Final Report

Description of the timeline of *Ceratomyxa shasta* myxospore maturation in adult Chinook salmon carcasses

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ABSTRACT

Ceratomyxa shasta is a myxozoan parasite of salmonids which causes extensive losses of outmigrant Chinook salmon smolts in the Klamath River. The parasite has an indirect life cycle: the actinospore stage develops in the freshwater polychaete *Manayunkia speciosa* and is infective to the salmonid. The myxospore stage develops in the salmonid and is infective to the polychaete. The lifecycle seems to have evolved to take advantage of anadromy exhibited by Pacific salmon. Returning adults are infected during their upstream migration and carry the parasite high up in the watershed. Completion of the lifecycle likely happens late in life or after death of the fish, at which time myxospores are released and polychaetes in the river are infected. Little is known about the chronology of myxospore production in returning adults and carcasses. In this study 40 spawned Chinook carcasses were obtained from Iron Gate Hatchery on the Klamath River in Hornbrook, CA and screened for infection by rectal swab. No myxospores were seen, but possible pre-sporogonic stages were seen. One carcass was placed in each of six artificial stream units at the Humboldt State University Fish Hatchery and allowed to decompose. Water samples were to be collected for genetic analysis to detect/quantify parasites as the carcasses decomposed in order to describe the chronology of myxospore production. The experiment was terminated after a week due to leaks in the sumps of four stream units as well as fungal growth which clogged the standpipes and caused overflow from the units. A secondary experiment involved incubation of pieces of intestine from Bogus Creek carcasses. Incubation was performed under different sets of conditions (temperature, pH, atmospheric gas, and added nutrient level) to investigate possible triggers for myxospore formation. Wet mounts were observed every day for two weeks. No myxospores were seen after field screening, and no changes were observed during incubation.

INTRODUCTION

Ceratomyxa shasta is a myxozoan parasite of salmonids that produces the disease ceratomyxosis. Infected fish can be found throughout the Klamath Basin but the stage infective to fish appears largely limited to the main stem of the Klamath River (Hendrickson et al. 1989, Stocking et al. 2006). Recent studies monitoring prevalence of selected fish pathogens in smolts sampled during outmigration implicated *C. shasta* as the direct cause of extensive losses in Chinook salmon (Williamson and Foott 1998, Foott et al. 1999, 2002, 2003). Foott et al. (2002) suggested that ceratomyxosis appeared to be the leading cause of disease and death in juvenile Chinook salmon in the Klamath River and estuary during summer of 2001. Williamson and Foott (1998) and Foott et al. (1999) suggested that reduced levels of infection in the Klamath River estuary as compared to the main river was due to death of infected fish prior to reaching the estuary.

Ceratomyxa shasta has an indirect life cycle (Bartholomew et al. 1997). Fish are infected by actinospores produced within the freshwater polychaete *Manayunkia speciosa.* Development in fish takes place primarily in the posterior intestine and ends in the production of myxospores which are released into the water. Myxospores are taken in by the polychaete. A rather complicated development takes place in the polychaete (Meaders and Hendrickson 2009, Bjork and Bartholomew 2008) leading to the production of the actinospore stage which is again infective to fish.

The life cycle seems to have evolved to take advantage of anadromy exhibited by Pacific salmon. Adult Chinook salmon returning to freshwater bring the infection back into freshwater with them or are more likely re-infected upon re-entry into freshwater. They carry early

developmental stages of *C. shasta* back to the top of the watershed and then spawn and die. Myxospores are likely produced only very late in life or after death. Myxospore development probably takes place in carcasses and myxospores are likely released as carcasses decompose. Slezak (2009) found that a high percentage of post-spawned adults were infected with prespore stages but only a few actually produced myxospores under his experimental protocol. Little is known about the chronology of myxospore production in returning adults and carcasses.

An unpublished pilot study by Bartholomew et al. (2009) investigated the effect of removing adult Chinook salmon carcasses on *C. shasta* levels in Bogus Creek, a tributary that enters the Klamath just below Iron Gate Dam. One hundred carcasses in reach 2 of Bogus Creek were sampled over four occasions for infection by visual identification of myxospores in intestinal scrapings. Infection prevalence ranged from 13% to 40% and averaged 30% overall. Scrapings from decomposed carcasses contained significantly higher numbers of myxospores than those from fresh ones. Subsamples from two sampling occasions, consisting of material from both myxospore positive and negative intestinal scrapings, showed higher prevalence of infection (56% overall) when assayed with qPCR. The investigators suggest that either a low number of myxospores were present and were missed visually, or that DNA from pre-sporogonic stages was present. They concluded that this may suggest parasite maturation is occurring within carcasses, or that some parasites are not completing their life cycle (not maturing to myxospores).

The primary purpose of our study is to describe the timeline of myxospore production in adult Chinook salmon carcasses. This includes when spore production begins, when it peaks, and when it ends. Tracking myxospore production through time will also allow us to estimate the total number of myxospores produced by a single infected spawned Chinook salmon carcass. This number, when applied to total escapement for Chinook in the basin and the overall infection

rate, will produce an estimate of the total myxospore production from adult carcasses in the basin (assuming roughly equal infection prevalence in all tributaries).

As a secondary task we will investigate possible triggers of sporogenesis in pre-sporogonic stages of *C. shasta* in adult carcasses. Variables to be investigated will be temperature, atmospheric gas, pH, and external nutrient concentration.

MATERIALS AND METHODS

Timeline of Myxospore Production

Artificial stream units at the Humboldt State University (HSU) Fish Hatchery were used to hold carcasses as they decomposed. They were located on a raised deck outside of the main hatchery building. There was no electronic temperature control, so temperature varied with daily ambient temperature as would occur in the natural environment. Each trough measured 15 feet 10 inches long, by 16 inches wide, by 7 inches deep. Gradient was very low. Water was pumped into the upper end through 3.5 inch inner diameter PVC pipe, flowed to the lower end, and drained to the sump through a 6 inch inner diameter PVC standpipe. There was a screen across the trough about 4 inches up from the standpipe to keep coarse materials from clogging the standpipe, and a finer screen over the opening of the standpipe. Each trough was covered by plastic screens on wooden frames that could be lifted off to access the trough. Water depth in the trough was about 6 inches. Sumps measured 46.5 inches long, by 14.5 inches wide, by 16 inches deep. Water was pumped from the sump to the upper end input by a Sweetwater 115/230 volt, 1 horsepower pump. Stream units were filled with hatchery water, which comes from a small pond in the redwood forest behind the hatchery.

On October 30, 2009 forty adult Chinook carcasses were obtained from Iron Gate Hatchery (Hornbrook, CA) immediately after being spawned, and transported back to HSU on ice. Sex was recorded for each carcass, and each was weighed, measured, and tagged with an individual ID number. Intestinal material was sampled from each carcass by rectal swab with a sterile bacterial loop. Wet mounts were prepared and screened for parasites. Infection was determined by presence of myxospores or pansporoblasts.

Six carcasses were selected and one was placed in each stream unit on October 31. The remaining carcasses were bagged and frozen. Once every three days, three one-liter water samples were to be collected from each stream unit and the unit would then be drained and refilled. Water samples were to be filtered through 5µm nitrocellulose filter membranes, which would be stored at -35°C pending genetic analysis. Real-time PCR would be used to detect and quantify *C. shasta* DNA in the samples as described by Hallett and Bartholomew (2006).

Triggers For Myxospore Formation

Twenty-two carcasses from Bogus Creek, a tributary which enters the Klamath River just below Iron Gate Hatchery, were screened for this part of the study. On November 18, 2009 a California Department of Fish and Game carcass crew was removing carcasses from the creek. As they did so, we took some aside and recorded sex, length, and weight. Wet mounts were prepared from intestinal material collected by rectal swab, and examined for myxospores. Four contained myxospores. The hindguts were removed from these fish and transported back to HSU on ice. Five others were also collected.

Back at HSU, intestines from infected fish were sliced open longitudinally and then cut into numerous small square pieces. Several 96-well PCR plates with 0.2 mL wells were cut into 4x6-

well sections and set up according to Figure 1. Each 4x6 plate contained three 2x2 blocks with samples and three 2x2 blocks left empty.

pH 4 (fish 1)	pH 6 (fish 1)	empty	empty	pH 4 (fish 3)	pH 6 (fish 3)
pH 8 (fish 1)	pH 10 (fish 1)	empty	empty	pH 8 (fish 3)	pH 10 (fish 3)
empty	empty	pH 4 (fish 2)	pH 6 (fish 2)	empty	empty
empty	empty	pH 8 (fish 2)	pH 10 (fish 2)	empty	empty

Figure 1. Arrangement of pH treatments within each 4x6-well plate. Each 2x2 block of pH treatments contained tissue pieces from a different fish. Three fish were used for the experiment. Sixteen replicate plates were set up, and each was incubated at either 5, 10, 15, or 20°C in a bag filled with either ambient air, carbon dioxide, oxygen, or nitrogen (16 temperature/gas combinations).

Gut tissue from one of three fish was placed in the pH wells. Thus each 4x6 plate contained tissue from three fish, and each of the tissues was treated with four levels of acidity. The same three fish were used for every 4x6 plate. Two of the fish showed possible prespores during screening, and one showed myxospores and a pansporoblast in addition to possible prespores. Sixteen 4x6 plates were set up and incubated in plastic sandwich bags. Four bags were filled with ambient air, four with carbon dioxide, four with oxygen, and four with nitrogen. Of the four bags filled with a given gas, one was incubated at 5°C, one at 10°C, one at 15°C, and one at 20°C. These plates were filled on November 21, 2009.

Two other plates were set up to investigate the effect of added nutrients. Plate set up is shown in Figure 2.

pH 4 , 0.1g/100mL	pH 4 , 0.2g/100mL	pH 4 , 0.4g/100mL
pH 6 , 0.1g/100mL	pH 6 , 0.2g/100mL	pH 6 , 0.4g/100mL
pH 8 , 0.1g/100mL	pH 8 , 0.2g/100mL	pH 8 , 0.4g/100mL
pH 10 , 0.1g/100mL	pH 10 , 0.2g/100mL	pH 10 , 0.4g/100mL

Figure 2. Arrangement of nutrient plates. Two 4x3 plates were prepared using tissue pieces from one fish. Four levels of acidity were paired with three concentrations of Difco™ Nutrient Broth. Both plates were incubated in sandwich bags filled with ambient air, one at 10°C and one at 20°C.

These plates were 4x3 and paired the same four pH treatments with three levels of added nutrients. Difco™ Nutrient Broth (powder; beef extract and peptone) was added to each well in a concentration of either 0.1g, 0.2g, or 0.4g per 100mL. One fish was used for both plates. During screening the fish showed myxospores and a pansporoblast in addition to possible prespores. Plates were incubated in sandwich bags filled with ambient air, one at 10°C and one at 20°C. These two plates were also set up on November 21, 2009.

Beginning on November 23, wet mounts were prepared daily from every well and examined for parasite development. This was continued for ten days.

RESULTS

Timeline of Myxospore Production

Out of the 40 carcasses, none yielded a wet mount containing myxospores. As a result, carcasses which contained possible pre-sporogonic stages were selected. It was decided that this experiment would serve to test the protocol, and if no results were generated the procedure could be repeated if everything else worked.

Problems with the procedure arose about a week after initial set-up and ultimately led to termination of the experiment. The sumps of four of the stream units began to leak four days into the experiment. Drips or puddles were not seen beneath the deck, so the leaks were not able to be specifically located. In four to six hours water level dropped from full down to a level too low for the pumps to re-circulate, which put the pumps at risk of burning out from running while empty. Sumps were refilled twice a day, but this meant that parasite production could not be accurately measured for any time period due to continual loss through the leaks.

Another problem was fungus. While the carcasses stayed mostly intact, fungus began to grow quickly in the stream units. Gobs of it clogged the screens over the standpipes, preventing water from flowing down to the sump to be re-circulated. This resulted in overflows as the water in the sump was continually pumped into the clogged troughs. This required constant attention, with fungus needing to be cleared every couple of hours. Losing water to overflow presented the same measurement problem as the leaking sumps.

As a result of these problems the experiment was terminated. One set of samples was collected, but not analyzed.

Triggers For Myxospore Formation

No changes were observed in any of the wells during the observational period. Possible prespores were seen. No myxospores were seen in tissue samples from the fish that contained them during screening.

DISCUSSION

Timeline of Myxospore Production

The problem of fungus in a re-circulating stream unit presents the greatest obstacle in the way of this type of experiment. In addition to clogging the stream units it poses other problems, such as clogging the filter membranes and potentially causing inhibition during qPCR. However, these problems are more manageable than overflowing troughs.

Due to the difficulties encountered during this experiment, we opted to carry out a similar experiment using juvenile Chinook salmon as surrogates for adults. Juveniles can be infected by exposure to Klamath River water, then reared in a laboratory setting. As the disease progresses and the fish die/decompose, water samples can be collected and processed as described for the experiment with adult carcasses. This will show if the fish is shedding the parasite prior to the time of death. It should also help delineate the timeline of parasite shedding during decomposition. An estimate of total parasite production per weight of fish can be generated, then scaled up by adult weight to estimate production in an adult carcass.

Triggers For Myxospore Formation

Not seeing any myxospores in the experiment after seeing them in wet mounts prepared in the field suggests that particular carcass was not heavily infected. Another possibility is that bacteria or fungus may have helped break down parasites, especially in the nutrient-enriched wells (no antibacterial/antifungal compounds were added). This experiment requires several carcasses heavily infected with prespores near the pansporoblast stage, as well as pansporoblasts. Collecting carcasses in the field does not guarantee that any number of ideal carcasses will be found.

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Final Report

Production of myxospores of *Ceratomyxa shasta* from juvenile Iron Gate Chinook salmon

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ABSTRACT

Ceratomyxa shasta is a myxozoan parasite of salmonids which causes extensive losses of outmigrant Chinook salmon smolts in the Klamath River. The parasite has an indirect life cycle: the actinospore stage develops in the freshwater polychaete *Manayunkia speciosa* and is infective to the salmonid. The myxospore stage develops in the salmonid and is infective to the polychaete. The lifecycle seems to have evolved to take advantage of anadromy exhibited by Pacific salmon. Returning adults are infected as they migrate upstream and carry the parasite high up in the watershed. Completion of the lifecycle likely happens late in life or after death of the fish, at which time myxospores are released and polychaetes are infected. Little is known about the chronology of myxospore production in returning adults and carcasses. In this study juvenile Iron Gate Chinook were held in the Klamath River for 3 days to expose them to *C. shasta*. They were held at Humboldt State University in 19L tanks at 20°C. Every day tanks were drained, rinsed, and refilled. One-liter water samples were drawn during water changes. Sampling schedule varied across four trials of the experiment. Water samples were filtered and filter membranes were analyzed for *C. shasta* DNA with real-time PCR. Parasite concentration in the water over time was used to delineate chronology of parasite shedding from the host. The sum of parasite loadings in tanks during the experiment was used to estimate total parasite production. This value, along with average weight of a juvenile Chinook intestine and estimated average weight of the intestine in an adult carcass were used to estimate total parasite production from an adult carcass. Production was estimated to be between 2.3 and 23.4 million myxospores. Average spawner escapement from 1978 to 2010 is 84,688. Estimated myxospore prevalence in carcasses is about 30%. At the estimated range of myxospore production, adult carcasses would contribute a total of about 58.4×10^9 to 59.5×10^{10} myxospores to the river.

INTRODUCTION

Ceratomyxa shasta is a myxozoan parasite of salmonids that produces the disease ceratomyxosis. Infected fish can be found throughout the Klamath Basin but the stage infective to fish appears largely limited to the main stem of the Klamath River (Hendrickson et al. 1989, Stocking et al. 2006). Recent studies monitoring prevalence of selected fish pathogens in smolts sampled during outmigration implicated *C. shasta* as the direct cause of extensive losses in Chinook salmon (Williamson and Foott 1998, Foott et al. 1999, 2002, 2003). Foott et al. (2002) suggested that ceratomyxosis appeared to be the leading cause of disease and death in juvenile Chinook salmon in the Klamath River and estuary during summer of 2001. Williamson and Foott (1998) and Foott et al. (1999) suggested that reduced levels of infection in the Klamath River estuary as compared to the main river was due to death of infected fish prior to reaching the estuary.

Ceratomyxa shasta has an indirect life cycle (Bartholomew et al. 1997). Fish are infected by actinospores produced within the freshwater polychaete *Manayunkia speciosa.* Development in fish takes place primarily in the posterior intestine and ends in the production of myxospores which are released into the water. Myxospores are taken in by the polychaete. A rather complicated development takes place in the polychaete (Meaders and Hendrickson 2009) leading to the production of the actinospore stage which is again infective to fish.

The life cycle seems to have evolved to take advantage of anadromy exhibited by Pacific salmon. Adult Chinook salmon returning to freshwater bring the infection back into freshwater with them or are more likely re-infected upon re-entry into freshwater. They carry early developmental stages of *C. shasta* back to the top of the watershed and then spawn and die. Myxospores are likely produced only very late in life or after death. Myxospore development

probably takes place in carcasses and myxospores are likely released as carcasses decompose. Slezak (2009) found that a high percentage of post-spawned adults were infected with prespore stages but only a few actually produced myxospores under his experimental protocol. Little is known about the chronology of myxospore production in returning adults and carcasses.

An unpublished pilot study by Bartholomew et al. (2009) investigated the effect of removing adult Chinook salmon carcasses on *C. shasta* levels in Bogus Creek, a tributary that enters the Klamath just below Iron Gate Dam. One hundred carcasses in reach 2 of Bogus Creek were sampled over four occasions for infection by visual identification of myxospores in intestinal scrapings. Infection prevalence ranged from 13% to 40% and averaged 30% overall. Scrapings from decomposed carcasses contained significantly higher numbers of myxospores than those from fresh ones. Subsamples from two sampling occasions, consisting of material from both myxospore positive and negative intestinal scrapings, showed higher prevalence of infection (56% overall) when assayed with qPCR. The investigators suggest that either a low number of myxospores were present and were missed visually, or that DNA from pre-sporogonic stages was present. The preliminary study conclusions state that this may suggest parasite maturation is occurring within carcasses, or that some parasites are not completing their life cycle (not maturing to myxospores).

Another aspect of the *C. shasta* life cycle is the potential production of myxospores in juvenile Chinook salmon which are infected during outmigration. As stated earlier, outmigrant smolts are subject to high rates of infection and mortality. Bjork and Bartholomew (2010) described entry, migration to the gut, and development of *C. shasta* in juvenile Iron Gate Chinook and Trout Lodge rainbow trout (*Oncorhynchus mykiss*. During their study they observed sporogenesis through all layers of the intestine at two weeks post-infection. Nichols

and True (2007) observed Iron Gate Chinook smolts infected with *C. shasta* within five days of being released from the hatchery. Infection prevalence peaked at 65% in the third week following release. They found no histological evidence of recovery during their study, and concluded that a significant portion of infected fish succumbed to disease before reaching the ocean.

The purpose of this study is to estimate the chronology of myxospore production in adult Chinook carcasses by using juvenile Iron Gate Chinook as surrogates. The high infection rates documented in outmigrating juveniles during the summer provide an opportunity to experimentally infect fish for study purposes. Juvenile Chinook carcasses, being far less massive than adults, should be easier to work with because they are not likely to overwhelm tanks with fungus. We will also estimate total myxospore production from individual juveniles, then scale up by weight to estimate that which would occur in an adult carcass.

MATERIALS AND METHODS

Trial 1: May-July 2010

Two trials of the experiment were performed during the summer of 2010. During the first trial, forty juvenile Chinook salmon were obtained from Iron Gate Hatchery on May 24 and transported in aerated coolers to Fisher's RV Park (16733 State Highway 96, Klamath River, CA 96050-9111). Fisher's RV Park is located just upstream from the confluence with Beaver Creek. At the RV park the fish were placed in cages and the cages were placed in the Klamath River for 72 hours to expose the fish to *Ceratomyxa shasta*. Water temperature was recorded each day during the exposure period. Water monitoring data showing *C. shasta* concentration in the river

at Beaver Creek during this time period were obtained from Gerri Buckles (Department of Microbiology, Oregon State University, Corvallis, Oregon).

On May 27 the fish were transported back to the Humboldt State University (HSU) Fish Pathology Laboratory in aerated coolers. About 45 minutes before reaching HSU nitrofurazone powder (Binox®) was added to the coolers as prophylaxis to combat other pathogens the fish may have been exposed to in the river, particularly *Flavobacterium columnare*, the causative agent of columnaris. A dose of one gram per five gallons, or approximately 52.6 milligrams per liter, was administered.

At HSU all fish were acclimated to 20°C and then placed in pairs in separate five-gallon tanks. Aeration in each tank was provided by compressed air diffused through a one-inch air stone. Fish were fed daily with hatchery feed from the HSU Fish Hatchery. Water came from the HSU Fish Hatchery and was tempered to 20°C prior to use.

Tanks had no water filtration system, so complete water changes were performed every day. Fish were placed in a separate, clean holding tub in clean water while their tanks were refilled. Tanks were drained by siphoning with a hose, and a fresh hose was used for each tank. Tanks were thoroughly rinsed with hot tap water and wiped with paper towels prior to being refilled. The holding tub was wiped with 10% bleach and rinsed between fish. At the end of each day the siphon hoses were rinsed with 10% bleach and hot tap water. For the first five days at HSU nitrofurazone treatment was continued. Time was recorded as days post-exposure (DPE), with the day of transport back to HSU being day zero. Nitrofurazone was added to the tanks through day four post-exposure, for a total of five days of treatment.

On day five post-exposure the tanks were rinsed several times and refilled without nitrofurazone. Beginning on day six and continuing every day until day 49 a one-liter water sample was taken from each tank during water changes. The hose was used to stir the water for 15 seconds prior to siphoning, and stirring continued while the sample was drawn. This was done to re-suspend any *C. shasta* myxospores which might have settled. Samples were siphoned directly into a one-liter graduated cylinder, then transferred to jars and placed in the refrigerator until processing. Between samples graduated cylinders were sprayed with 10% bleach and left for five minutes, then rinsed with hot tap water. Samples were processed within 24 hours of collection.

Water samples were processed by vacuum filtration through Millipore MF™ nitrocellulose membrane filters with 5µm pores. Filter membranes were frozen in individual 2mL tubes at -35°C. Between samples the parts of the vacuum filter apparatus were rinsed with hot tap water, then rinsed with distilled water, then placed in an ultraviolet light sterilization box for at least 30 minutes. Sampling continued until both fish in a tank had died and fully decayed. At 42 DPE the surviving fish were euthanized with MS-222. Gut contents were collected from all mortalities by rectal swap and wet mounts were made to look for *C. shasta.* Carcasses which were not decomposed at that time were left in tanks, and decomposition was completed by day 49.

Upon completion of sample collection, filter membranes were sent to the department of microbiology at Oregon State University for analysis. Each membrane was dissolved with acetone to remove the membrane material. The remaining material, which consisted of the particles trapped during filtration, was processed via the Qiagen DNeasy® protocol for extraction and purification of DNA from animal tissue. The purified extract was assayed for *C.*

shasta with real-time PCR (qPCR) according to the procedure outlined in Hallett and Bartholomew (2006).

Trial 2: July-September 2010

In the second trial, forty juvenile Chinook were obtained from Iron Gate Hatchery on July 20 and exposed to Klamath River water in cages for 72 hours as in the first trial. Water monitoring data were again supplied by Gerri Buckles (Department of Microbiology, Oregon State University, Corvallis, Oregon). The same transportation, holding, and sampling protocols were repeated with minor alterations. Instead of medicating prophylactically for the first five consecutive days, the fish were treated on days 2, 4, 6, 8, and 10 post-exposure. This allowed for sampling during the first days out of the river, and sampling was continued every other day for the duration of the experiment. At day 47 post-exposure all living fish were euthanized and preserved. Carcasses present at that time were allowed to finish decomposing, and this was completed by day 53.

At the end of the study, membranes were sent to the department of microbiology at Oregon State University for qPCR like in the first trial.

Trial 3: June 2011

One hundred juvenile Chinook salmon were obtained from Iron Gate Hatchery on June 17 and transported in aerated coolers to Fisher's RV Park. At Fisher's RV Park the fish were placed in cages which were placed in the Klamath River for 72 hours. Each day during exposure

three one-liter water samples were taken immediately up-current from the cages at 0800, 1200, and 1600 to estimate the approximate *C. shasta* concentration at the exposure site. Samples were filtered in the field using the same 5µm membranes used throughout the study, and membranes were stored at ambient temperature in 95% ethanol.

After exposure, fish were transported back to the Humboldt State University (HSU) Fish Pathology Laboratory according to the same protocol outlined for earlier trials. Prior to returning, 35 additional fish were obtained from Iron Gate Hatchery to be used as control fish. They were transported and prophylactically treated with nitrofurazone powder the same as the exposed fish.

At HSU all fish were acclimated to 20°C. Five exposed fish and five unexposed fish were arbitrarily selected and placed individually in separate five-gallon tanks. Aeration in each tank was provided by compressed air diffused through a one-inch air stone. Fish were fed daily with hatchery feed from the HSU Fish Hatchery. Tanks lacked water filtration system, so complete water changes were performed on a daily basis. Water in the tanks came from the HSU Fish Hatchery and was tempered to 20°C prior to use. The same every-other-day schedule of prophylaxis described for trial 2 was used for this trial.

Water samples were drawn from tanks as described for earlier trials, except this time two one-liter water samples were taken from each tank instead of one. Water samples were vacuum-filtered through Millipore MF nitrocellulose membrane filters with 5µm pores according to the same protocol described for earlier trials.

At 17 DPE the experiment was terminated because no fish showed any signs of ceratomyxosis. Under the holding conditions we expected to see signs by this time, so fish were

euthanized and gut tissue samples were frozen for genetic analysis. Arrangements were made to repeat the procedure two weeks later with a new batch of fish.

Trial 4: July-August 2011

One hundred thirty juvenile Chinook salmon were obtained from Iron Gate Hatchery on July 22 and transported in aerated coolers to Fisher's RV Park. At Fisher's RV Park the fish were placed in cages which were placed in the Klamath River for 72 hours. On the second and third days of exposure three one-liter water samples were taken immediately up-current from the cages at 0800 and 1400 to estimate the approximate *C. shasta* concentration at the exposure site. Samples were filtered in the field using the same 5µm membranes used throughout the study, and membranes were stored at ambient temperature in 95% ethanol.

After exposure, fish were transported back to the Humboldt State University (HSU) Fish Pathology Laboratory according to the same protocol outlined for earlier trials. Prior to returning, 40 additional fish were obtained from Iron Gate Hatchery to be used as control fish. They were transported and prophylactically treated with nitrofurazone powder the same as the exposed fish.

At HSU all fish were acclimated to 20°C. Five exposed fish and five unexposed fish were arbitrarily selected and placed individually in separate five-gallon tanks. Aeration, feeding, and water changes were performed according to the same protocol described for earlier trials. HSU Fish Hatchery water tempered to 20°C was used. The same every-other-day schedule of prophylaxis described for trials 2 and 3 was to be used for this trial, but two mortalities attributed

to columnaris on the second day in the lab prompted continual treatment through day six. Water sampling was performed as described for the third trial.

Wet mounts were made from gut contents of all mortalities via rectal swab and checked for *C. shasta*. Wet mounts were made as close as possible to the time of death. A second rectal swab was smeared into a 2mL tube and preserved with 95% ethanol for genetic analysis. Carcasses were left in tanks until fully decomposed, and water sampling continued until that time. On day 31 post-exposure the last samples were taken. Shortly thereafter the surviving fish were euthanized with MS-222 and preserved. A small section of hindgut was removed from each carcass and frozen for genetic analysis prior to preservation.

At the end of the study all samples (smears, tissue, and filter membranes) were sent to the department of microbiology at Oregon State University for qPCR. Since two exposed fish died early and were discarded, two control fish were randomly selected and their samples were not analyzed. This left three exposed and three control fish.

RESULTS

Trial 1: May-July 2010

Fish used in the first trial were in the 180 per pound size class. Water temperature during exposure ranged from 11°C to 13°C. Parasite concentration in the river at this time was approximately 1-10 spores per liter as determined visually from a graph provided by Gerri Buckles at Oregon State University in Corvallis, Oregon (Figure 1). Concentration is inversely related to Cq, with a Cq of 32.5 corresponding to a *C. shasta* concentration of one spore per liter.

These data are interpreted as ranges which encompass an order of magnitude each. For every 3.3 decrease in Cq, *C. shasta* concentration increases tenfold. Thus a Cq of 29.2 corresponds to 10 spores per liter, a Cq of 25.9 corresponds to 100 spores per liter, and so on.

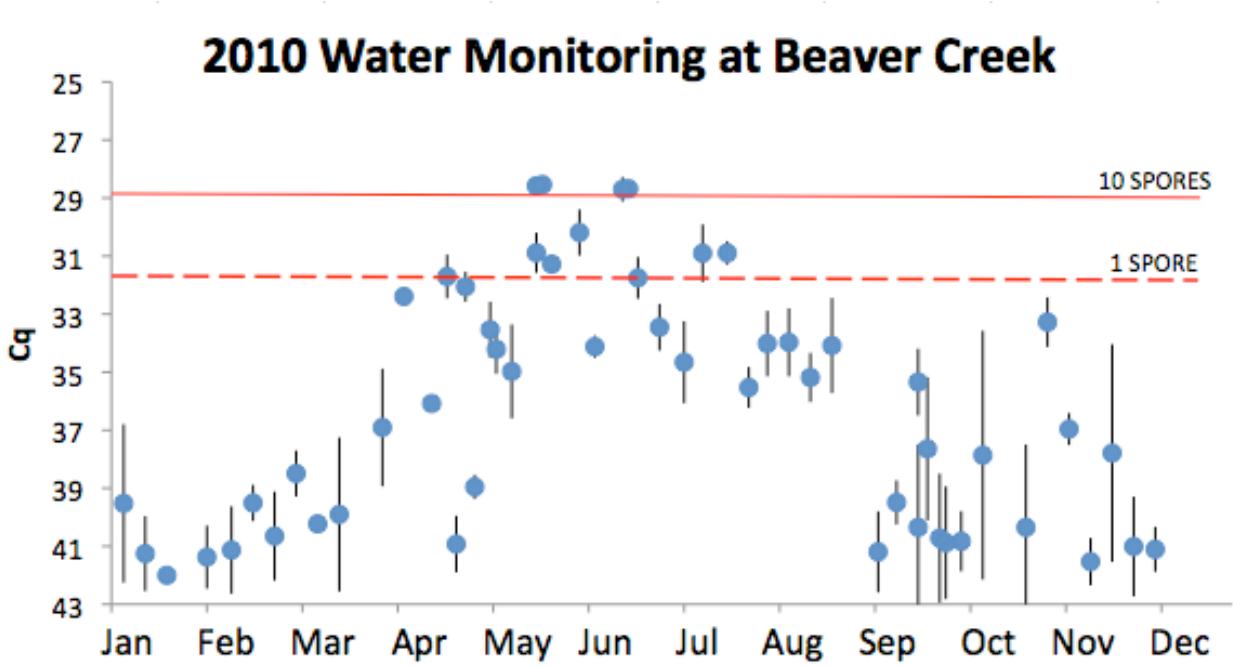

Figure 1. Concentration of *Ceratomyxa shasta* in 1L water samples collected in the Klamath River near the confluence with Beaver Creek during 2010. Courtesy of Gerri Buckles, Oregon State University, Corvallis, Oregon.

The first mortality was observed at 13 days post-exposure (dpe). Average time to death was 21 days. The last mortality occurred on day 39, and the remaining fish were euthanized on day 42. Thirty-two out of 40 fish died naturally during the experiment (80%). Twenty-five of those showed *C. shasta* myxospores in wet mounts of intestinal material (62.5% of all fish). One died due to observer error, and seven were euthanized.

Five days worth of water samples were analyzed in order to gain a general idea of the timeline of parasite release from the fish. Each sample day consisted of a one-liter water sample from every tank in use at the time, so as fish died and decomposed the number of samples on a

given day decreased. Since fish were paired in tanks, some tanks ended up with two mortalities and some ended up with one. Parasite concentration in water samples taken from tanks where both fish ultimately died is presented in Figure 2. The data point for day one does not represent samples, and is thus set at Cq = 42 to represent a concentration of absolutely zero.

The time at which fish began dying (13 dpe) corresponds with increasing parasite concentration, and the average time to death (21 dpe) corresponds with peak parasite concentration. The highest mean concentration observed was in the 10 to 100 spore per liter range, but one standard deviation above that value reaches nearly 10,000 spores per liter. After day 25 parasite concentration drops rapidly to just over one spore per liter on day 33, then to less than a spore per liter by day 39.

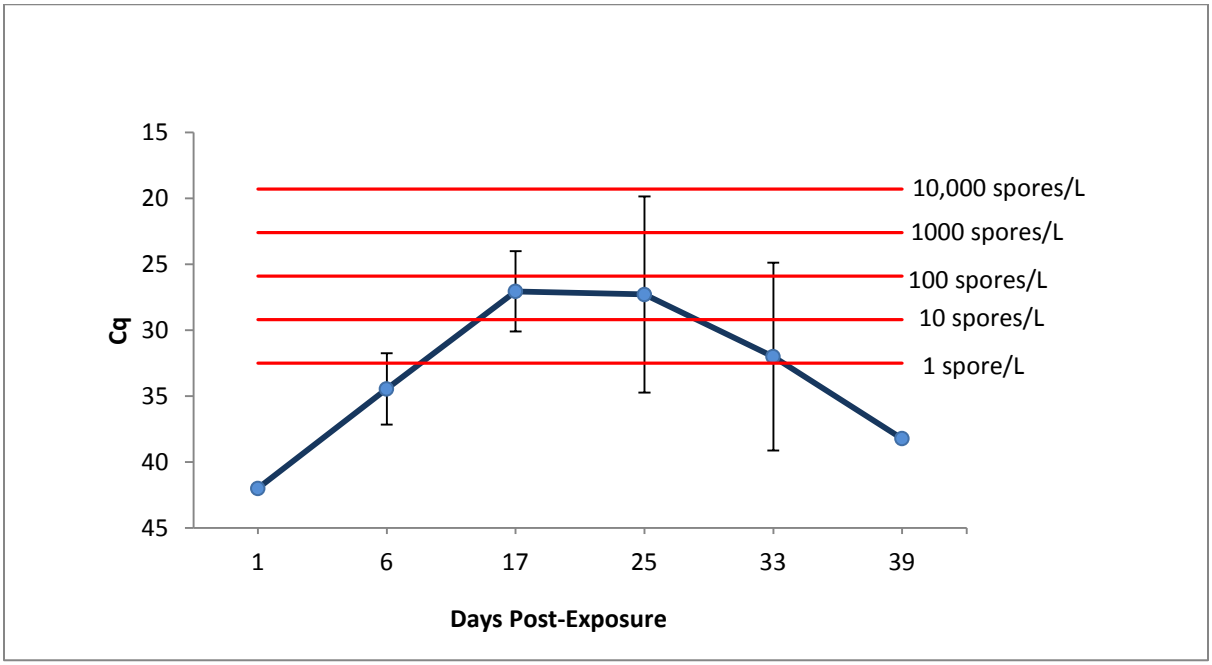

Figure 2. Concentration of *C. shasta* myxospores in 1L water samples drawn from tanks holding pairs of exposed fish which both ultimately died. Each data point represents the mean Cq over all samples taken that day (as fish died and decomposed, tanks were emptied and sampling from them ceased). Each point is accompanied by error bars showing one standard deviation above and below the mean. No samples were taken on day one, so the value was set at Cq = 42 which represents an unquestionably negative sample. Parasite concentrations in spores per liter are shown as horizontal red lines at their corresponding Cq values.

Figure 3 shows parasite concentration in water samples taken from tanks where one fish died naturally and the other was either euthanized at the termination of the experiment or died during the study due to observer error. Of the euthanized fish, only one had myxospores in a wet mount of intestinal material. The accidental mortality had none. The general pattern of parasite concentration over time is the same in these tanks as in the ones where both fish died, except it does not reach the same peak. Highest observed mean concentration for this group of tanks was just below 10 spores per liter on day 17, and one standard deviation above the mean on day 25 reached the 100 to 1000 spore per liter threshold.

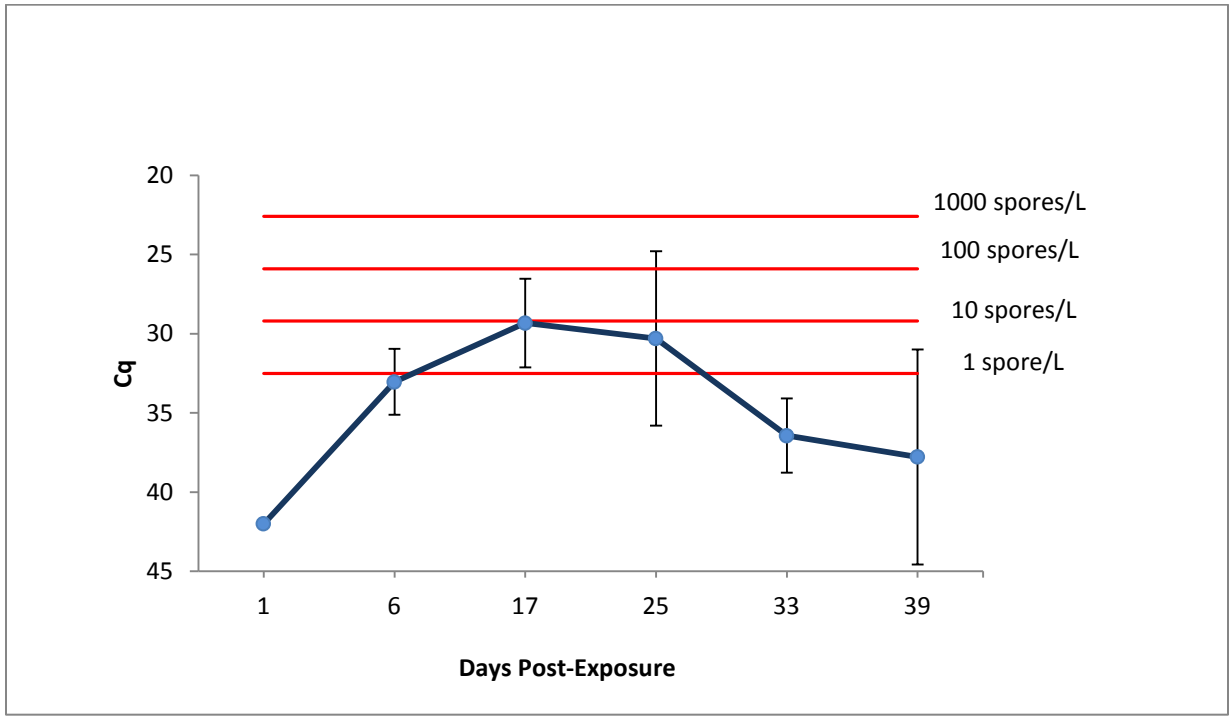

Figure 3. Concentration of *C. shasta* myxospores in 1L water samples drawn from tanks holding pairs of exposed fish where one died and the other was euthanized at the end of the study or was accidentally killed during the study. Each data point represents the mean Cq over all samples taken that day. Each point is accompanied by error bars showing one standard deviation above and below the mean. No samples were taken on day one, so the value was set at Cq = 42 which represents an unquestionably negative sample. Parasite concentrations in spores per liter are shown as horizontal red lines at their corresponding Cq values.

Trial 2: July-September 2010

Fish used in the second trial were in the 50 per pound size class. Water temperature during exposure ranged from 21°C to 24°C. Parasite concentration in the river at this time was below the one spore per liter threshold as determined visually from the graph in Figure 1, though it had been in the 1-10 spore per liter range in mid-July, just prior to exposure.

The first mortality was observed at 10 days post-exposure. Average time to death was 17 days. The last mortality occurred on day 41, and the remaining fish were euthanized on day 47. Thirty-five out of 40 fish died naturally during the experiment (87.5%). Twenty-eight of those showed *C. shasta* myxospores in wet mounts of intestinal material (70% of all fish). Two died due to observer error, and three were euthanized.

Six days worth of water samples were analyzed. Each sample day consisted of a one-liter water sample from every tank in use at the time, so as fish died and decomposed the number of samples on a given day decreased. Since fish were paired in tanks, some tanks ended up with two mortalities and some ended up with one. Parasite concentration in water samples taken from tanks where both fish ultimately died is presented in Figure 4. Since prophylaxis was performed every other day during this trial, samples were collected on the first day post-exposure.

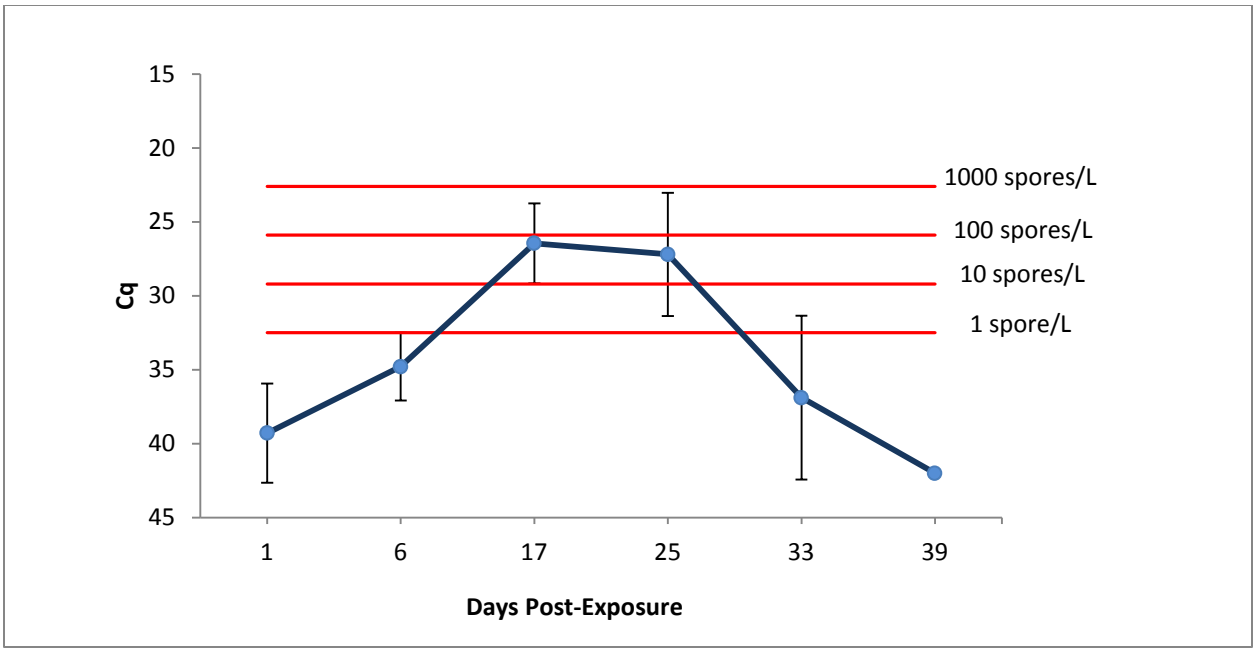

Figure 4. Concentration of *C. shasta* myxospores in 1L water samples drawn from tanks holding pairs of exposed fish which both ultimately died. Each data point represents the mean Cq over all samples taken that day. Each point is accompanied by error bars showing one standard deviation above and below the mean. Parasite concentrations in spores per liter are shown as horizontal red lines at their corresponding Cq values.

The time at which fish began dying (10 dpe) corresponds with increasing parasite concentration, and the average time to death (17 dpe) corresponds with the beginning of peak parasite concentration. The highest mean concentration observed was in the 10 to 100 spore per liter range, but one standard deviation above the highest values reaches into the range of 100 to 1000 spores per liter. After day 25 parasite concentration drops to below one spore per liter on day 33, then to nothing on day 39.

Figure 5 shows parasite concentration in water samples taken from tanks where one fish died naturally and the other was either euthanized at the termination of the experiment or died during the study due to observer error. Euthanized fish were preserved whole without being checked for myxospores. The two accidental mortalities were checked and neither had

myxospores. The general pattern of parasite concentration over time is the same in these tanks as in the ones where both fish died, except it does not reach the same peak on day 17 and the standard deviations of the two highest concentrations reach into the 1000 to 10,000 spore per liter range. One tank in the group had quite high parasite concentrations on days 17 and 25 (nearly 1000 spores per liter and barely less than 10,000 spores per liter, respectively), and another had a very high concentration on day 17 (nearly 10,000 spores per liter). These values contribute to the high standard deviations around the means on these two days.

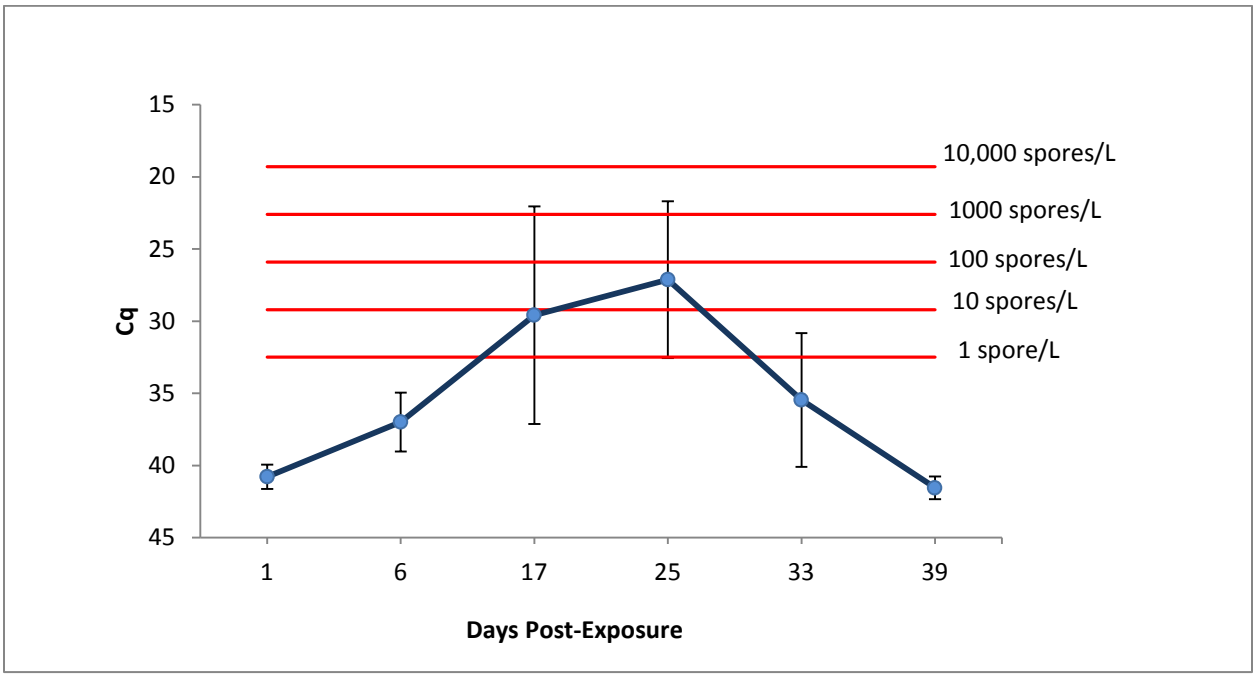

Figure 5. Concentration of *C. shasta* myxospores in 1L water samples drawn from tanks holding pairs of exposed fish where one died and the other was euthanized at the end of the study or was accidentally killed during the study. Each data point represents the mean Cq over all samples taken that day. Each point is accompanied by error bars showing one standard deviation above and below the mean. Parasite concentrations in spores per liter are shown as horizontal red lines at their corresponding Cq values.

Trial 3: June 2011

Fish used in the third trial were in the 125 per pound size class. Water temperature during exposure ranged from 15°C to 18°C. Temperature at the time of each water sample

collection during exposure is presented in Figure 6. Parasite concentration in the river during exposure fluctuated within the one to 10 spore per liter range, dropping below one spore per liter on the final sampling occasion. At 0800 on 19 June 2011 the variation among samples produced a standard deviation which reaches above the mean into the 10 to 100 spore per liter range. At each sampling occasion three one-liter samples were collected. Average parasite concentration at each occasion is presented in Figure 7.

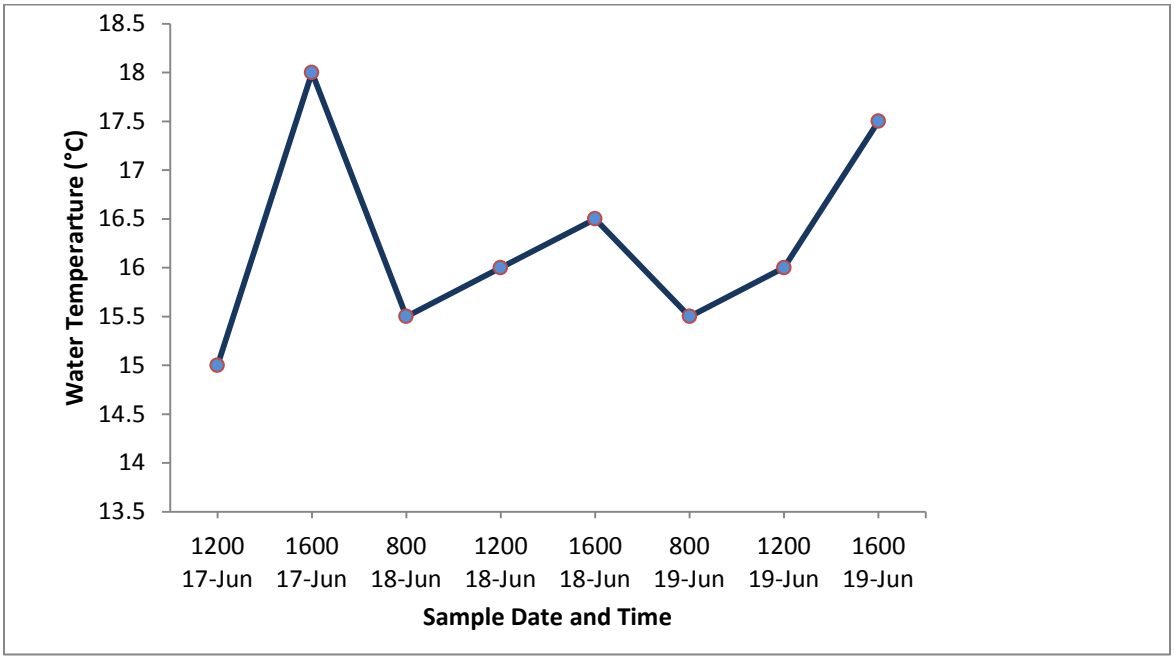

Figure 6. Water temperature in degrees Celsius during June 2011 exposure of fish in the Klamath River. Water temperature was measured at 0800, 1200, and 1600. Fish were put in the river in the late morning on the 17th and removed on the morning of the 20th.

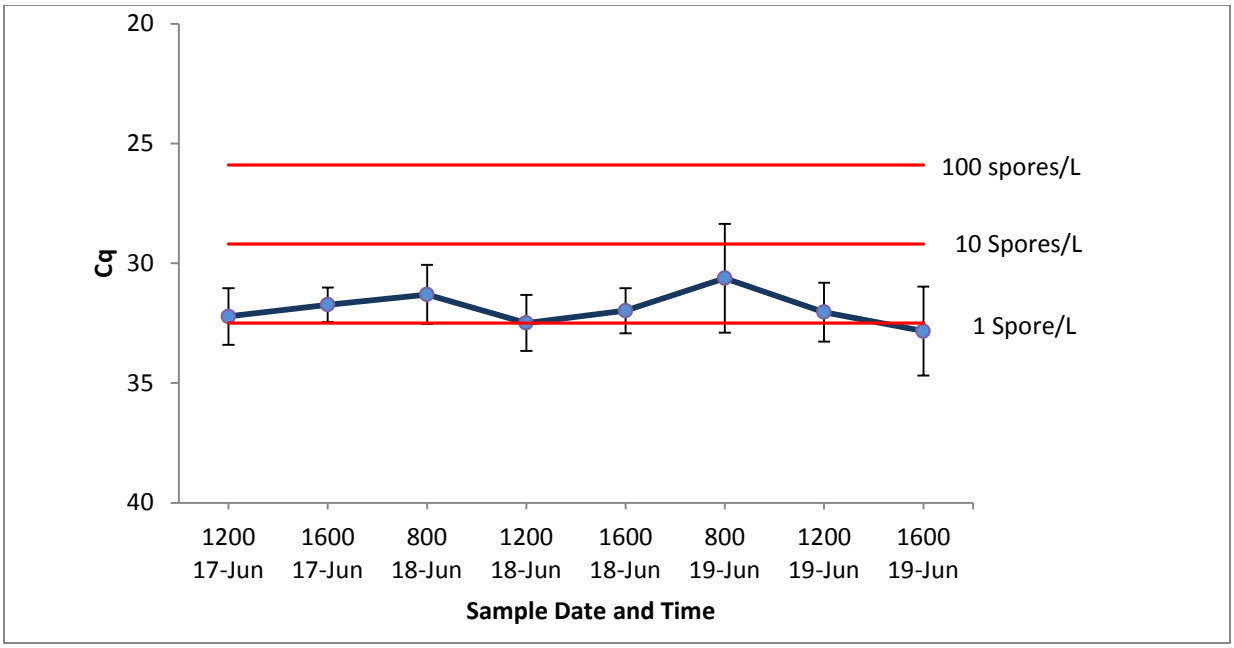

Figure 7. Concentration of *C. shasta* in the Klamath River during exposure of fish in June 2011. Fish were put in the river in the late morning on the 17th and removed on the morning of the 20th. Each point represents the average Cq of three one-liter water samples drawn at the date/time indicated. Error bars represent one standard deviation above and below the mean.

No mortalities were observed in this group of fish. At 17 days post-exposure no clinical signs of ceratomyxosis were observed in exposed fish. During the 2010 trials mortality was observed at 10-13dpe (with clinical signs), so the decision was made to terminate this trial and re-attempt exposure with a new group of fish. The group was euthanized, a wet mount was prepared from intestinal material from each fish, and a small section of hindgut was frozen for genetic analysis. All fish were negative by wet mount. All exposed fish were positive by qPCR (Figure 8).

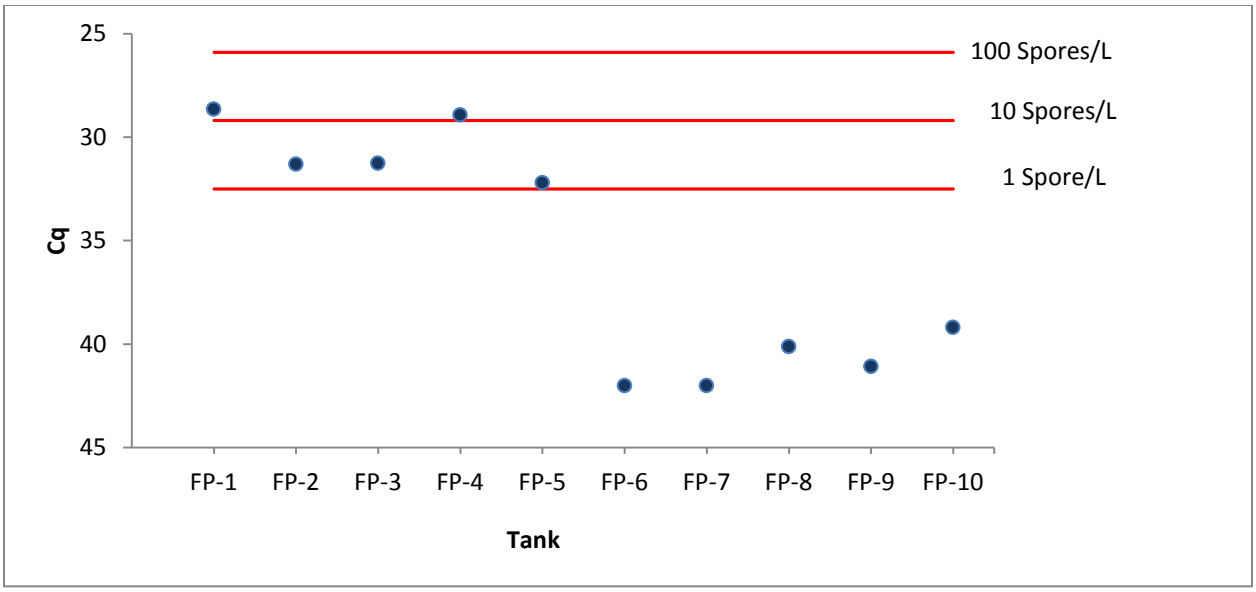

Figure 8. Results of genetic diagnosis of *C. shasta* infection in fish from the June 2011 exposure group. Tanks FP-1 through FP-5 each contained one exposed fish, and tanks FP-6 through FP-10 each held one unexposed control fish. Fish were euthanized at 17dpe and a small section of hindgut was removed from each one. No clinical signs of *C. shasta* were seen at this time, and microscopic examination of intestinal material showed no parasites. All exposed fish were positive for the parasite by qPCR, and all control fish were negative.

Trial 4: July-August 2011

Fish used in the fourth trial were in the 60 per pound size class. Water temperature during exposure ranged from 20.5°C to 22°C. Water temperature during exposure is presented in Figure 9. Parasite concentration in the river during exposure fluctuated within the one to 10 spore per liter range. At each sampling occasion three one-liter samples were collected. Average parasite concentration at each occasion is presented in Figure 10.

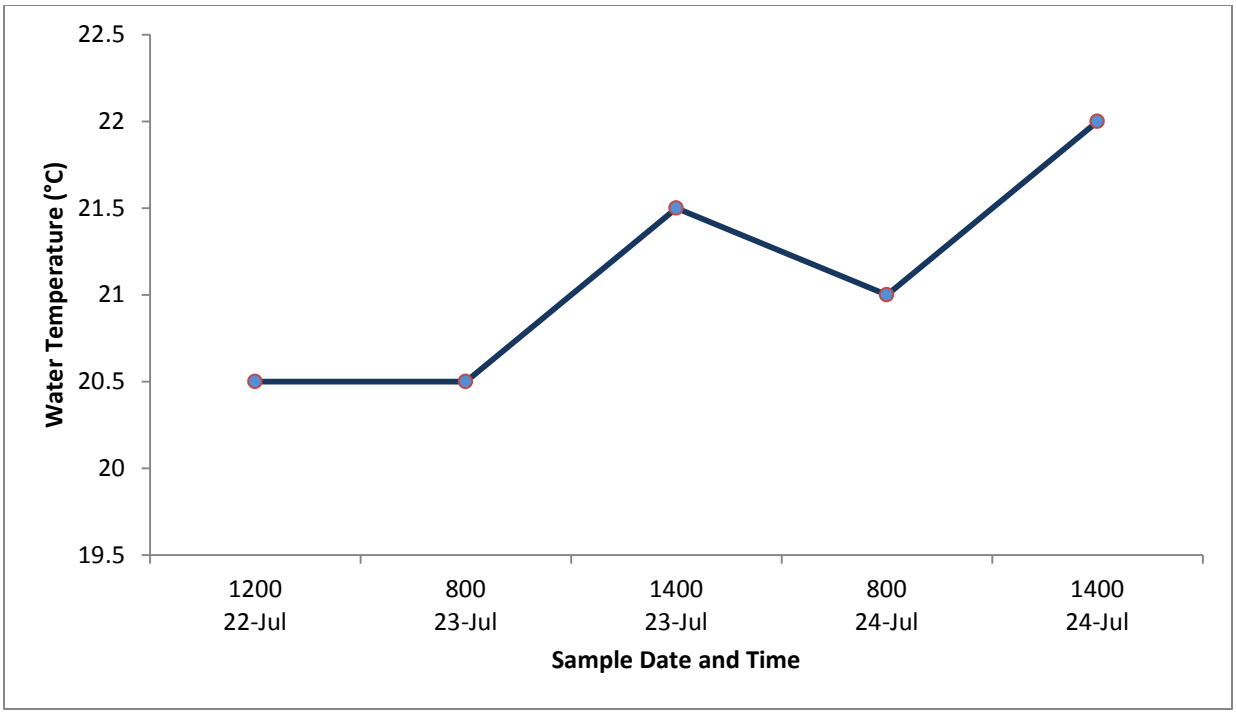

Figure 9. Water temperature in degrees Celsius during July 2011 exposure of fish in the Klamath River. Water temperature was measured at 0800 and 1400. Fish were put in the river in the late morning on the 22nd and removed on the morning of the 25th.

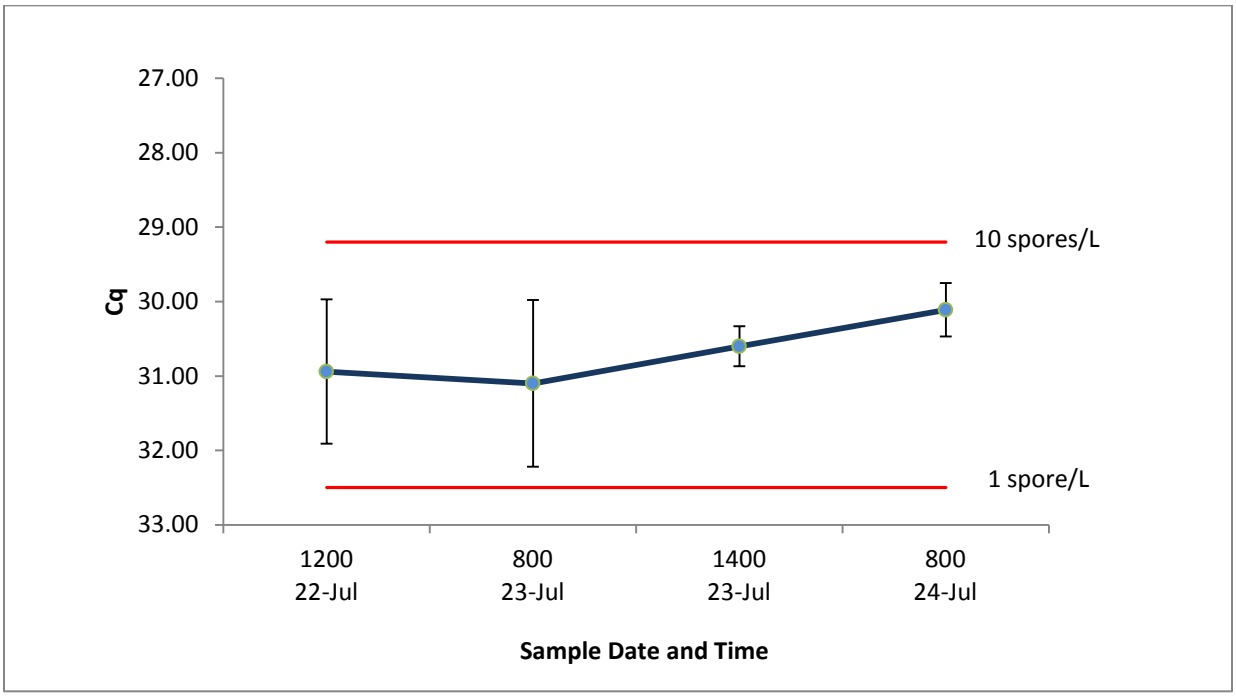

Figure 10. Concentration of *C. shasta* in the Klamath River during exposure of fish in July 2011. Fish were put in the river in the late morning on the 22nd and removed on the morning of the 25th. Each point represents the average Cq of three one-liter water samples drawn at the date/time indicated. Error bars represent one standard deviation above and below the mean.

On the second day of lab rearing (2dpe) two exposed fish died. One had a large saddleback lesion characteristic of columnaris and the other had a large lesion on the caudal peduncle. The caudal fin was badly shredded when it was first placed in the tank, presumably due to aggressive behavior of other fish. On the second day the caudal fin was gone and the lesion covered the whole posterior of the fish. Both mortalities were attributed to columnaris, but confirmatory cultures were not prepared. The carcasses were discarded to prevent spread of the disease. Wet mounts prepared from intestinal material prior to disposal did not show presence of *C. shasta*.

Three exposed fish remained, along with the five unexposed control fish. The first mortality due to ceratomyxosis occurred in tank FP-5 at 15 days post-exposure. The fish had been passing bloody fecal casts, and it had ascites. A wet mount of intestinal material showed *C. shasta* myxospores. A very small amount of intestinal material was removed by rectal swab and smeared into a 2mL tube, which was filled with 95% ethanol for preservation. The sample was assayed by qPCR and yielded a Cq of 22.62 (100-1000 spores/L). The carcass decomposed to skin scraps, spine, eye parts, and fungus by day 23 and water sampling ceased at that time. Parasite concentration in FP-5 over time is shown in Figure 11.

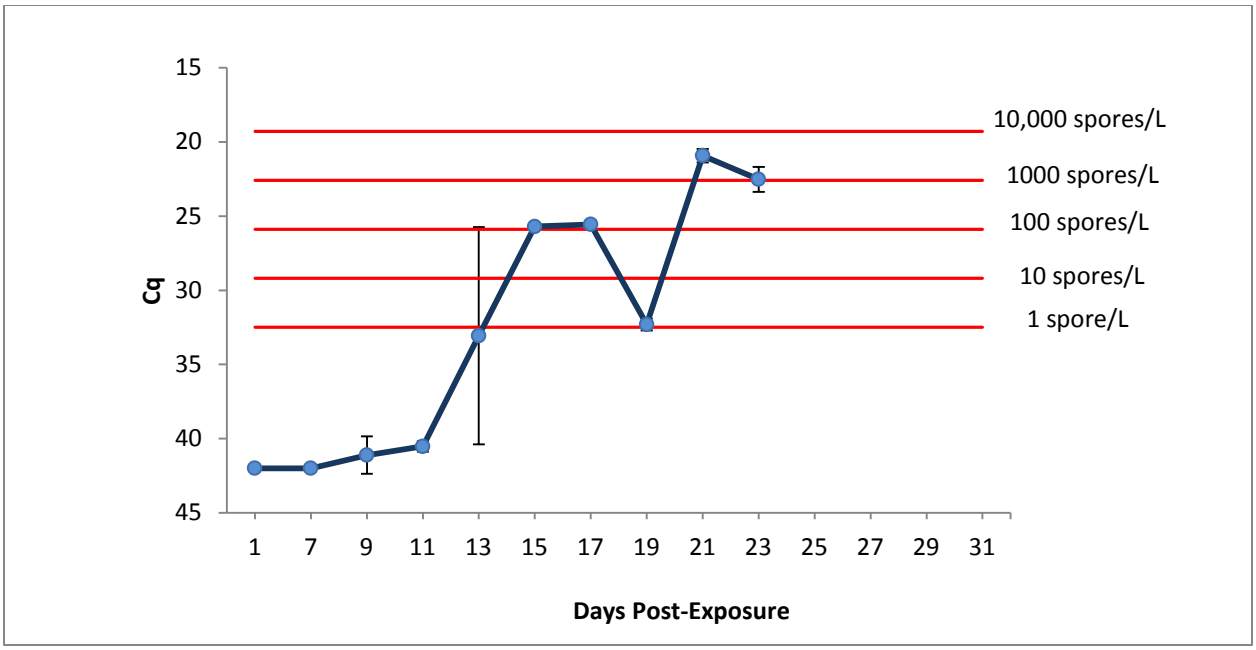

Figure 11. *C. shasta* concentration in tank FP-5 over time. Each point represents the average of two one-liter water samples. Error bars show one standard deviation above and below the mean. Parasite concentration is shown by the horizontal red lines. The fish died on day 15 and had decomposed to skin scraps, spine, eye parts, and fungus on day 23.

The second mortality due to ceratomyxosis occurred in tank FP-1 at 21 days post-exposure. This fish had a swollen red vent and ascites. A wet mount of intestinal material did not show *C. shasta* myxospores. A very small amount of intestinal material was removed by rectal swab and smeared into a 2mL tube, which was filled with 95% ethanol for preservation. The sample was assayed by qPCR and yielded a Cq of 22.65 (100-1000 spores/L). The carcass decomposed to skin scraps and bone by day 31 and water sampling ceased at that time. Parasite concentration in FP-1 over time is shown in Figure 12.

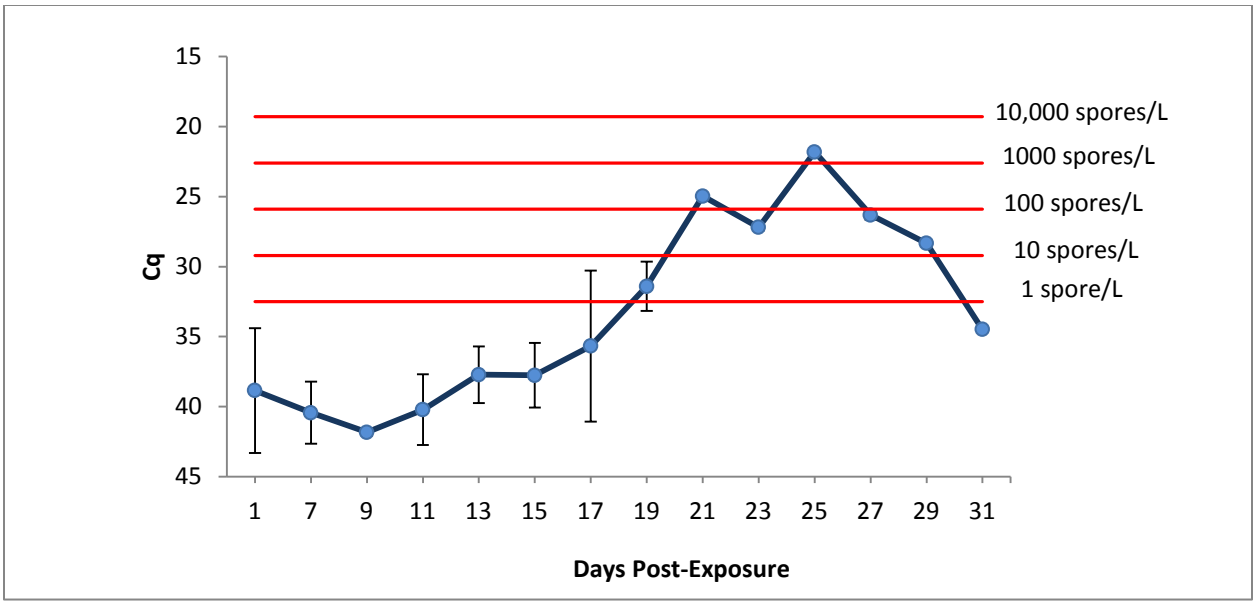

Figure 12. *C. shasta* concentration in tank FP-1 over time. Each point represents the average of two one-liter water samples. Error bars show one standard deviation above and below the mean. Parasite concentration is shown by the horizontal red lines. The fish died on day 21 and had decomposed to skin scraps and bone on day 31.

The last exposed fish (tank FP-4) showed no clinical signs of ceratomyxosis and was euthanized along with the control fish on day 36. It was negative for *C. shasta* by wet mount, however a section of intestine which was removed and assayed with qPCR yielded a Cq of 31.07 (one to 10 spores per liter). All water samples from this tank came up negative, except that on day nine one standard deviation above the mean barely crossed into the one to 10 spore per liter range. No signs of ceratomyxosis were observed in the control fish. All water samples from control tanks were negative for *C. shasta* when assayed with qPCR.

To estimate total spore production from the experimental fish, daily Cq values were converted to ranges of spore concentration per liter, scaled up by tank volume (19L), and summed. Since samples were taken every other day, the sum of all samples was doubled to estimate the total. Table 1 shows data for the first mortality (FP-5) and Table 2 shows data for the second (FP-1).

Table 1. Daily Cq values (mean of two samples) for tank FP-5. Each Cq falls into a range of spore concentrations per liter. Upper and lower bounds of these ranges are shown, along with upper and lower bounds for the total number of spores in the 19L tank at the time of sampling. At the bottom the bounds for the total number of spores captured during sampling is given, along with an estimate of total production generated by doubling the number captured.

DPE	Cq	spores/L (low)	spores/L (high)	spores/19L (low)	spores/19L (high)
1	42.00	0	0	0	0
7	42.00	0	0	0	0
9	41.11	0	0	0	0
11	40.52	0	0	0	0
13	33.06	0	0	0	0
15	25.70	100	1000	1900	19000
17	25.56	100	1000	1900	19000
19	32.30	1	10	19	190
21	20.92	1000	10000	19000	190000
23	22.52	1000	10000	19000	190000
			TOTAL:	41819	418190
			2x TOTAL:	83638	836380

Table 2. Daily Cq values (mean of two samples) for tank FP-1. Each Cq falls into a range of spore concentrations per liter. Upper and lower bounds of these ranges are shown, along with upper and lower bounds for the total number of spores in the 19L tank at the time of sampling. At the bottom the bounds for the total number of spores captured during sampling is given, along with an estimate of total production generated by doubling the number captured.

DPE	Cq	Spores/L (low)	Spores/L (high)	Spores/19L (low)	Spores/19L (high)
1	38.85	0	0	0	0
7	40.43	0	0	0	0
9	41.82	0	0	0	0
11	40.21	0	0	0	0
13	37.72	0	0	0	0
15	37.76	0	0	0	0
17	35.67	0	0	0	0
19	31.41	1	10	19	190
21	24.97	100	1000	1900	19000
23	27.20	100	1000	1900	19000
25	21.82	1000	10000	19000	190000
27	26.31	10	100	190	1900
29	28.34	10	100	190	1900
31	34.48	0	0	0	0
			TOTAL:	23199	231990
			2x TOTAL:	46398	463980

To estimate production of myxospores from an adult fish, total production estimates from juveniles were converted to spores per gram according to average juvenile intestine weight and scaled up to adult intestine weight (Table 3). Average weight of the intestine of the experimental fish was determined from the other fish obtained from the hatchery which were not selected for this study. They were euthanized in batches over an 18 day period for another study. The intestine was removed and weighed to the nearest 0.0001 gram. Average weight was 0.0520g (StDev=0.0362, n=141).

Adult intestine weight was estimated from a set of carcass data collected during fall 2011. Intestines were removed from carcasses at the Shasta River weir and Bogus Creek Weir and weighed to the nearest 0.01g. Average weight was 1.87g (StDev=0.86, n=22).

Table 3. Estimates of spore production from adult carcasses, obtained by scaling up estimated production from juveniles. Total production from juveniles is estimated as twice the number of spores captured during sampling and detected with qPCR. Upper and lower bounds of juvenile production are standardized by gram of intestine, then scaled up by adult intestine weight. All values rounded to the nearest whole number. Data used to estimate average intestine weight for juvenile and adult fish was provided by Nick Campise (Humboldt State University).

Tank:	FP-5	FP-1	Average	St Dev
Total Spores (low):	83,638	46,398	65,018	26,333
Total Spores (high):	836,380	463,980	650,180	263,327
Mean Juv. Intestine Weight (g):	0.0520	0.0520	0.0520	NA
Spores/g (low):	1,608,423	892,269	1,250,346	506,397
Spores/g (high):	16,084,231	8,922,692	12,503,462	5,063,972
Mean Adult Intestine Weight (g):	1.87	1.87	1.87	NA
Estimated Adult Spores (low):	3,007,751	1,668,543	2,338,147	9,469,623
Estimated Adult Spores (high):	30,077,512	16,685,435	23,381,473	94,69,628

DISCUSSION

In the unpublished pilot study by Bartholomew et al. (2009), 30% of all adult carcasses sampled in Bogus Creek between October 24 and November 14, 2008 produced intestinal scrapings in which myxospores were detected. Myxospore load ranged from 3000 to 14.7 million spores per gram of scraping. Fresh carcasses had lower myxospore loads than older, decomposed ones. Subsamples showed that some scrapings in which no myxospores were in fact positive by qPCR. Together, this information seems to point to development of *C. shasta* after the death of the host.

The fourth trial of this study provided the most useful information, although the sample size is so small that the findings need more support. Nonetheless, an interesting pattern did emerge. Water sampling data from the two fish that ultimately died with clinical signs of ceratomyxosis showed parasite concentration increasing to or past the one spore per liter mark two days before death and reaching a peak on the day of death. After death there was a decrease in concentration, followed by another peak that exceeded the initial one. This could be due to parasite development, or it could be due to physical breakdown of the carcass causing parasites to be washed out. In light of the observation in the pilot study that older carcasses had higher myxospore loads, the possibility of a temporal gap between death of the host and peak parasite production/release should be investigated further.

In their discussion, the authors mention unpublished data gathered by the California-Nevada Fish Health Center which indicates that many (up to 80%) fall-run adults are infected with *C. shasta*, but that myxospores are mostly seen in carcasses. Many infections are only detected by qPCR, with a few detected by visual identification of pre-sporogonic stages. Slezak

(2009) also found high infection with pre-sporogonic stages, with myxospores in 34% of the carcasses he examined from Bogus Creek, the Klamath mainstem, and the Shasta River. Again, this points to the question of whether *C. shasta* is undergoing sporogony in carcasses. Another possibility is that the parasite can mature fully if it is at or past a certain developmental stage by the time the host dies.

The juvenile Chinook in this study produced between 65,018 and 650,180 total myxospores. This is obviously a wide range, but using qPCR alone to detect and quantify spore load prevents extremely precise estimation. Any given Cq value can only be placed into a range of spore concentrations spanning one order of magnitude, meaning estimates become more uncertain as parasite concentration increases. A Cq between 32.5 and 29.2 equals about one to 10 spores per liter, but a Cq between 29.2 and 25.9 equals about 10 to 100 spores per liter. The range of Cq values is 3.3 in both cases, but the range of spore concentrations goes from nine to 90. The advantage of the method for this study was being able to detect parasites in the water with a live, infected fish.

Uncertainty was also introduced to the estimate of spore production in the juvenile fish by the fact that data was collected every other day. Each sample contains spores released during about a one day period, but each day between sample days is unknown. Since tanks were emptied and rinsed every day, each sample taken represents a quantity of parasites captured and removed. On the other days an unknown quantity of parasites was thrown out. Therefore the total of all samples equals the parasites produced during half of the study time, but not necessarily half of the total parasites. However, doubling the values means both the high and low values were doubled, which averages out to intermediate values. Also, given the fact that within a given range a set of Cq values will be interpreted as representing the same range of

spore concentrations, there is some leeway when deciding how to deal with days between samples.

Moving from estimates of total spore production to estimates of spores produced per gram of intestine, the range is about 1.25 million to 12.5 million. Toward the end of the unpublished pilot study by Bartholomew et al. there is mention of some unpublished data from the California-Nevada Fish Health Center 2008 prognosis study. A smear was made from the contents of the lower intestine, and the rest was held at 18°C for 48 hours. After the holding period intestines contained six to nine million myxospores per gram, which is within the range estimated by this study.

Scaling up from the estimate of myxospores per gram of juvenile intestine to total myxospores per whole adult intestine yields a range of 2.3 million to 23.4 million. It is uncertain whether parasite numbers are simply proportional to intestinal mass. An infected juvenile Chinook in the Klamath River typically experiences higher water temperatures than an adult because the juvenile is there in the spring and summer, while the adult is there in the fall or winter. This study held fish at 20°C, which matches summertime high temperatures. This affects both stress level of the fish (susceptibility) and development rate of the parasite. Infectious load in the river typically peaks in the late spring through mid-summer, then drops off through the fall and remains low during the winter. Adults are therefore exposed to a lower parasite concentration, at a lower temperature. During the spawning migration, however, the adults are dying. During outmigration, juveniles are developing. All of these factors have the potential to affect the progress of *C. shasta*.

In the pilot study on Bogus Creek, decomposed carcasses had a median myxospore load of 182,109 and fresh carcasses had a median myxospore load of 30,656. This was determined by careful dilution of intestinal contents and hemocytometer counts. The estimated range of 2.3 million to 23.4 million far exceeds this. This may be due to the fact that the carcasses were in colder water. The average water temperature before they began sampling carcasses was 8.6°C (minimum 5.3°C, maximum 11.5°C). This was on October 22. This comes back to the fact that adults migrate through cooler water with lower *C. shasta* concentration than juveniles. Therefore, scaling up from estimates of parasite production generated at 20°C may not accurately reflect production in adult carcasses. Alternatively, production could be similar in magnitude in adults, but it might happen much slower. This brings up the question of long-term parasite survival in highly degraded tissues or in sediments, which cannot be addressed in light of the present data.

Chesney and Knechtle (2011) released a California Department of Fish and Game annual report on recovery of fall-run Chinook and coho salmon at Iron Gate Hatchery. In it they provide counts of Chinook (adults and jacks) returning to the Klamath basin overall, as well as the portions returning to the hatchery and to Bogus Creek. The average spawner escapement in the Klamath basin from 1978 through 2010 is 84,688. Going with this number, if 30% were infected with *C. shasta* and produced myxospores, that would be about 25,406 infected fish. If each one of those produced 2.3 million to 23.4 million myxospores, the total load released into the river by their carcasses would be about 58.4×10^9 to about 59.5×10^{10} myxospores. Many factors affect this, however. An important consideration is variation in myxospore production between hatchery-spawned fish and fish that migrate to tributaries. Slezak saw myxospores in only 4% of the Iron Gate spawned adults he sampled. Timing of release is also important.

Spores released during periods of high flow may be less likely to contact polychaetes and perpetuate the lifecycle.

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