Brood stock rainbow trout at Leaburg SFH in 2011 exhibiting severe secondary infections.
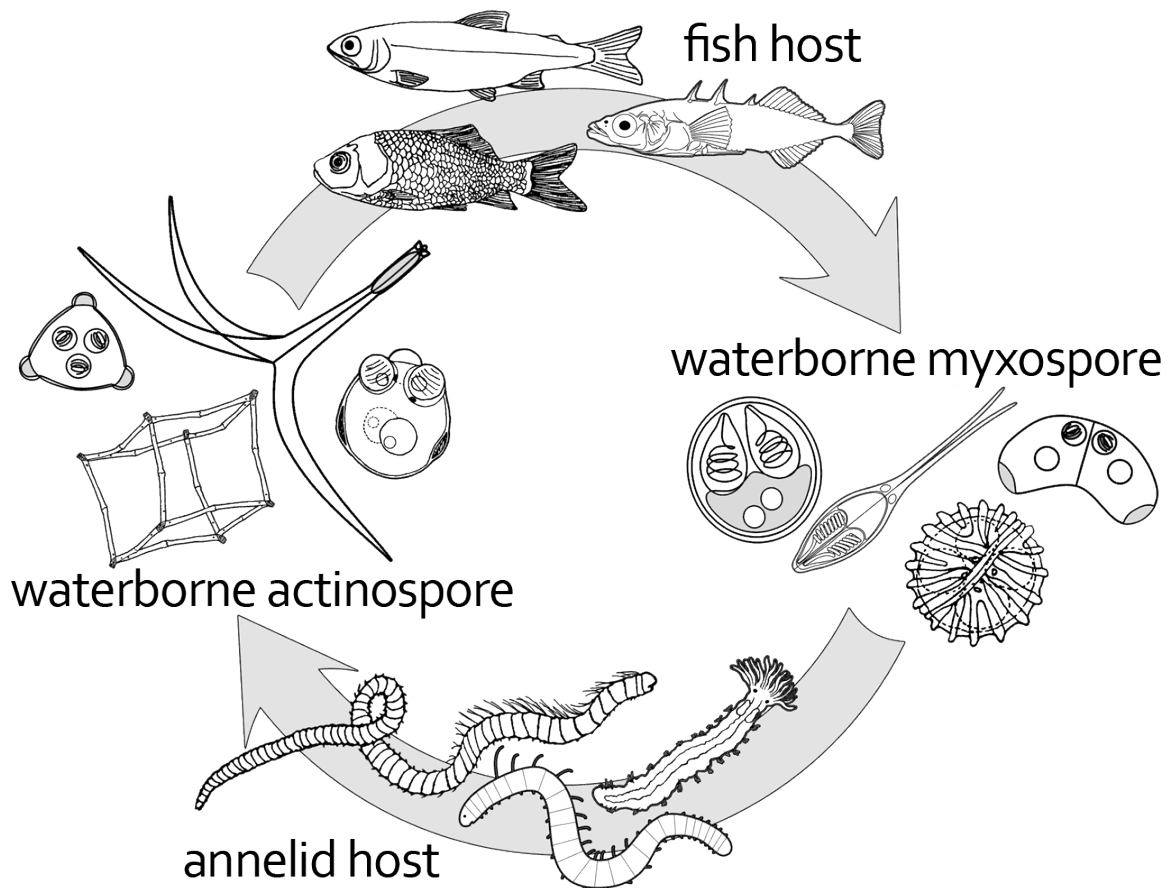
CHAPTER 2: Epidemiology of *Myxobolus squamalis* at Two Oregon hatcheries

INTRODUCTION

Myxobolus squamalis (Iversen 1954) is a Myxozoan parasite of trout and salmon and contributes to lowered fitness of hatchery and wild fish stocks in the Pacific Northwest of North America. The parasite creates cysts in the scale pockets that give fish a wart-like, inflamed appearance commonly described as "salmon pox." The cysts can rupture and create white pustules or lesions (Figure 1) (Iversen 1954). The damaged skin may affect the market value of the fish (Kent et al. 1994) and ruptured cysts may permit secondary pathogens (Amandi, Oregon Department of Fish and Wildlife, pers. comm.) (Figure 5). The parasite has been described from 4 host species, *Oncorhynchus keta* (chum salmon) (Iversen 1954, Hoskins et al. 1976), *O. kisutch* (coho salmon) (Iversen 1954, Hoskins et al. 1976, Olson 1978), *O. mykiss* (rainbow or steelhead trout) (Iversen 1954, Lom and Noble 1984) and *O. tshawytscha* (Chinook salmon) (Olson 1978). Early detection and prevention of Myxozoan infections is crucial to hatchery and wild fish management, as there is no documented treatment for infected fish. The life cycle of *M. squamalis* is unknown, but other *Myxobolus* species are known to have complex life cycles that involve obligate host vertebrates (fish) and invertebrates (oligochaete worms), with two waterborne spore stages (Kent et al. 2001) (Figure 6).

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Figure 6. General Myxozoan lifecycle courtesy of Dr. Stephen Atkinson, PhD thesis, 2011.

fish host

waterborne myxospore

waterborne actinospore

annelid host

We recently provided a supplemental description of *M. squamalis* from rainbow trout, *Oncorhynchus mykiss* (Polley et al. 2012). Our morphological and morphometric data were consistent with the original description, which we augmented with small subunit ribosomal gene (SSU) sequence data. We showed that the parasite SSU sequence was identical in rainbow and steelhead trout (both *O. mykiss*) from different river basins.

In the present study, we used our SSU sequence data to develop a specific polymerase chain reaction (PCR) assay for identification of *M. squamalis* DNA in water and tissue samples. *M. squamalis* is morphologically similar to other myxospore types (e.g. *M. cerebralis* Hofer 1904, *M. kisutchi* Yasutake & Wood 1957, *M. neurotropus*

Hogge *et al.* 2004), which may lead to misidentification. Thus a *M. squamalis*-specific

assay would be beneficial as a molecular diagnostic tool. PCR analysis of water samples

offers an economic alternative to sentinel fish studies for Myxozoan detection. The assay

will be applied to water samples collected from Leaburg and McKenzie SFH for *M.*

squamalis detection. Parasite levels of the influent and effluent of the hatcheries were

compared, providing estimates of hatchery infection and effects on the adjoining

McKenzie River system through spore release.

MATERIALS & METHODS

M. squamalis-**specific primer design**

We used our *M. squamalis* consensus sequence (GenBank accession number pending) (Chapter 1) to design four specific primers (Table 2). The primers were designed manually in BioEdit (Hall 1999) to anneal to variable regions of the SSU. Primer sequences were BLAST searched against the GenBank database to test for sequence homology with non-target taxa. Primers were tested with *M. squamalis* positive control (71T10 sample) and with water samples from Leaburg and McKenzie SFH. We chose two primers (MS193, MS684) on which to base our assay.

Sampling localities

Water was sampled above and below two Oregon state salmonid hatcheries that lie within 5 km of each other on the McKenzie River, Oregon, USA (Figure 7). McKenzie SFH rears Chinook salmon, while Leaburg SFH, which lies upstream, rears primarily rainbow and steelhead trout, with some Chinook salmon. Leaburg SFH has a long history of *M. squamalis* infection in juvenile and adult rainbow trout and steelhead. Water enters Leaburg SFH from the adjacent Leaburg Reservoir (L1) and flows through a concrete channel before entering the raceways (L2) (Figure 8). We sampled water from either end of this channel to determine if the parasite was originating from the reservoir or from the open channel. We also sampled the total outflow of Leaburg (L4) and the partial outflow (L5) to detect any differences between raceway spore productions. McKenzie SFH receives water diverted from the Leaburg reservoir, the Leaburg Canal (M1), and a

separate source, Cogswell Creek (M2) (Figure 9). The total outflow of McKenzie SFH was also sampled (M6).

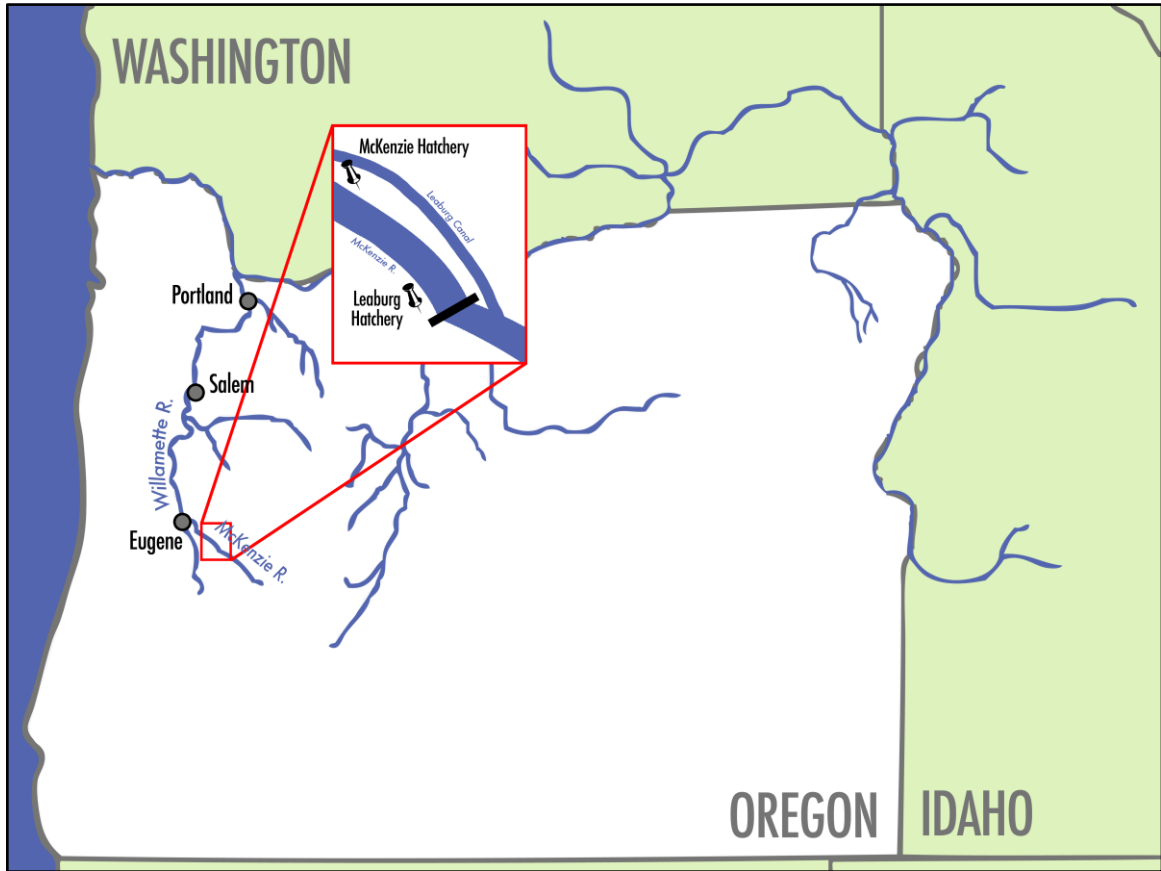**Figure 7**. Oregon map showing state fish hatchery localities.

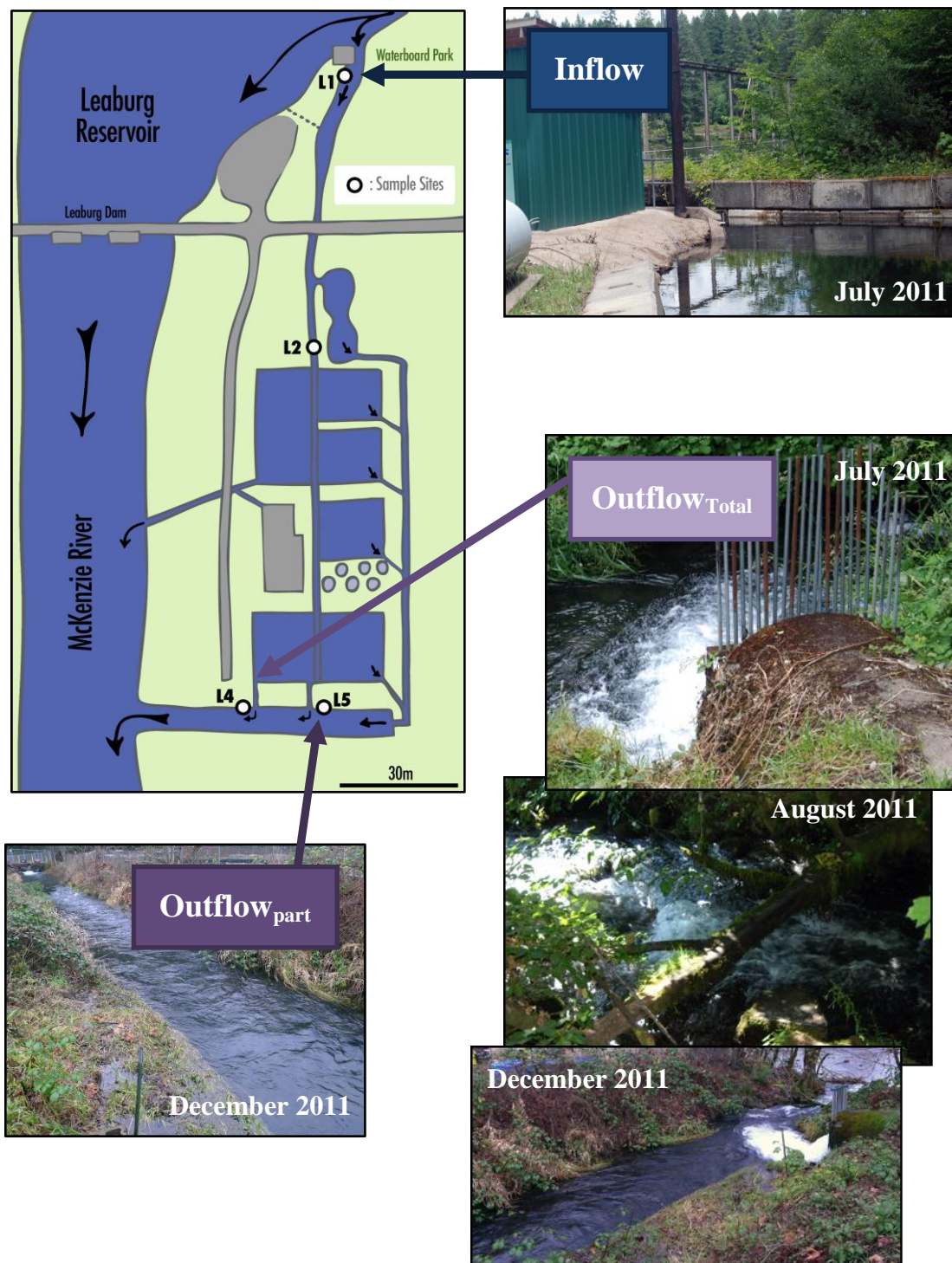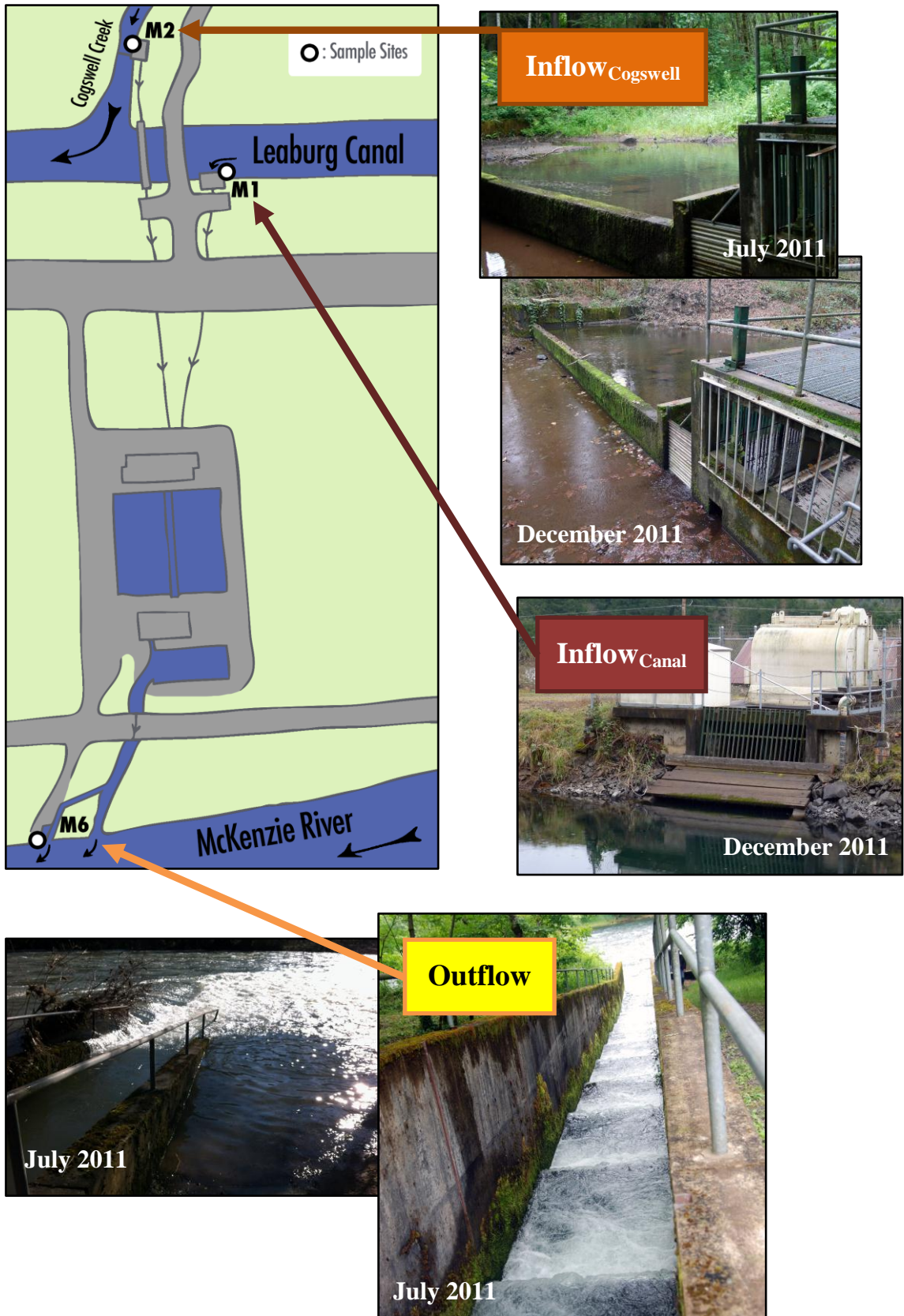**Figure 8**. Leaburg SFH sample sites. Outflow site is pictured during the winter and early and late summer.

Figure 9. McKenzie SFH sample sites. The inflow site is pictured in both summer and winter.

Environmental water sample collection

Water samples were taken monthly at approximately the same time of the day from April 2011 through March 2012. Water temperature was noted at time of collection; additional temperature data were obtained from Leaburg and McKenzie hatchery. Photoperiod data were obtained from the Online-Photoperiod Calculator (Lammi 2008). Triplicate one-liter water samples were collected in plastic bottles from each site and kept cool during return to the laboratory.

Water sample filtration and acetone treatment

Samples were filtered within 24-hours of collection according to the protocol of Hallett and Bartholomew (2006). Briefly, each 1 L sample was vacuum-filtered through a 47 mm diameter, 5 μm nitrocellulose filter (MF-Millipore); the filter was then folded and placed in a 2 mL microcentrifuge tube and stored frozen. Prior to DNA extraction, filters were processed according to the acetone dissolution protocol of Hallett et al. (2012).

DNA extraction of water samples

Total DNA was extracted from acetone-treated filter disks using a DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA) following the Animal Tissue: Spin-Column Protocol. DNA was recovered from columns with 2 x 60 μL elutions of buffer AE and stored at -20°C.

Spore standards

Two sets of spore standards were fashioned by depositing single and ten myxospores in microcentrifuge tubes by pipette, to determine the sensitivity of the PCR assay and to estimate DNA losses from the acetone treatment of the filter paper. One group of one and ten myxospores was processed using only the DNA tissue extraction protocol with only myxospores present in the tube. The other group was processed with an addition of a 5 μm x 47 mm filter (MF-Millipore) and roughly 0.1 gram of sediment for acetone treatment and DNA extraction, to simulate the water sample processing.

Water sample PCR analysis

Primers MS 193 (forward, 5` CCAACTACCGGCGTAACG 3`) and MS 684 (reverse, 5` CTATTTGATGTTGAAGCA 3`) were used to assay all water samples (Table 2). Reactions were performed in 20 μL: 1 μL DNA, 0.5 μL each primer (10 μM), 0.25 μL GoTaq Flexi polymerase (5 U/ μL), 0.4 μL dNTPs (10 mM each), 0.5 μL bovine serum albumin (10 mg/mL), 1.0 μL Rediload dye stock, 1.2 μL $MgCl_2$ (25 mM), 4.0 μL GoTaq Flexi clear buffer and 10.65 μL water. PCRs were carried out on a MJ Research PTC-200 thermocycler using cycling conditions: primary denaturation 180s at 95°C; followed by 35 cycles of 94°C for 30s, 58°C for 30s, and 72°C for 45s; then 72°C for 600s for terminal elongation. The PCR was repeated using first round amplicons as template. PCR products were electrophoresed on 1% TAE gels stained with SYBRsafe (Life Technologies, Carlsbad, CA) to visualize amplification on an ultraviolet imaging device.

RESULTS

M. squamalis was detected using the specific PCR assay on environmental water samples and positive controls. The assay was also found to be easily sensitive to one myxospore.

M. squamalis-specific primer design

Chosen primer sequences resulted in no BLAST recognition with other common Myxozoa. All combinations of forward and reverse primers were tested to ascertain which pair gave optimal amplification. Primer set MS193 and MS684 created a 491 bp amplicon and was chosen for all subsequent water sample detections. The alternative primer set, MS205 and MS655, when tested against water samples, resulted in unknown, multiple band amplification and questionable sensitivity. Amplicons were sequenced from several water samples to verify consistency and specificity of the primers to *M. squamalis*. A two-round PCR proved more sensitive than a single-round PCR for water samples. Primer specificity was not assessed *in vitro* on samples of other commonly co-occurring Myxozoans, including *M. cerebralis*, *Ceratomyxa shasta* and *M. insidiosus* and should be done in future work.

Spore standards

The tissue extraction protocol of the spores resulted in lower *M. squamalis* DNA loss than the acetone or filter protocol (Table 3) (Figure 10). Tissue extractions of the one-spore samples resulted in two of the three samples being amplified. The acetone and filter protocol on the one-spore samples resulted in zero of the three samples detected, meaning
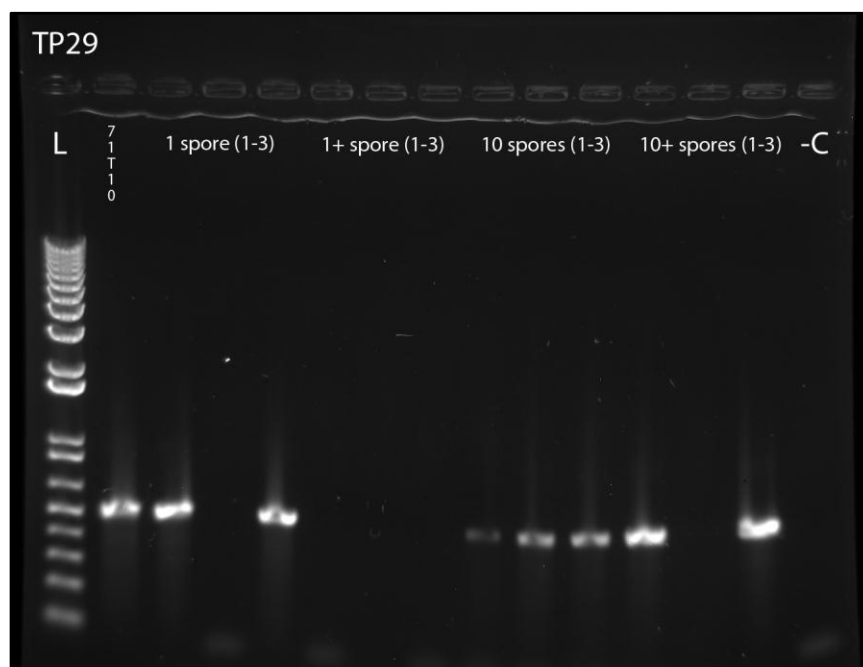
sufficient amplified DNA was lost to render the remainder undetectable by the PCR assay

(may be detectable using qPCR). The ten spore samples showed variable DNA loss: the

tissue extraction had 3/3 amplify to detectable levels, while the ext.+filter/soil extraction

had 2/3 detected. All acetone and filter protocol samples had higher spore loss than their

tissue extraction comparisons.

Table 3. Spore standard results: comparison between 1 and 10 spore DNA extraction
protocols.

1 Spore						10 Spores					
Tissue Extraction			Ext.+Filter/Soil			Tissue Extraction			Ext.+Filter/Soil		
+	+	-	-	-	-	+	+	+	+	+	-

+	Positive
-	Negative

Figure 10. Spore standard PCR gel image. 1 and 10 spore samples from tissue extraction
and the 1+ and 10+ spore samples contain a filter and soil from the acetone protocol and
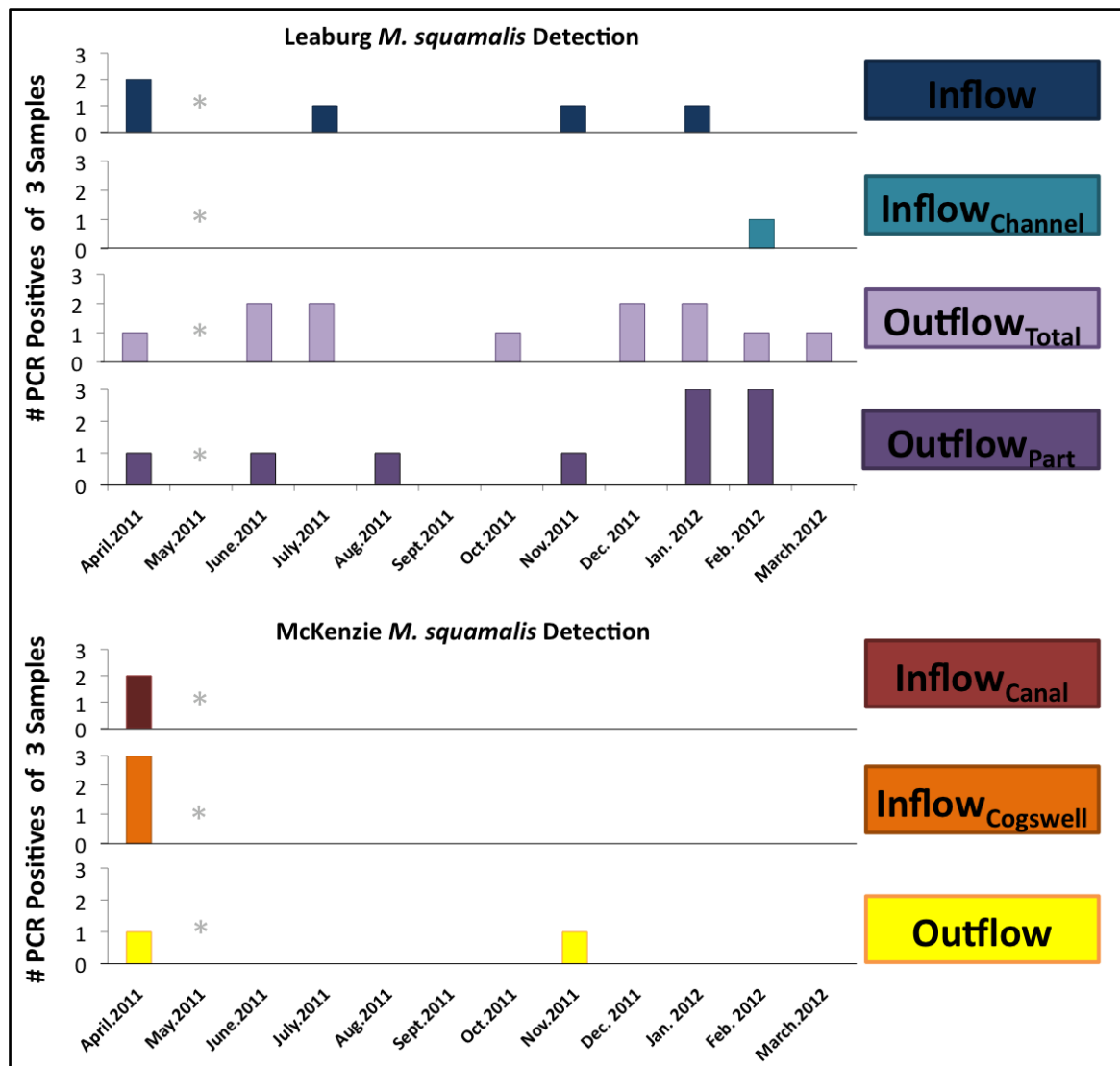extraction.

Sampling localities

Detection of *M. squamalis* in water samples varied between McKenzie and Leaburg SFH, with 0/3 L being none, 1/3 L being low, 2/3 L being medium, and 3/3 L being high detection. McKenzie SFH rears only Chinook salmon and *M. squamalis* detection was consistently low, with a single, large spike in detection in April 2011 (Figure 11). *M. squamalis* was detected in McKenzie SFH in two of the eleven months sampled. In April 2011 Cogswell Creek, one of the two influent water sources used by McKenzie SFH, demonstrated high detection of *M. squamalis*, and Leaburg Canal, the second inflow to the facility, showed medium detection (Figure 8). McKenzie SFH outflow is released directly into the McKenzie River and had a low detection in April 2011 and November 2011 only; but no parasite was detected in the inflow in November. Adult rainbow trout also were observed ascending Cogswell Creek in November 2011, and salmonid fry have been seen upstream of the pump station. In contrast, *M. squamalis* was detected from both the influent and effluent of Leaburg SFH during nine of the eleven months sampled (Figure 11). Leaburg SFH also holds a resident population of susceptible rainbow trout all year, both juveniles and adults. The rainbow trout brood stocks were heavily infected with *M. squamalis* (winter 2010 & 2011) with visible white lesions (Figure 1).

Spatial & temporal distribution at two hatcheries

Water from the influent and effluent of both McKenzie and Leaburg hatcheries was sampled monthly for *M. squamalis* detection with the PCR assay. Water temperature was also recorded concurrently with the water sampling. *M. squamalis* was detected at least once from all sampled sites over the eleven month sample set (April 2011 − March 2012),

and Leaburg hatchery showed significantly more assay positives than McKenzie (Figure 10). There was no obvious correlation between *M. squamalis* detection, temperature, or photoperiod of the coinciding sampling localities (Figure 11-13).

Figure 11. Spatial and temporal distribution of *M. squamalis* at two hatcheries on the McKenzie River.

* May 2011 not sampled.

Figure 12. Recorded temperatures at two state fish hatcheries on the McKenzie River.
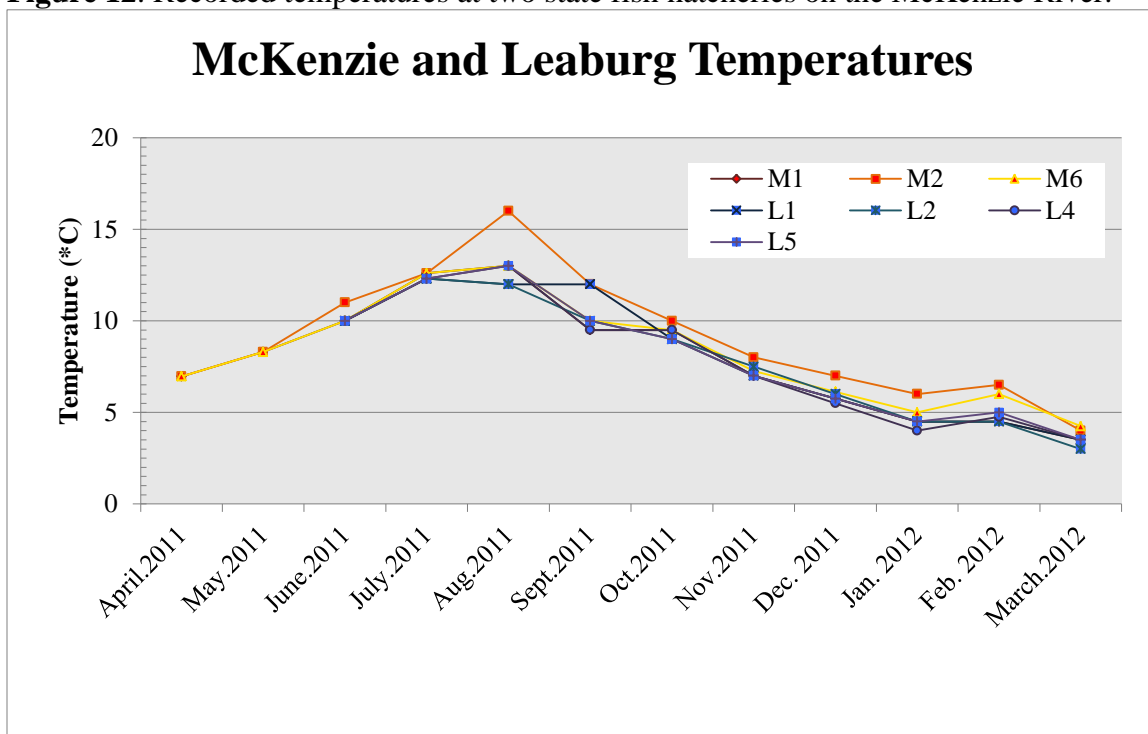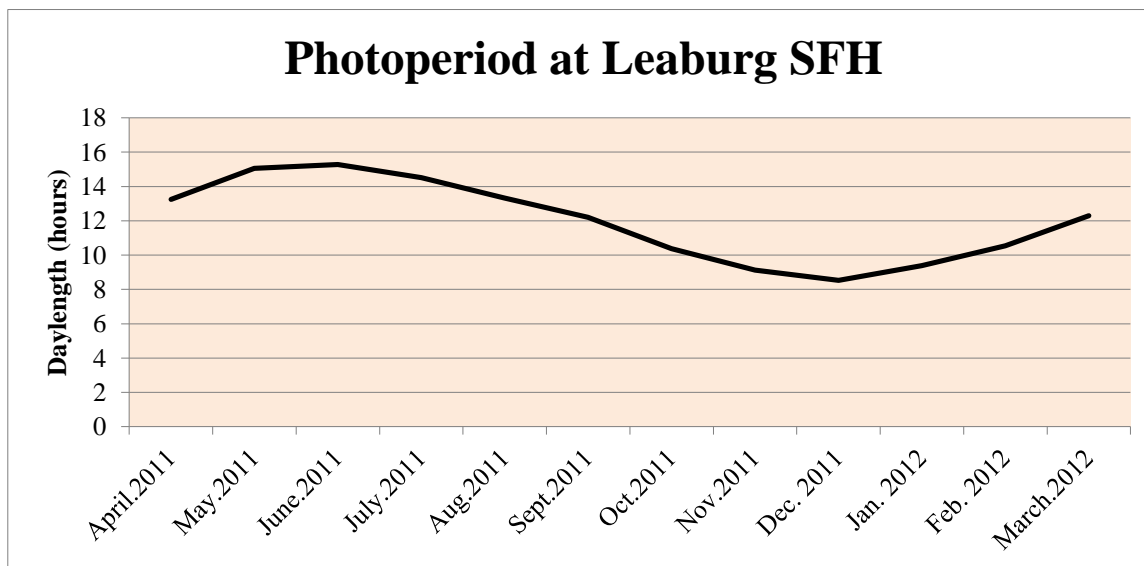

Figure 13. Recorded photoperiod data at Leaburg SFH on the McKenzie River.

DISCUSSION

M. squamalis **salmonid host specificity**

McKenzie and Leaburg SFH were chosen for our sampling localities because of their history of *M. squamalis* infections in *O. mykiss* and their current practice of rearing Chinook salmon. Juvenile and adult Chinook have never been seen as hosts to *M. squamalis* at either McKenzie or Leaburg SFH, even when raised on the same water supply as infected *O. mykiss* (Leaburg SFH). These data lead us to believe that McKenzie river Chinook salmon are not susceptible to parasitism by *M. squamalis*.

M. squamalis-**specific primer design**

A *M. squamalis* PCR assay was used to amplify parasite DNA from infected fish and environmental water samples. Primer specificity was determined by BLAST analysis with available gene sequences on GenBank; however, specificity was not assessed *in vitro* on samples of other commonly co-occurring Myxozoans, including *M. cerebralis*, *Ceratomyxa shasta*, and *M. insidiosus*, and is future work that will need to be done. DNA amplified in *M. squamalis*-positive water samples was sequenced to assess specificity and resulted in accurate alignment to the previously described *M. squamalis* consensus sequence (Polley *et al*. 2012).

Hatchery contribution

The number of positive water samples was higher in the outflow than the inflow of Leaburg SFH, indicating amplification of the parasite in the hatchery. Detection of M.

squamalis DNA changed seasonally, with higher detection spring. The high-density raceways of infected fish release *M. squamalis* myxospores constantly. Juvenile *O. mykiss* did not show severe pathology even with high spore counts obtained in their skin scrape, allowing infected fish to continue shedding spores undetected.

Utility of the PCR assay

A PCR assay that can be applied to water samples quickly and economically will allow hatcheries to estimate *M. squamalis* levels within and around hatcheries or water systems. Currently, only qPCR is utilized for Myxozoan detection in water samples, and both the machine and reagents are expensive. PCR detection of *M. squamalis* would provide a lower cost alternative to parasite detection.

Spore standards

DNA is lost during the acetone treatment of the Millipore filters shown by the spore standards. Even with the loss of DNA with the acetone protocol for water sample processing, the PCR assay demonstrated a detection limit of about ten spores per liter of river water sampled when DNA loss is taken into account.

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