CERATOMYXA SHASTA: TIMING OF MYXOSPORE RELEASE FROM JUVENILE CHINOOK SALMON

by

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ABSTRACT

Ceratomyxa shasta: timing of myxospore release from juvenile Chinook salmon

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Ceratomyxa shasta is a myxozoan parasite of salmonid fishes endemic to river systems of the Pacific northwest of North America. In the lower Klamath River, California, *C. shasta* has caused significant losses of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) during summer outmigration for more than a decade. Population dynamics of fall-run Chinook salmon in the Klamath are affected by *C. shasta*. My study investigates the timing of *C. shasta* myxospore release from juvenile Chinook salmon. It will help determine whether juvenile Chinook salmon play a role in perpetuating the infectious cycle in the river, and also provide insight into *C. shasta* production in spawned adult Chinook salmon carcasses. During the summers of 2010 and 2011 juvenile Chinook salmon from Iron Gate Hatchery were held in cages in the Klamath River for 3 days, then reared at the Humboldt State University Fish Pathology Laboratory. Water samples were collected from the holding tanks and tested for *C. shasta* DNA with QPCR. Parasite DNA was mainly detected around the time of death and one week after death. Observations suggest the parasite is released passively from fish. Out-migrating juvenile Chinook salmon are capable of producing about 500 billion myxospores, but migration timing may place the spores at an area low enough in the river that they do not contribute significantly to the infectious cycle.

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF TABLES (continued)

Table Page

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

Figure Page

LIST OF FIGURES (continued)

Figure Page

INTRODUCTION

Ceratomyxa shasta is a freshwater myxozoan which infects salmonid fishes. It was first described by Noble (1950) after an epizootic in rainbow trout (*Oncorhynchus mykiss*) at Crystal Lake Hatchery in Shasta County, California in 1948. Since that time *C. shasta* has been found to be endemic to a handful of river systems in the Pacific Northwest of the United States and Canada (Margolis and Evelyn 1975, Ratliff 1981, Ching and Munday 1984, Hoffmaster et al. 1988,Hendrickson et al. 1989).

Ceratomyxa shasta has a two-stage life cycle involving a salmonid fish and the fresh water polychaete *Manayunkia speciosa* (Bartholomew et al. 1997). The actinospore stage is infective to the salmonid host, and the myxospore stage is infective to the polychaete. Bjork and Bartholomew (2010) described infection in Chinook salmon (*Oncorhynchus tshawytscha*) at 18°C. The actinospore attaches to the gill lamellae and releases a sporoplasm between the gill epithelial cells within 5 minutes of exposure. After 1 to 2 days parasites are present in blood vessels of the gill filaments, and after 3 days they are in blood vessels of the gill arches. Within the blood vessels of the gills the parasite passes through five developmental stages. The fifth stage is referred to as a secondary cell. Mature secondary cells, along with some of the earlier stages, are found in blood vessels of the intestine at 4 and 5 days after infection. Secondary cells enter a replicative stage in intestinal villi 5 days after infection. By 1 week post-infection the pansporoblast stage is present in the intestinal epithelium, and by 2 weeks some mature

myxospores are seen in all layers of the intestine. After proliferation in the intestine *C. shasta* may also be detected in the liver, pyloric cecae, kidney, and spleen (Bjork and Bartholomew 2010).

Meaders and Hendrickson (2009) described infection of the polychaete *Manayunkia speciosa* by *C. shasta* and development of the actinospore stage. The polychaete is a filter feeder, and myxospores are ingested during feeding. Within 3 hours of infection myxospores attach to the gut lining. Each myxospore releases a sporoplasm between epithelial cells. Between 3 hours and 7 days post infection the sporoplasm multiplies within cells of the gut epithelium and musculature, as well as in the septal nerves. This produces a proliferative stage of the parasite, and after 8 hours it is seen in the epidermis of the polychaete. A complex series of developmental and proliferative processes continue in the epidermis until mature actinospores are formed. Under the experimental protocol of Meaders and Hendrickson (2009) spore development from initial infection took 49 days at an average temperature of 17.3°C.

Severe *C. shasta* infection in the salmonid host leads to the disease ceratomyxosis which is characterized by swelling, hemorrhaging and necrosis of the posterior intestine, and sometimes accumulation of ascites in the body cavity (Bartholomew et al. 1989). This condition is typically fatal to the host. Susceptibility of salmonids to infection by *C. shasta* and the risk of succumbing to ceratomyxosis are affected by multiple factors (Bartholomew 1998 and references therein) including fish species and strain, water temperature (Ray et al. 2012), duration of exposure to water containing actinospores,

actinospore concentration in the water, and genetics of the host. Different genotypes of *C. shasta* have also been described and have specific affinities for different species of salmonids (Atkinson and Bartholomew 2010).

Ceratomyxa shasta has been responsible for extensive losses of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) in the Klamath River below Iron Gate Dam (California, USA) for over a decade (Foott et al. 1999, Foott et al. 2002, Nichols and Foott 2005, Nichols et al. 2007). Nichols and True (2007) observed Iron Gate Hatchery Chinook salmon smolts infected with *C. shasta* within five days of being released into the river. Infection prevalence in the cohort peaked at 65 percent in the third week following release. During their study they found no histological evidence of fish recovering from the disease, and concluded that a significant portion of infected fish succumbed to disease before reaching the ocean. They also noted that the disease risk to wild juvenile Chinook salmon rearing in the Klamath River prior to out-migrating was likely to be higher than that of the hatchery-reared fish due to longer exposure time. Bolick et al. (2012) reported an infection prevalence of 54 percent (diagnosed by histology) among fish of mixed (hatchery and wild) origin during the summer of 2009. The average infection prevalence from 2006 through 2011 was 25 percent by histology and 32 percent by real-time polymerase chain reaction (QPCR).

Fujiwara et al. (2011) demonstrated that ceratomyxosis has an effect on the population dynamics of fall-run Chinook salmon in the Klamath River basin. They examined survival to age 2 (in the ocean) of Chinook salmon released from Klamath River basin hatcheries in the spring, and also numbers of returning adults. After

accounting for environmental factors, they found that Chinook salmon released from Trinity River Hatchery (Lewiston, California) had significantly higher survival to age 2 than those released from Iron Gate Hatchery (Hornbrook, California). Trinity River Hatchery fish do not migrate through the highly infectious zone from the Shasta River confluence to Seiad Valley (Hallet and Bartholomew 2006, Stocking et al. 2006), but fish from Iron Gate Hatchery do. Age-specific spawning abundance data for Bogus Creek, Shasta River, Scott River, Salmon River, and Trinity River were analyzed using multivariate time series analysis to isolate any effect of disease. Using this model, Fujiwara et al. detected a short period of increased mortality in juvenile Bogus Creek and Shasta River Chinook salmon during downstream migration. It was not detected in fish from the other tributaries. Bogus Creek and Shasta River fish migrate through the entire infectious zone, so the increase in mortality is presumed to be linked to *C. shasta* infection. Detection of a disease effect at the population level indicates that *C. shasta* mortality is significant among juvenile Klamath River Chinook salmon.

High mortality among juvenile Chinook salmon in the river is a concern because of the potential impacts on tribal communities on the river and on the fisheries based out of California and Oregon (Bartholomew et al. 2011). In 2006 there was a 90 percent decrease in commercial landings of adult Chinook salmon, leading to a closure of the troll industry. The closure resulted in the loss of 1,100 jobs and $28 million in Oregon. The cost to California was about twice as much. The closure was a result of low numbers of returning adult Chinook salmon to the Klamath River, and one of the main causes of low returns was loss of juveniles to disease. Stock origin of individual fish landed at sea

cannot be determined by fishermen, so the whole fishery was closed to conserve the Klamath River fish (Bartholomew et al. 2011).

Reduction of polychaetes in the river has been considered as a strategy to reduce ceratomyxosis in Chinook salmon. However, prevalence of *C. shasta* infection in polychaete populations has been found to be very low (Stocking and Bartholomew 2007, Jordan 2012). Stocking and Bartholomew (2007) observed an overall prevalence of 0.27 percent. The two highest rates of infection were 4.9 percent and 8.3 percent, observed in populations just below Iron Gate Dam. With relatively few infected individuals responsible for the actinospore loading in the river, removal efforts would essentially need to eliminate all polychaetes to be effective in reducing disease in salmon.

Another management tool that has been considered is removal of adult Chinook salmon carcasses after spawning. Prevalence of *C. shasta* myxospores in adult carcasses has been monitored for several years in the main stem Klamath River, Shasta River, and Bogus Creek (Foott et al. 2009a, Foott et al. 2009b, Foott et al. 2010, Fogerty et al. 2012). The general finding of these surveys has been an infection prevalence of about 80 percent in returning adult Chinook salmon. However, only 30 percent of these fish contained myxospores. Further, only a few carcasses with myxospores contain extremely high numbers. Observations suggest that roughly 10 percent of adult carcasses contribute about 90 percent of the myxospores to the river. Therefore nearly all carcasses would need to be removed in order to eliminate the highest myxospore producers and reduce infection among polychaetes. One observation that emerged in these myxospore surveys is that older (more decomposed) carcasses tended to have higher myxospore counts than

fresh carcasses. Foott et al. (2009b) stated that one area of future research should be investigation of the timing of spore release from carcasses to determine when during the spawning season carcass removal would be effective at preventing myxospore input to the river.

The main purpose of this study was to investigate the timing of *Ceratomyxa shasta* myxospore release from juvenile Iron Gate Hatchery Chinook salmon during the summer months in the lower Klamath River. Information on timing of release will provide insight into possible locations in the river where myxospores are contributed by migrating juveniles, which may indicate whether juveniles are contributing to the infectious cycle by infecting areas of dense polychaete populations. Timing of myxospore release from juveniles may also provide insight into the timing of myxospore release from adult carcasses. In addition to timing, this study will also provide an estimate of the total number of myxospores produced from an individual juvenile Chinook salmon. Because studies indicate that *C. shasta* proliferation is proportional to available tissue (Bjork and Bartholomew 2009, Ray 2010), this estimate can be scaled up by intestine weight to estimate myxospore production from an adult Chinook salmon. Estimates of *C. shasta* production from individual fish will be scaled up in order to compare potential myxospore input to the river each year by hatchery cohorts released in the spring to that of spawning runs of adults returning in the fall. Together, these pieces of information will indicate approximately when, where, and how many *C. shasta* myxospores are released into the Klamath River by Chinook salmon at different life stages, and the biological significance of myxospore release timing.

MATERIALS AND METHODS

Sample Fish

All procedures involving live fish were approved through Humboldt State University Institutional Animal Care and Use Committee permit 09/10.F.39-A. Juvenile Chinook salmon from Iron Gate Fish Hatchery on the Klamath River (8638 Lakeview Road, Hornbrook, California 96044) were used in this study. The experiment was repeated four times: May 2010, July 2010, June 2011 and July 2011. Table 1 shows average weight of fish used and exact dates of exposure.

Fish were netted from an outdoor raceway and divided among two to three coolers ranging from about 40 to 100 liters in volume. Coolers were filled with hatchery water and aerated with 2.5 cm air stones. Fish were driven directly to the exposure site, about 30 minutes away.

Exposure

The exposure site was about 0.8 km upstream from the confluence of Beaver Creek with the Klamath River. This was at Fisher's Klamath River RV Park (16733 State Highway 96, Klamath River, California, Figure 1). Upon arrival, fish were immediately transferred from aerated coolers to cages in the river. Cages were semi-cylindrical two-piece plastic minnow traps 42 cm long and 23 cm wide in the center, tapering to 15 cm wide at the ends. Mesh size was 6 mm. Holes at the ends of the traps

Table 1. Average weight and exposure dates of juvenile Chinook salmon obtained from Iron Gate Fish Hatchery (Hornbrook, California) during 2010 and 2011. Fish were obtained from the hatchery and placed in cages in the Klamath River on the first day of exposure. They were removed from the river and transported to Humboldt State University (Arcata, California) on the last day of exposure.

Experimental Group	Average Weight (g)	Exposure Dates
May 2010	2.5	May 24 – May 27
July 2010	9.1	July 20 – July 23
June 2011	3.6	June 17 – June 20
July 2011	7.6	July 22 – July 25

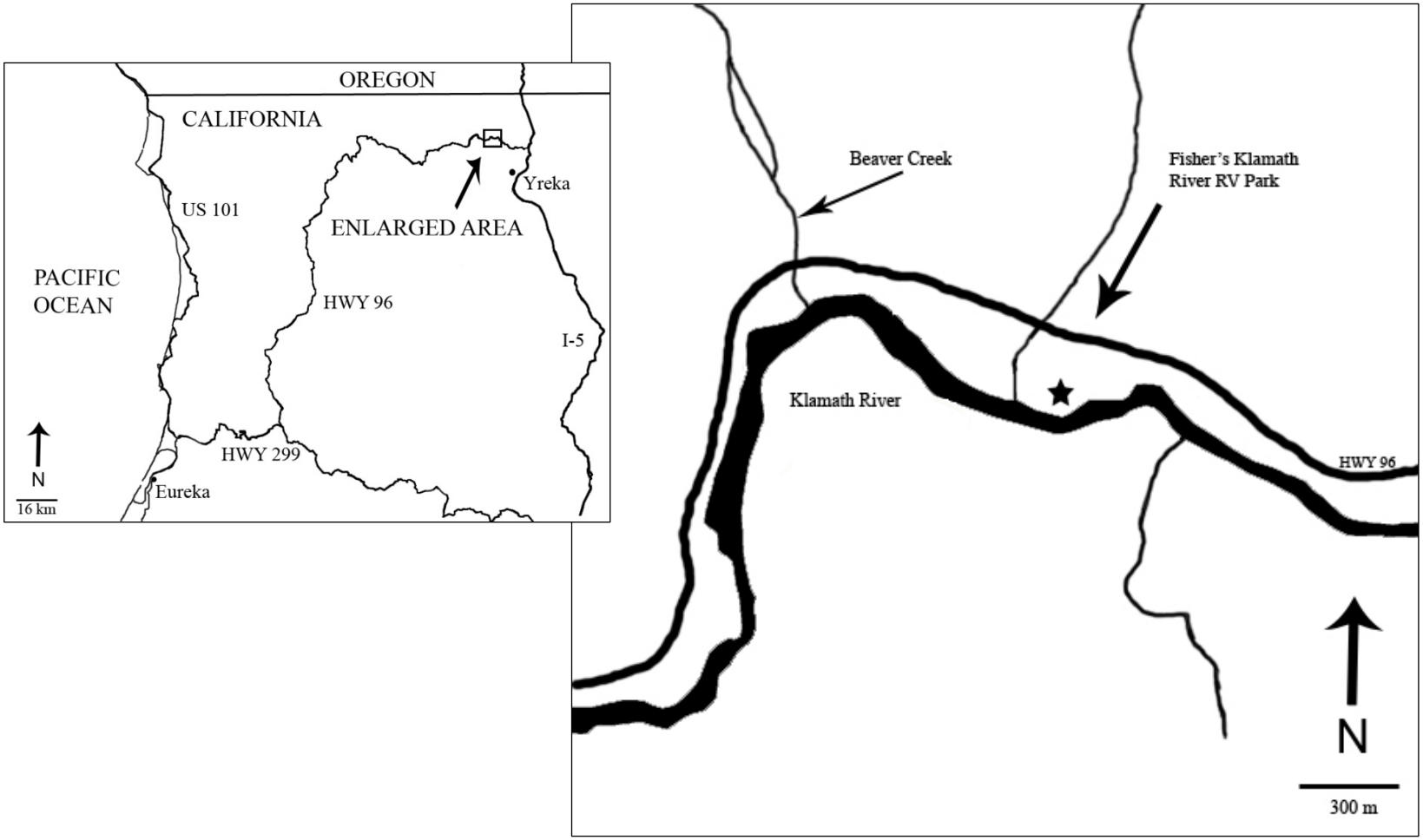

Figure 1. Map showing the site where juvenile Chinook salmon from Iron Gate Hatchery were held in the Klamath River to expose them to *Ceratomyxa shasta*. The black star is at Fisher's Klamath River RV Park, 16733 State Highway 96, Klamath River, California. Fish were held for 72 hours in cages along the bank of the RV park, approximately 800 meters upstream of the confluence with Beaver Creek.

were plugged to prevent escape of fish. A light coat of brown paint was applied to make the cages less apparent from shore. Number of fish per cage ranged from six to 15, depending on fish size class (up to 15 smaller fish, as few as 6 larger fish).

Once supplied with fish, cages were tied to shoreline vegetation with parachute cord. The specific site of exposure was an area about 1.2 m deep with overhanging willow trees. Cages naturally floated just under the surface and were tied in positions that provided constant water flow. Due to a bend in the river just upstream, the flow direction along this bank was reversed. Velocity was not measured, but cages were not tossed about by turbulence so their placement was determined not to be unnecessarily stressful.

Cages were left in place for 72 hours. During that time water temperature was measured three times per day with a handheld thermometer. During the 2010 experiments, concentration of *C. shasta* in the Klamath River at Beaver Creek was monitored independently by researchers from Oregon State University. Those data were provided by Gerri Buckles (personal communication, G. Buckles 2010. Department of Microbiology, Nash Hall 528, Oregon State University, Corvallis, OR, 97331). During the 2011 experiments, water samples were taken each day at the exposure site to quantify *C. shasta* concentration. Three 1 liter samples were taken with a graduated cylinder at each collection. Samples were filtered through 5 µm nitrocellulose membranes. Membranes were stored at ambient temperature in tubes of 95 percent ethanol. During the June experiment, samples were collected three times per day (0800, 1200, and 1600). During the July experiment samples were collected twice per day (0800 and 1400).

Transport From Exposure Site To Laboratory

In the 2011 experiments, a second group of fish (controls) was collected from Iron Gate Hatchery prior to removing cages from the river. They were transported in an aerated cooler, in hatchery water.

Transport coolers for exposed fish were filled with water from the river at the exposure site. Cages were removed from the river one by one and fish were gently poured into coolers. Air stones (2.5 cm) were placed in the coolers for aeration during transport. Fish were driven back to the Fish Pathology Laboratory, Humboldt State University (Arcata, California). Transport took about five hours. About 45 minutes from the laboratory, nitrofurazone powder (Binox®) was added to all coolers (including controls in 2011) as prophylactic treatment against other pathogens the fish may have been exposed to in the river, particularly *Flavobacterium columnare*, causative agent of columnaris (personal communication, R. Stone 2010. California-Nevada Fish Health Center, 24411 Coleman Fish Hatchery Road, Anderson, CA 96007). Dosage was 52.6 milligrams per liter. This drug is not known to affect *C. shasta*.

Laboratory Rearing

Aerated coolers were placed in the laboratory with lids open to allow water temperature to adjust to 20°C, the temperature at which fish would be held for the rest of the experiment. Fish were then transferred to separate glass tanks measuring 40.6 cm long, 20.3 cm wide, and 26.7 cm deep. Each tank was filled with 19 liters of water from the Humboldt State University Fish Hatchery and aerated with a 2.5 cm air stone. In the

2010 experiments two exposed fish were placed in each of 20 tanks. In 2011, five exposed fish and five controls were held individually in tanks. Remaining fish were placed in outdoor tanks as part of a different study. Fish were fed *ad libidum* each day with 1 mm BioDiet™ trout pellets.

Tanks lacked filtration and water flow, so complete water changes were performed every day. Fish were placed in a separate, clean holding tub in clean water. Tanks were drained by siphoning with a hose. A clean hose was used for each tank. In the 2011 experiments, each tank had its own labeled hose to prevent mixing water from exposed fish with control tanks. Tanks were thoroughly rinsed with hot tap water (58°C) and wiped with paper towels prior to being refilled. Tanks were filled most of the way, fish were returned to tanks along with water from the holding tub, and water level in each tank was then topped off to 19 liters. The holding tub was wiped with 10 percent bleach and rinsed between fish. At the end of each day siphon hoses were rinsed with 10 percent bleach and hot tap water.

Prophylactic nitrofurazone treatment was continued in all tanks for five days after Klamath River exposure. The drug was added to tanks with each water change. The May 2010 group was held continuously in water with nitrofurazone (Binox®) from the dose given during transport through the fourth water change. Both the July 2010 and June 2011 groups were treated every other day from the second day after removal from the river through the tenth day. The July 2011 group had two mortalities attributed to columnaris on the second day, prompting continual treatment of the remaining fish through day six. The two carcasses were discarded to prevent further losses.

Water Sampling And Filtration

The day fish were transported to the laboratory and placed in tanks was counted as day zero. From this point time was counted in days post-exposure (dpe). In the May 2010 experiment one water sample was taken from each tank every day starting at 6 dpe and continuing until 49 dpe. In the July 2010 experiment one sample was taken from each tank every other day from 1 dpe through 53 dpe. In the June 2011 experiment two samples were taken from each tank from 1 dpe through 15 dpe. In the July 2011 experiment two samples were taken from each tank 1 dpe, then every other day from 7 dpe through 31 dpe.

All water samples from tanks were collected 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 to the bottom. Each sample was 1 liter of water siphoned directly into a graduated cylinder, then transferred to a jar and placed in the refrigerator until processing. Between samples graduated cylinders were sprayed with 10 percent 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 2 ml tubes at -35°C. Between samples, parts of the vacuum filter apparatus were rinsed with hot tap water, then rinsed with distilled water and placed in an

ultraviolet light sterilization box for at least 30 minutes. For field samples, vacuum filter parts were soaked in 10 percent bleach, rinsed with tap water, and left in the sun to dry for at least 30 minutes. Ethanol was used for preservation of filtered samples in the absence of a low temperature freezer.

Treatment Of Mortalities

When a fish died, a sample of gut contents was collected via rectal swab and the carcass was promptly returned to its tank. Wet mounts of gut contents were viewed at 100x total magnification to check for *C. shasta* myxospores. Fish showing no signs of infection after disease-related mortality in the group ceased were euthanized with MS-222 (250ppm) and wet mounts of gut contents were examined. Any carcasses remaining at this time were allowed to finish decomposing, but euthanized fish were discarded.

In the June 2011 group there were no signs of infection among exposed fish at 17 days post-exposure. Thus, the whole group was euthanized and gut contents were examined. Sections of hindgut were also removed and frozen at -35°C for genetic analysis. In the July 2011 group, in addition to viewing wet mounts from recently deceased fish, rectal swabs were smeared into 2mL tubes and preserved with 95 percent ethanol for genetic analysis. When fish were euthanized at the end of the experiment, sections of hindgut were removed and frozen for analysis.

Analysis Of Samples

Filter membranes, smears, and tissues were sent to the Department of Microbiology, Oregon State University for analysis by laboratory manager Gerri Buckles and her assistants. They dissolved the filter membranes with acetone to remove the membrane material. The remaining material, which consisted of 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). Details of sample processing procedures are described by Hallett et al. (2012). Tissues and smears were processed the same way, but without the acetone treatment. The output from QPCR is a number called a Cq, which is inversely related to concentration of DNA in a sample. In this study the DNA being detected was that of *C. shasta*.

Due to the large number of samples collected during the 2010 experiments, a subset of samples was chosen for analysis. From the May group, all samples collected on days 6 (16 samples), 17 (20 samples), 25 (12 samples), 33 (10 samples), and 39 (8 samples) were analyzed. The number of samples varied from day to day because tanks were emptied and sampling ceased as fish died and decomposed. A temporary shortage of supplies limited the number of samples collected on day 6. From the July group, all samples collected on days 1 (20 samples), 6 (20 samples), 17 (20 samples), 25 (17 samples), 33 (10 samples), and 39 (6 samples) were analyzed.

The only samples analyzed from the June 2011 group were sections of hindgut removed from the fish after euthanasia. All water samples from exposed fish in the July 2011 group were analyzed, except samples drawn 1 dpe from the tanks of fish that died on the second day from presumptive columnaris. These were discarded. Samples from three controls were randomly selected to be analyzed with samples from the three exposed fish.

Data Analysis

A Cq of 32.5 represents a *C. shasta* density of 1 spore per liter (personal communication, G. Buckles 2010. Department of Microbiology, Nash Hall 528, Oregon State University, Corvallis, OR, 97331). From a Cq of 32.5, each subsequent decrease of 3.3 in Cq represents a tenfold increase in *C. shasta* density. Therefore a Cq of 29.2 equates to 10 spores per liter, a Cq of 25.9 equates to 100 spores per liter, and so on. This numerical relationship of Cq to *C. shasta* concentration only applies to the analysis of water samples, not to tissues or smears. A Cq under 32.5 from a non-water sample is considered positive for *C. shasta*, but it does not indicate a specific concentration.

For the May 2010 group, average Cq was plotted against time (days post-exposure) to show timing of *C. shasta* myxospore release. One graph shows average Cq from all tanks where both fish died with clinical signs of ceratomyxosis, and one shows tanks where only one fish died. The same plots were generated for the July 2010 group. Because no fish died or showed clinical signs of ceratomyxosis in the June 2011 group, a table of Cq values from gut samples taken after euthanasia was compiled to determine

presence or absence of *C. shasta*. For the July 2011 group, Cq was plotted against time for each of the three exposed fish to show more precisely the timing of *C. shasta* release. Average Cq of the controls was plotted against time to confirm the absence of *C. shasta*.

A standard curve was generated using R software to convert Cq values from water samples into estimates of *C. shasta* density (spores per liter). The standard curve was generated using parasite density ranging from one up to 10,000 spores per liter, and the corresponding Cq values. Cq was plotted against the logarithm (base 10) of spore concentration. Taking the logarithm of spore concentration normalized the values, which were otherwise right-skewed. A linear regression was performed to generate the equation: $\log_{10}(\text{spores/l}) = \alpha + b(\text{Cq})$. Exponentiating both sides of the equation yields the expression $\text{spores/l} = 10^{\alpha} \times 10^{b(\text{Cq})}$. Letting the value 10^{α} be called 'a' gives the standard curve: $\text{spores/l} = a \times 10^{b(\text{Cq})}$, where a=7,054,802,311 and b=-0.3030303. Because the Cq values associated with each spore concentration were provided as fixed values, there is no way to calculate a prediction interval (measure of uncertainty) around this standard curve.

Using the standard curve and the data collected in the fourth trial (July 2011), *C. shasta* density was calculated for each tank on each sample day. Density in each tank was converted to total number of parasites by scaling up the concentration by the total tank volume of 19 liters. Daily values were summed, and the sum was scaled up by a factor equal to the number of days the fish was in the tank, N, divided by the number of sample days, n, to estimate the total number of spores released during the experiment. Variance and sampling variance were calculated and used to construct 95% confidence

intervals according to equations for estimation of a total (Thompson 2002). To estimate the total parasite contribution to the river by out-migrant juvenile Chinook salmon, the average number of spores released per fish was multiplied by the Iron Gate Hatchery juvenile Chinook production goal (based on Chesney and Knechtle 2011) and the estimated infection rate among emigrating juvenile Chinook (based on Bolick et al. 2012).

To estimate the number of myxospores that might be produced in an adult Chinook salmon carcass, the number of spores produced per gram of intestine from juvenile fish was multiplied by average weight of an adult Chinook salmon intestine. Average weight of a juvenile Chinook salmon intestine was calculated from 141 fish obtained at Iron Gate Hatchery along with the fish used in this study. The intestines were removed and weighed to the nearest 0.0001 gram on an analytical balance. The number of spores produced per gram of intestine was calculated using the number of spores released per fish in this study. Average weight of an adult Chinook salmon intestine was estimated from a set of data collected from 22 carcasses during fall 2011. Intestines were removed from carcasses at the Shasta River and Bogus Creek weirs and weighed on a digital balance in the field to the nearest 0.01g.

Estimates of number of spores released by adult fall run Chinook salmon were generated using the number of spores per carcass, spawner escapement estimates (based on Chesney and Knechtle 2011), and infection prevalence among adult carcasses as estimated by several myxospore surveys reported by the California-Nevada Fish Health Center (Foott et al. 2009a, Foott et al. 2009b, Foott et al. 2010, Fogerty et al. 2012).

RESULTS

Water temperature during the May 2010 exposure ranged from 11°C to 13°C, ($\bar{x}$=11.5°C). Parasite concentration at the site of exposure in the river ranged from approximately 1-10 spores/l (Figure 2). The first mortality during laboratory rearing was observed at 13 dpe. Average time to death was 21 days. The last mortality occurred at 39 dpe. The remaining fish were euthanized on day 42. Thirty-two out of 40 fish (80 percent) died naturally during the experiment. In twenty-five of those (62.5 percent of all fish) *C. shasta* myxospores were observed in wet mounts of intestinal material. One fish was accidentally killed during a water change, and seven fish were euthanized.

Parasite concentration in water samples taken from tanks where both fish died of ceratomyxosis is presented in Figure 3. 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. After day 25, parasite concentration drops rapidly to just over 1 spore per liter on day 33, and less than 1 spore per liter by day 39.

Figure 4 shows parasite concentration in water samples taken from tanks where one fish died naturally and the other was either euthanized or killed accidentally. One euthanized fish yielded a wet mount of intestinal material which contained myxospores.

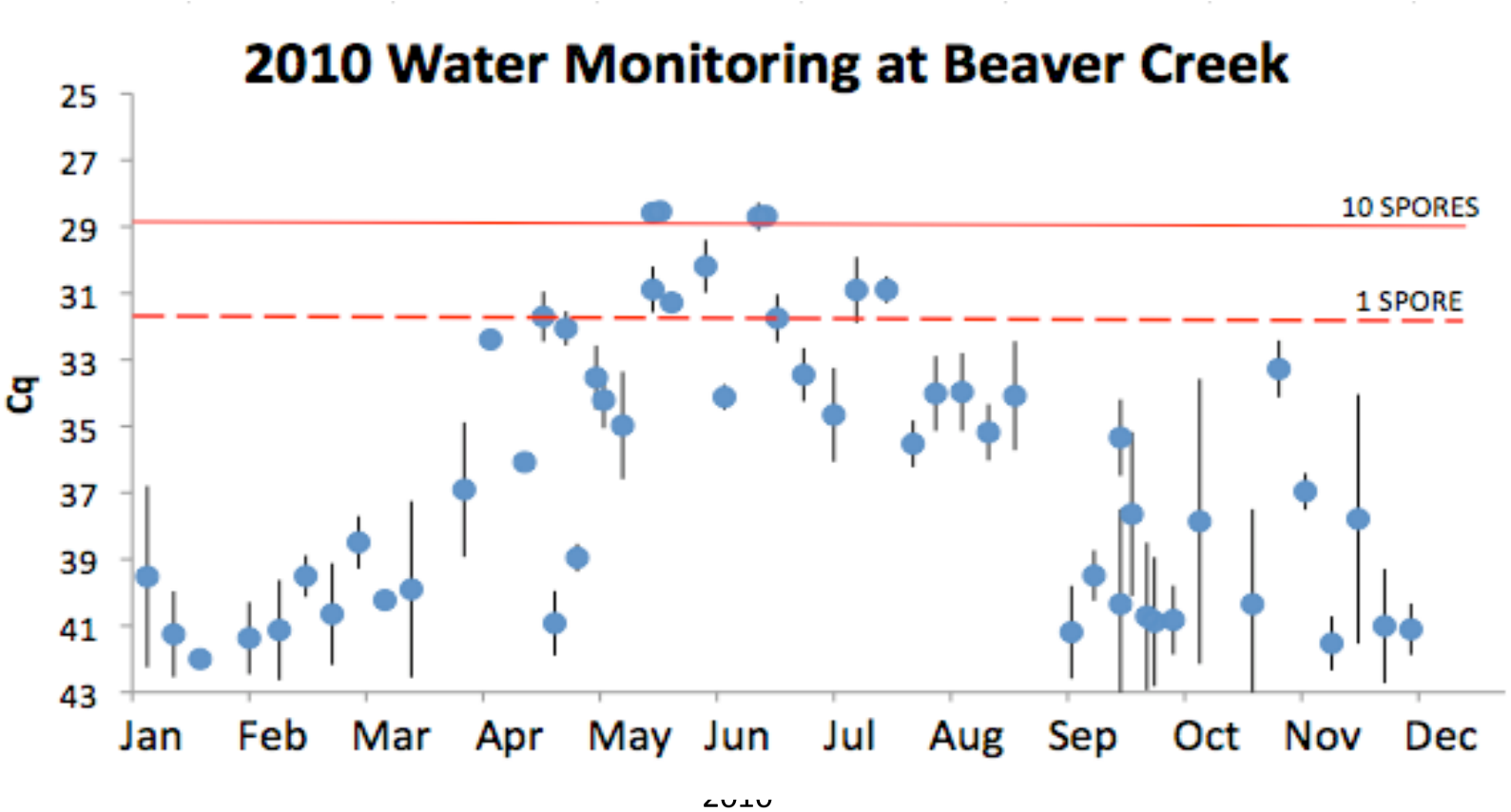

Figure 2. Concentration of *Ceratomyxa shasta* DNA in the Klamath River at the confluence with Beaver Creek throughout 2010. Each point represents the average Cq from three 1-liter water samples, the whiskers represent one standard deviation. Standard parasite DNA concentrations, given in spores/l, are shown as horizontal lines at their corresponding Cq values. The lowest (dashed) line represents 1 spore/l, and the solid line above indicates a tenfold increase. Courtesy of Gerri Buckles, Oregon State University, Corvallis, Oregon.

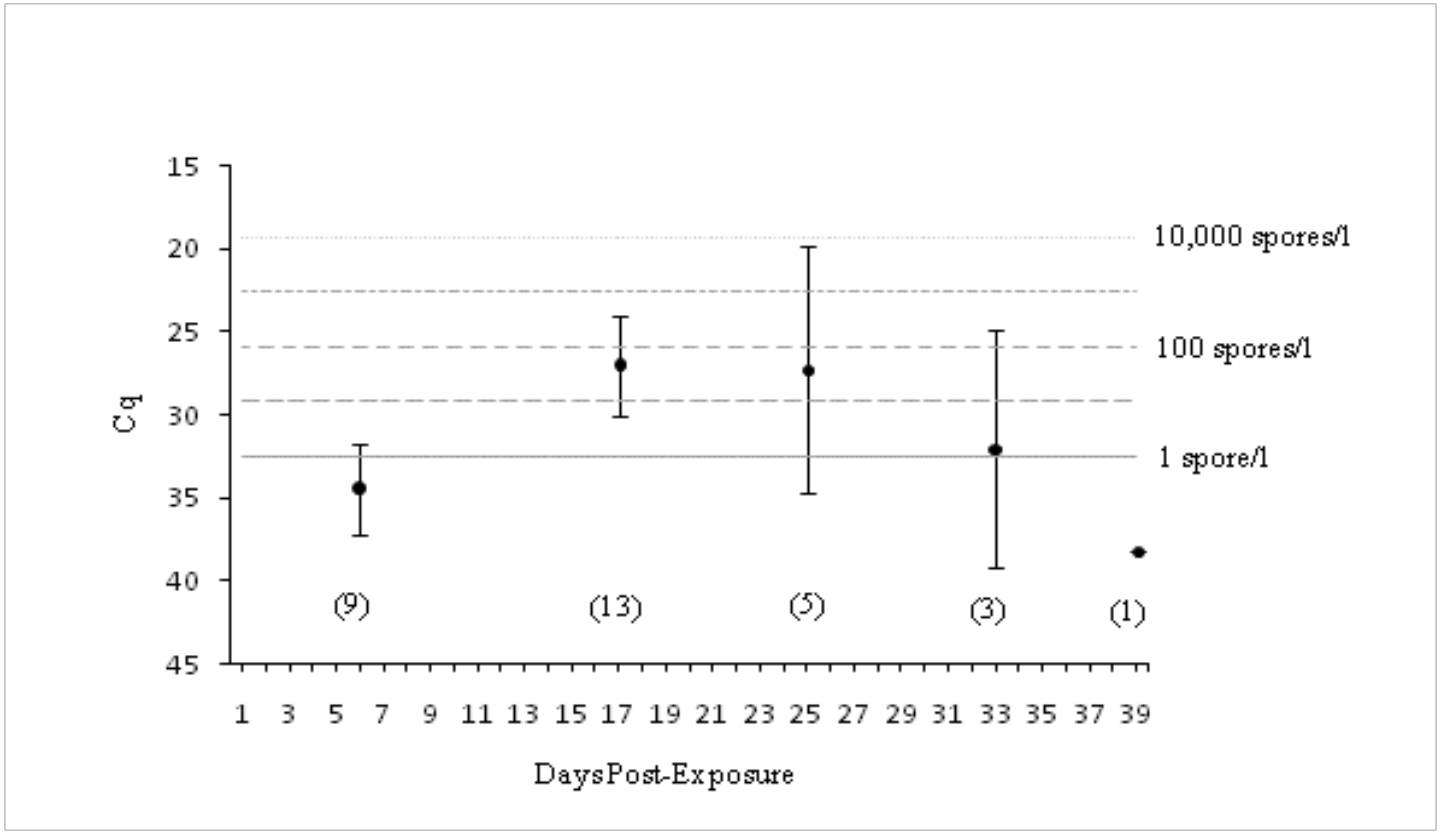

Figure 3. Concentration of *Ceratomyxa shasta* DNA (presumably myxospores) in 1 liter water samples drawn from tanks holding pairs of exposed Chinook salmon which both ultimately died. Exposure was conducted May 24-27, 2010. One sample was drawn from each tank, and each data point represents the mean Cq over all samples (number of samples shown in parentheses). Error bars represent one standard deviation. Standard parasite DNA concentrations, given in spores/l, are shown as horizontal lines at their corresponding Cq values. The lowest (solid) line represents 1 spore/l, and each subsequent line above indicates a tenfold increase.

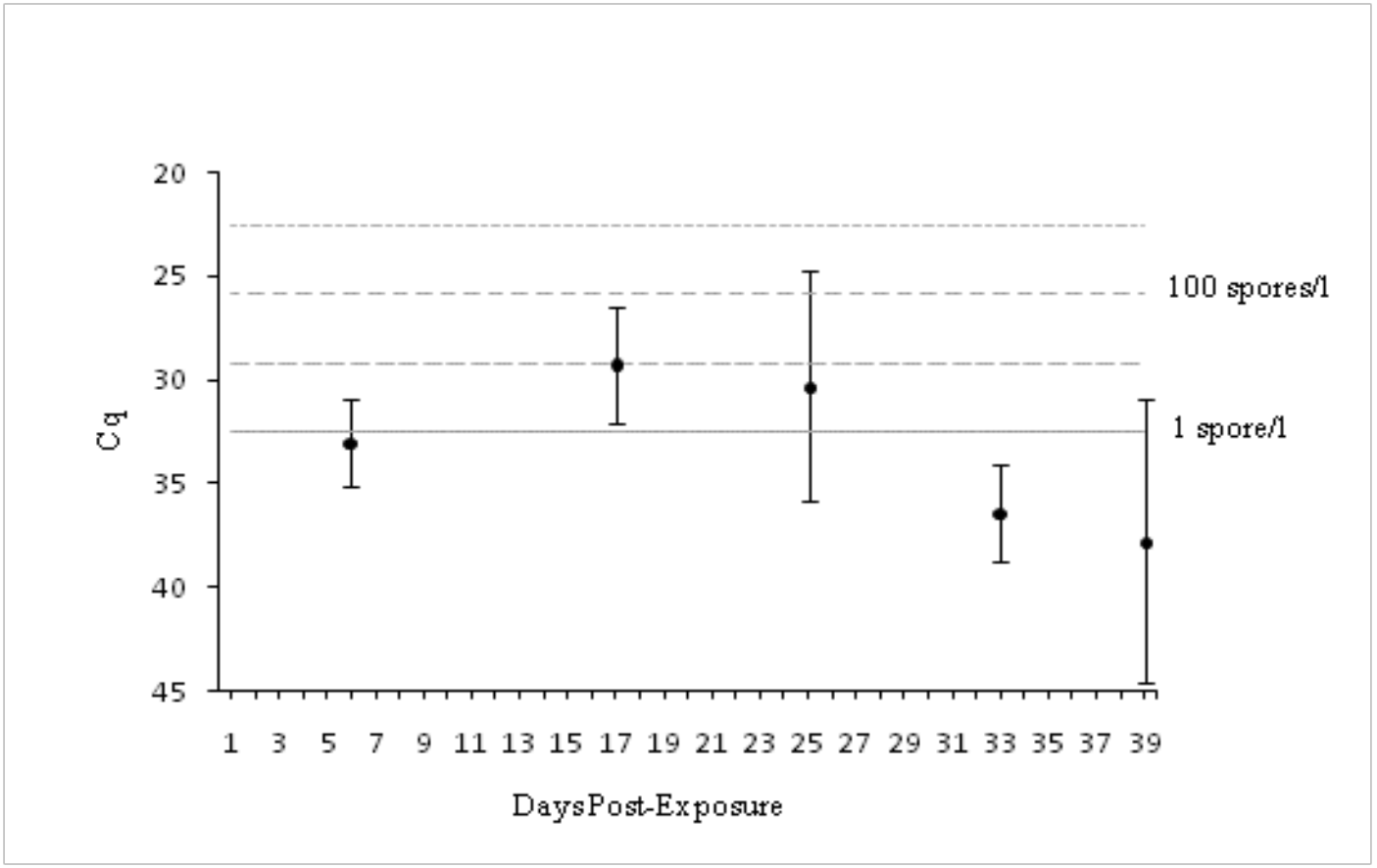

Figure 4. Concentration of *Ceratomyxa shasta* DNA (presumably myxospores) in 1 liter water samples drawn from tanks holding pairs of exposed Chinook salmon in which only one fish died with signs of ceratomyxosis. Exposure was conducted May 24-27, 2010. One sample was drawn from each tank, and each data point represents the mean Cq over seven samples. Error bars represent one standard deviation. Standard parasite DNA concentrations, given in spores/l, are shown as horizontal lines at their corresponding Cq values. The lowest (solid) line represents 1 spore/l, and each subsequent line above indicates a tenfold increase.

The general pattern of parasite concentration over time is the same as in those tanks where both fish died. However, the maximum daily spore production is less. The highest mean concentration observed for this group was just below the 10 spore/l threshold. From day 25 to day 33, parasite concentration dropped below one spore/l and remained there through day 39.

Water temperature during the July 2010 exposure ranged from 21°C to 24°C, ($\bar{x}$=22.3°C). Parasite concentration at the site of fish exposure in the river was below the one spore/l threshold (Figure 2), although it had been in the 1-10 spore/l range in mid-July, just prior to exposure. The first mortality during lab rearing was observed at10 dpe. Average time to death was 17 days. The last mortality occurred on day 41. Remaining fish were euthanized on day 47. Thirty-five out of 40 fish (87.5 percent) died with signs of ceratomyxosis during the experiment. In twenty-eight of those (70 percent of all fish) *C. shasta* myxospores were seen in wet mounts of intestinal material. Two fish were killed accidentally during the experiment, and three were euthanized.

Parasite concentration in water samples taken from tanks where both fish ultimately died is presented in Figure 5. The time at which fish began dying (10 dpe) corresponds with increasing parasite concentration. The average time to death (17 dpe) again corresponds with the beginning of peak parasite concentration. The highest mean concentration observed was in the 10 to 100 spore/l range. From day 25 to day 33, parasite concentration dropped to below 1 spore/l. Parasite DNA was not detected on day 39.

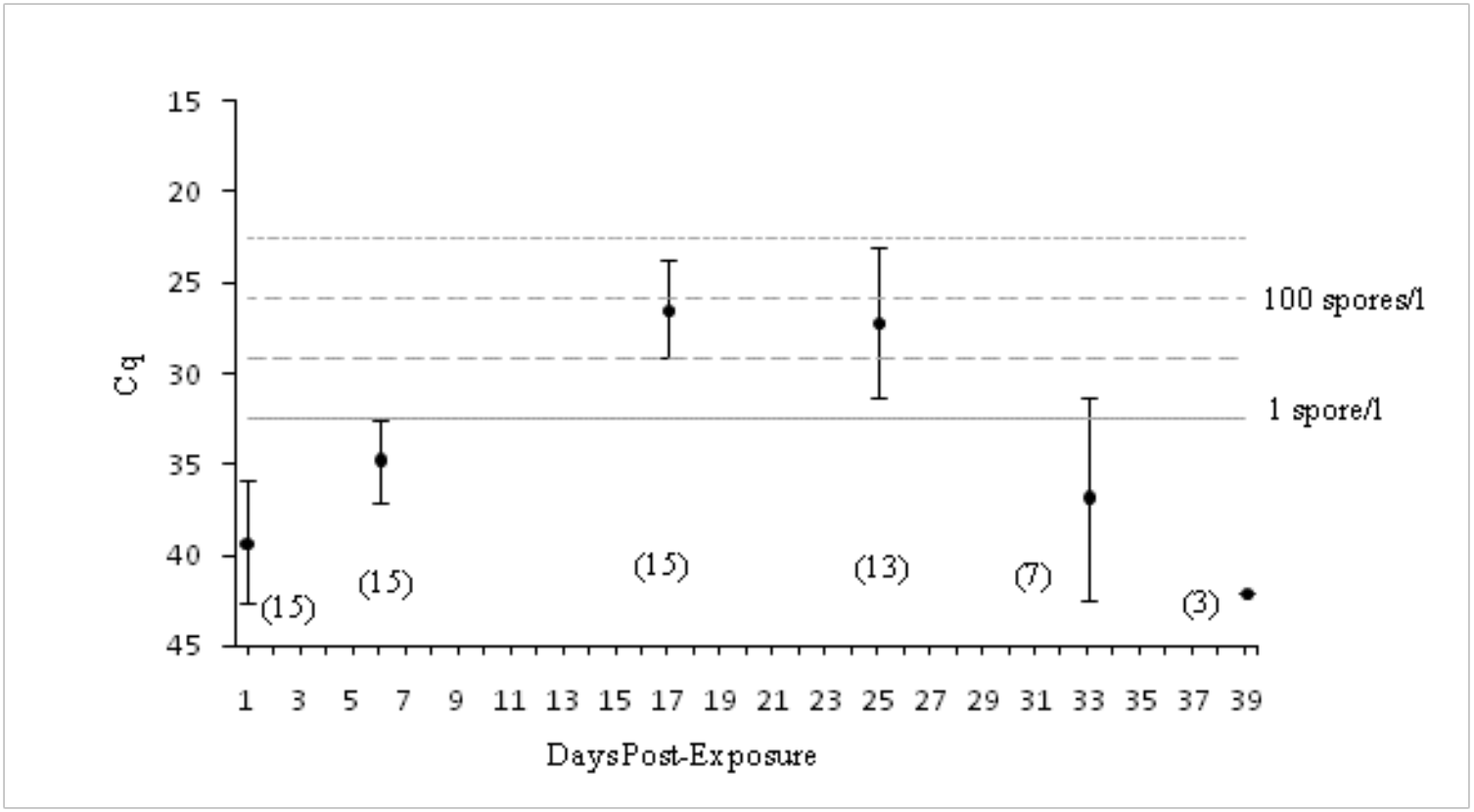

Figure 5. Concentration of *Ceratomyxa shasta* DNA (presumably myxospores) in 1 liter water samples drawn from tanks holding pairs of exposed Chinook salmon which both ultimately died. Exposure was conducted July 20-23, 2010. One sample was drawn from each tank, and each data point represents the mean Cq over all samples (number of samples shown in parentheses). Error bars represent one standard deviation. Standard parasite DNA concentrations, given in spores/l, are shown as horizontal lines at their corresponding Cq values. The lowest (solid) line represents 1 spore/l, and each subsequent line above indicates a tenfold increase.

Figure 6 shows parasite concentration in water samples taken from tanks where one fish died naturally and the other was euthanized at the termination of the experiment or killed accidentally. The general pattern of parasite concentration over time is the same here as in tanks where both fish died, however maximum daily spore production on day 17 is less. One tank in the group had quite high parasite concentrations on days 17 and 25 (nearly 1000 spores/l and just less than 10,000 spores/l, respectively). Another had a very high concentration on day 17 (nearly 10,000 spores per liter). These values contributed to the high standard deviations around the means on these two days.

Euthanized fish were preserved whole without being checked for myxospores. The two accidental mortalities were checked by wet mount of intestinal material and neither had myxospores.

Water temperature during the June 2011 exposure ranged from 15°C to 18°C ($\bar{x}$=16.1°C). The average parasite concentration at the site of fish exposure in the river fluctuated within the one to 10 spores/l range (Figure 7), based on sets of three 1 liter water samples collected throughout exposure. Concentration dropped below one spore/l on the final sampling occasion.

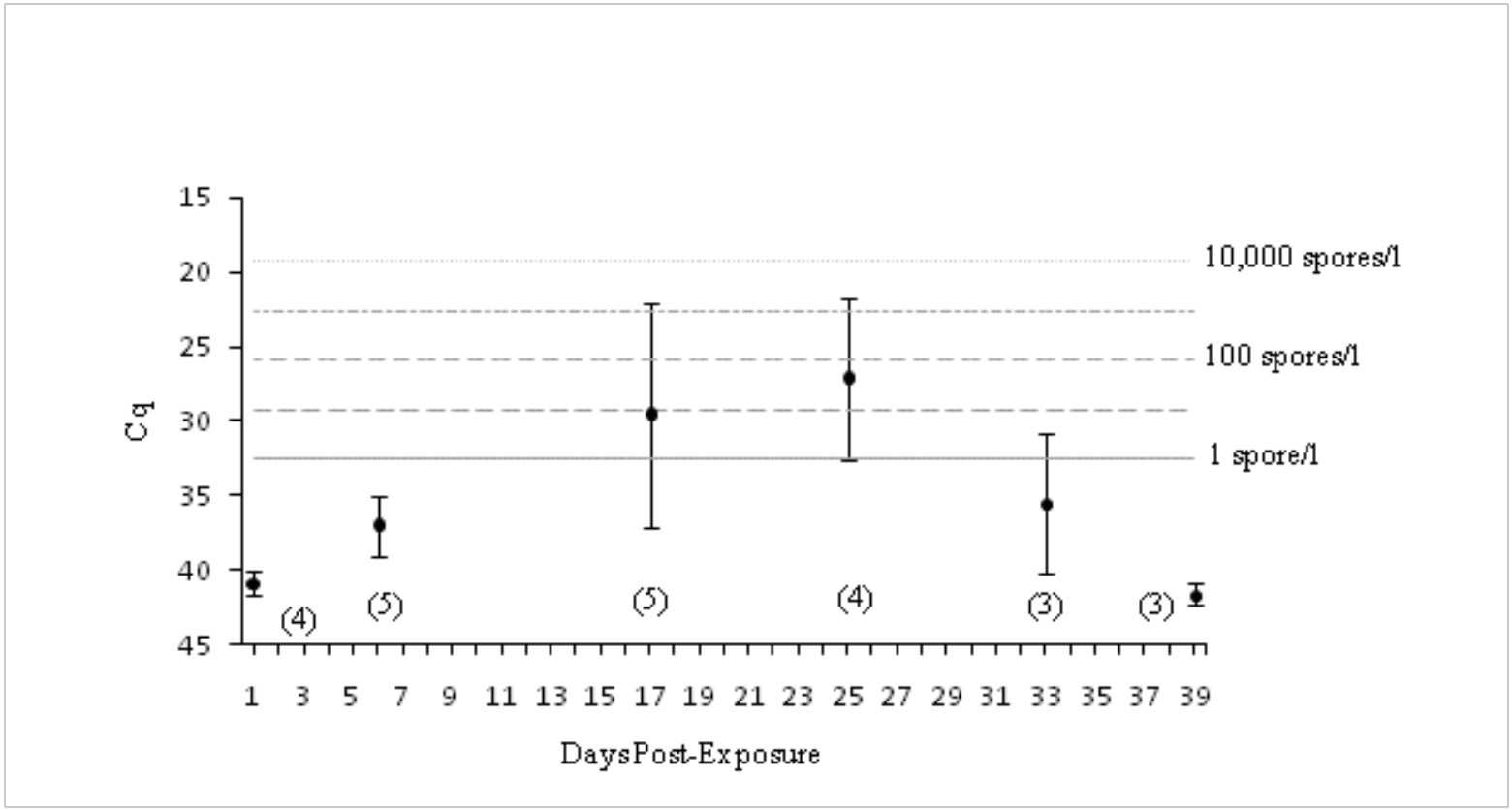

Figure 6. Concentration of *Ceratomyxa shasta* DNA (presumably myxospores) in 1 liter water samples drawn from tanks holding pairs of exposed Chinook salmon in which only one fish died with signs of ceratomyxosis. Exposure was conducted July 20-23, 2010. One sample was drawn from each tank, and each data point represents the mean Cq over all samples (number of samples shown in parentheses). Error bars represent one standard deviation. Standard parasite DNA concentrations, given in spores/l, are shown as horizontal lines at their corresponding Cq values. The lowest (solid) line represents 1 spore/l, and each subsequent line above indicates a tenfold increase.

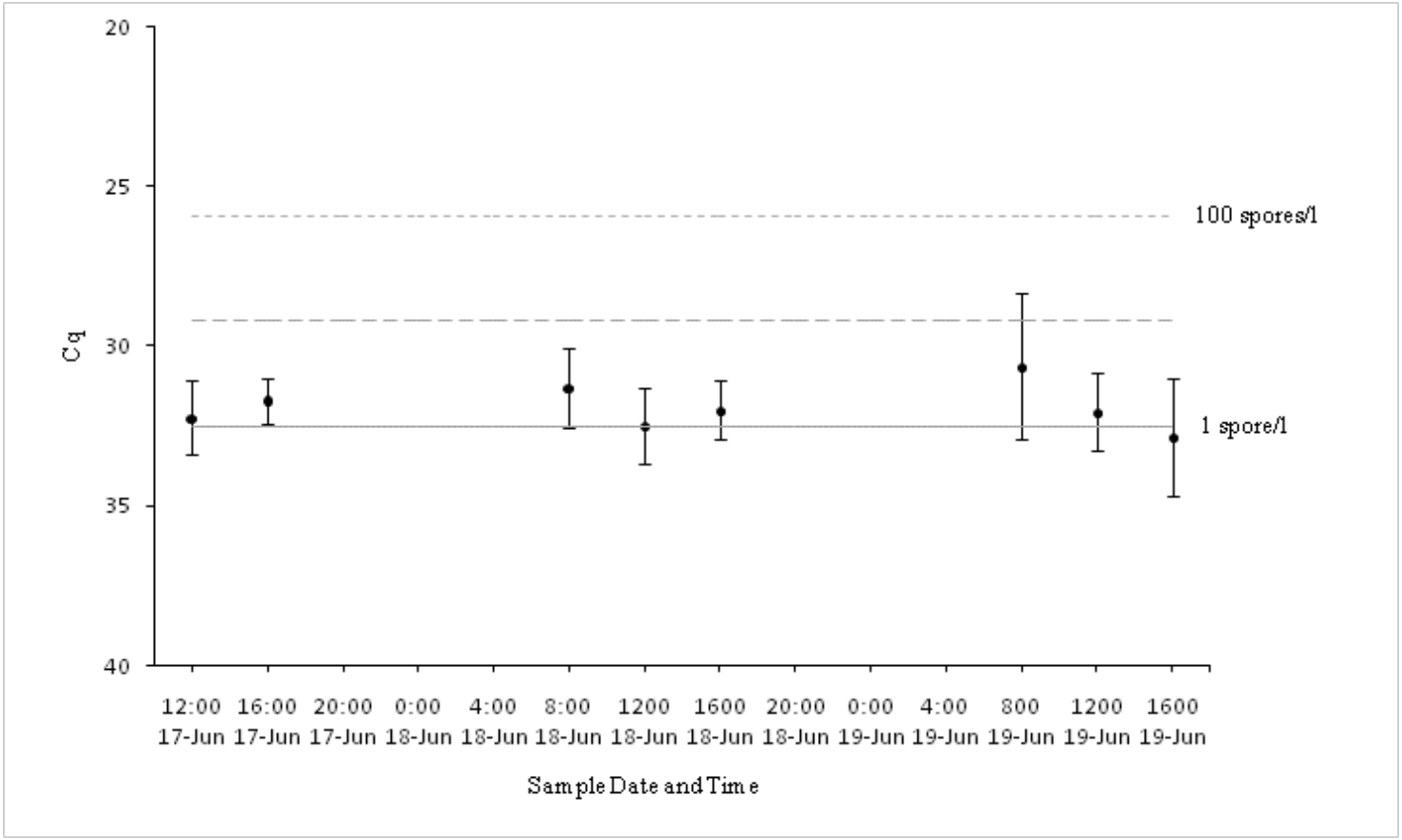

Figure 7. Average concentration of *Ceratomyxa shasta* DNA in the Klamath River during exposure of juvenile Chinook salmon June 17-20, 2011.Water samples were taken at the exposure site, Fisher's Klamath River RV Park (about 800m upstream from the confluence with Beaver Creek). Each point represents the average Cq of three one-liter water samples drawn at the date/time indicated along the x-axis. Error bars represent one standard deviation. Standard parasite DNA concentrations, given in spores/l, are shown as horizontal lines at their corresponding Cq values. The lowest (solid) line represents 1 spore/l, and each subsequent line above indicates a tenfold increase.

No mortalities were observed in this group of fish. At 17 dpe no clinical signs of ceratomyxosis were observed in exposed fish. During the 2010 experiments mortality was observed at 10-13dpe (with clinical signs), so this trial was terminated. The fish were euthanized, individual wet mounts were prepared from intestinal material from each one, and a small section of hindgut from each fish was frozen for genetic analysis. No myxospores were detected in any of the wet mounts. All exposed fish were positive for *C. shasta* by QPCR (Table 2).

Water temperature during the July 2011 exposure ranged from 20.5°C to 22°C ($\bar{x}$=21.1°C). The average parasite concentration in the river, based on sets of three 1 liter water samples collected throughout exposure, is presented in Figure 8. Parasite concentration in the river during exposure fluctuated within the one to 10 spores/l range.

On the second day of lab rearing (2dpe) two exposed fish died unexpectedly. 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 during exposure and transport. 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. 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*.

Table 2. Results of genetic diagnosis of *Ceratomyxa shasta* infection in juvenile Chinook salmon 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 and frozen for genetic analysis. 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 *C. shasta* DNA by QPCR (Cq<32.5). All control fish were negative for *C. shasta* DNA.

Tank	Treatment	Hindgut Tissue Cq
FP-1	Exposed	28.65
FP-2	Exposed	31.31
FP-3	Exposed	31.26
FP-4	Exposed	28.93
FP-5	Exposed	32.2
FP-6	Control	42
FP-7	Control	42
FP-8	Control	40.12
FP-9	Control	41.08
FP-10	Control	39.19

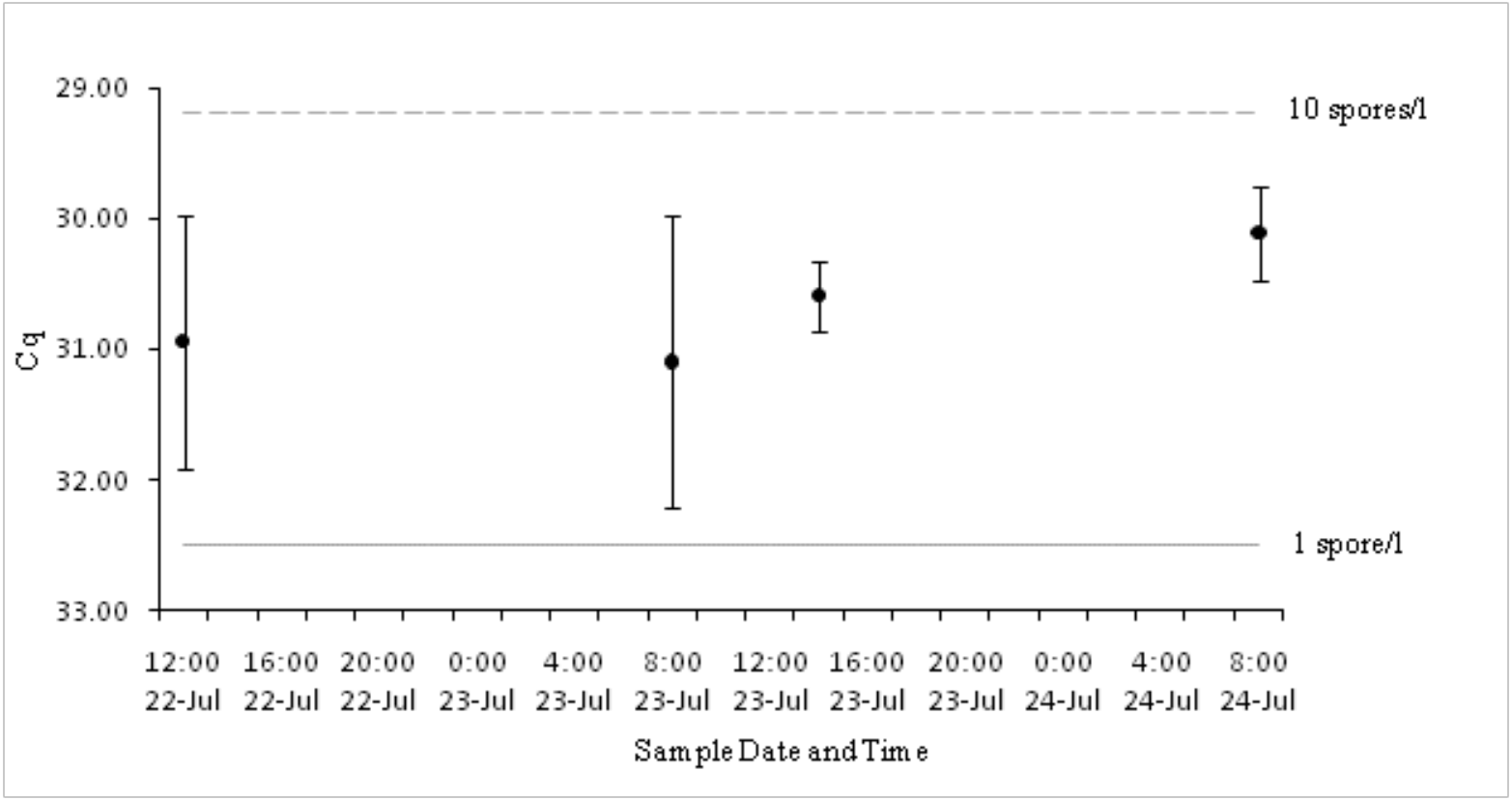

Figure 8. Average concentration of *Ceratomyxa shasta* DNA in the Klamath River during exposure of juvenile Chinook salmon July 22-25, 2011.Water samples were taken at the exposure site, Fisher's Klamath River RV Park (about 800m upstream from the confluence with Beaver Creek). Each point represents the average Cq of three 1 liter water samples drawn at the date/time indicated along the x-axis. Error bars represent one standard deviation. Standard parasite DNA concentrations, given in spores/l, are shown as horizontal lines at their corresponding Cq values. The solid line represents 1 spore/l, and the dashed line represents 10 spores/l.

Three exposed fish remained, along with the five unexposed control fish. The first mortality due to ceratomyxosis occurred in tank FP-5 at 15 dpe. The fish had been passing bloody fecal casts, and showed ascites. These are both signs of ceratomyxosis. A wet mount of intestinal material contained *C. shasta* myxospores. A very small amount of intestinal material was removed by rectal swab and smeared into a 2 ml tube, which was filled with 95 percent ethanol for preservation. The sample was assayed by QPCR and yielded a Cq of 22.62, indicating that *C. shasta* DNA was present. 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 9.

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, again both signs of ceratomyxosis. 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 2 ml tube, which was filled with 95 percent ethanol for preservation. The sample was assayed by QPCR and yielded a Cq of 22.65, indicating that *C. shasta* DNA was present. The carcass decomposed to skin scraps and bone by day 31. Water sampling ceased at that time. Parasite concentration in FP-1 over time is shown in Figure 10.

The last exposed fish (tank FP-4) showed no clinical signs of ceratomyxosis and was euthanized along with the control fish on day 36. *Ceratomyxa shasta* was not detected by wet mount, but a section of intestine which was removed and assayed with QPCR was positive for *C. shasta* DNA (Cq = 31.07). All water samples

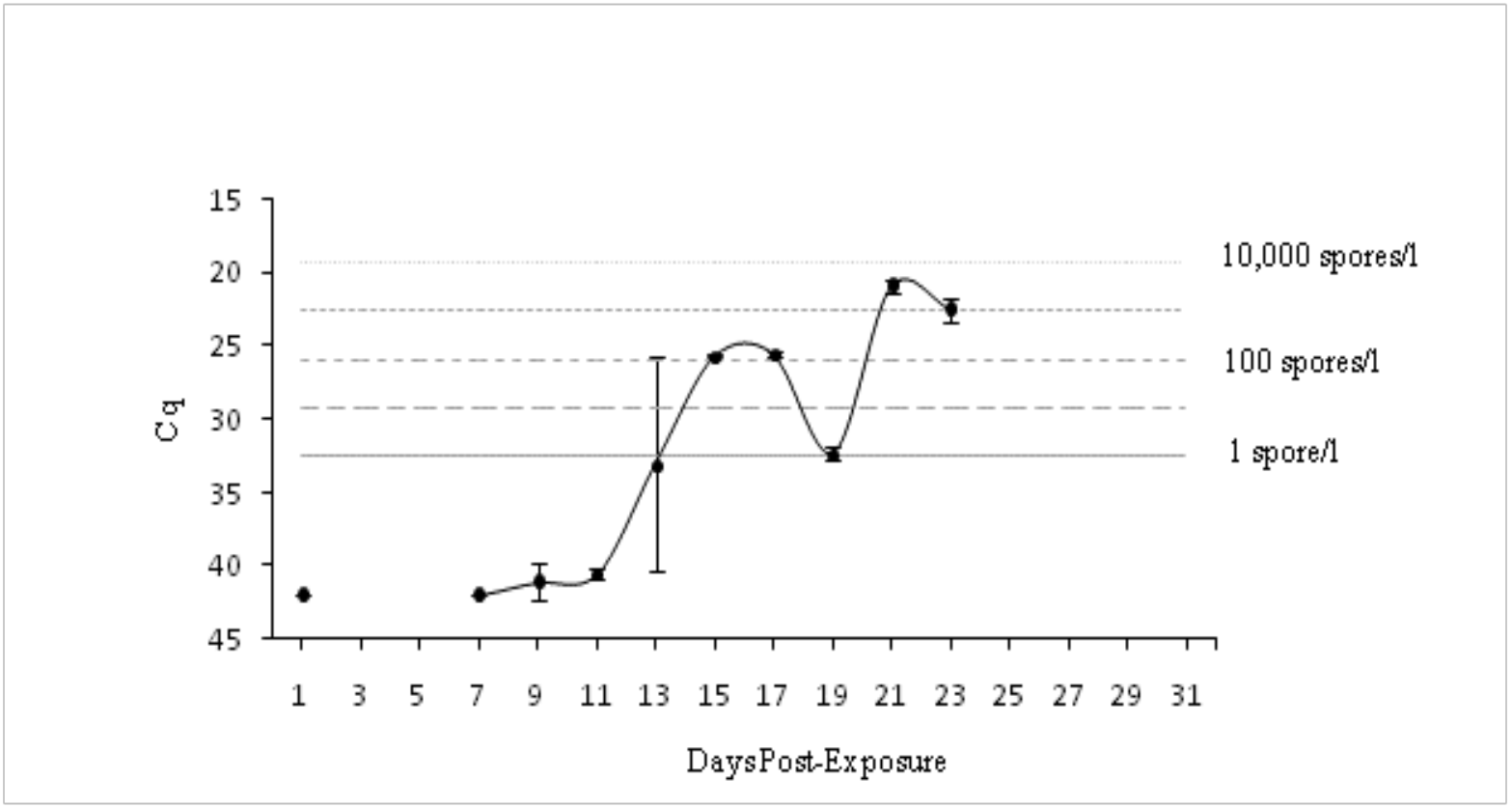

Figure 9. Concentration of *Ceratomyxa shasta* DNA, presumably myxospores, in tank FP-5 over time. Each point represents the average of two one-liter water samples. Error bars represent one standard deviation. Standard parasite DNA concentrations, given in spores/l, are shown as horizontal lines at their corresponding Cq values. The lowest (solid) line represents 1 spore/l, and each subsequent line above indicates a tenfold increase. The fish died on day 15 and was fully decomposed on day 23.

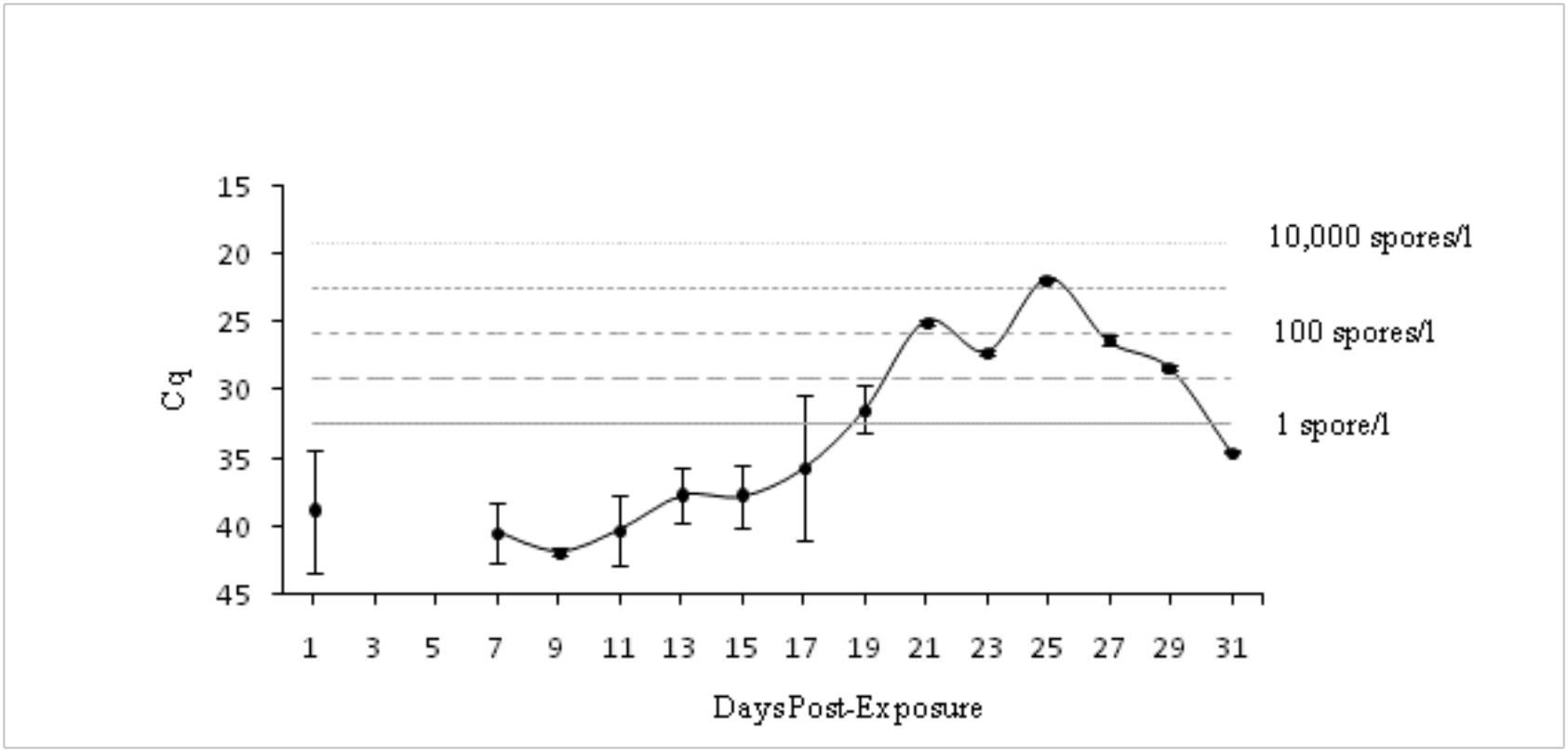

Figure 10. Concentration of *Ceratomyxa shasta* DNA, presumably myxospores, in tank FP-1 over time. Each point represents the average of two one-liter water samples. Error bars represent one standard deviation. Standard parasite DNA concentrations, given in spores/l, are shown as horizontal lines at their corresponding Cq values. The lowest (solid) line represents 1 spore/l, and each subsequent line above indicates a tenfold increase. The fish died on day 21 and was fully decomposed on day 31.

from this tank yielded estimates of less than one spore per liter. No signs of ceratomyxosis were observed in any control fish. All water samples from control tanks were negative for *C. shasta* DNA when assayed with QPCR.

Table 3 summarizes the estimated number of *C. shasta* myxospores in tanks holding exposed fish (FP-1, FP-4, and FP-5) on each day of sampling. Table 4 displays the sum of daily myxospore estimates for each tank, along with scaled-up estimates of total myxospore production and associated 95 percent confidence intervals.

Using the data from tanks FP-1 and FP-5, the average number of spores produced from an infected juvenile Chinook that dies of ceratomyxosis is estimated to be 141,975 (standard deviation = 78,841). According to Chesney and Knechtle (2011) the Iron Gate Hatchery production goal for juvenile Chinook salmon is 5,100,000 smolts, which are to be released from May through June. Table 5 summarizes infection prevalence in Chinook salmon smolts from May through July based on monitoring conducted in reaches above the confluence with the Trinity River from 2006 through 2011 by the California-Nevada Fish Health Center (Bolick et al. 2012). Bolick et al. provide data on Iron Gate Hatchery fish, identified by coded wire tags, as well as pooled data for marked and unmarked fish. Table 5 also shows estimates of the total spore contribution from 5,100,000 hatchery smolts based on data from fish in this study. The estimate assumes each infected fish dies of ceratomyxosis.

Table 6 presents an estimate of myxospore production from a post-spawned Chinook salmon carcass. If a juvenile Chinook salmon produces about 141,975 myxospores, and assuming parasite production is proportional to intestinal weight, an

Table 3. Estimated number of *Ceratomyxa shasta* myxospores in tanks FP-1, FP-4, and FP-5 on each sampling day. Each tank contained one exposed juvenile Chinook salmon. Estimates are based on *C. shasta* DNA concentration in water samples. Average Cq value from each day was converted to estimated spore concentration (spores/l) with a regression equation, then scaled up by total tank volume (19 liters). Values were rounded to the nearest spore. No samples were taken on days 3 and 5. Sampling in tank FP-5 ceased after day 23.

DPE	Estimated Myxospores (FP-1)	Estimated Myxospores (FP-4)	Estimated Myxospores (FP-5)
1	0	0	0
3	--	--	--
5	--	--	--
7	0	0	0
9	0	0	0
11	0	0	0
13	0	1	13
15	0	0	2,184
17	2	0	2,412
19	41	0	22
21	3,633	0	61,306
23	769	1	20,030
25	32,723	0	--
27	1,426	0	--
29	347	0	--
31	5	0	--

Table 4. Total *C. shasta* myxospore production estimates for each tank holding an exposed fish (FP-1, FP-4, and FP-5).Daily spore production estimates were summed, and the sum was scaled up by a factor equal to the ratio of the number of days the fish was in the tank, N, to the number of sample days, n. The scaled sum is the estimated total number of myxospores released by the fish during the experiment. Sample variance of the sum of daily values, sampling variance of the estimated experiment total, and associated statistical values were used to calculate 95 percent confidence bounds according to Thompson (2002).

Measure	FP-1 values	FP-4 values	FP-5 values
Sum:	38,946	3	85,967
N:	31	31	23
n:	14	14	10
Scale Factor:	2.214	2.214	2.3
Scaled Total:	86,226	7	197,724
Variance:	75,261,397	0.181	381,242,476
Sampling Variance:	2,833,054,029	6.825	11,399,150,019
df:	13	13	9
t:	2.16	2.16	2.262
95% Lower Bound:	-28,743	1	-43,783
95% Upper Bound:	201,195	13	439,231

Table 5.*Ceratomyxa shasta* infection prevalence (percent) and myxospore contribution from out-migrant juvenile Chinook salmon in the Klamath River. Infection prevalence data is from Bolick et al. (2012) and represents monitoring of fish above the confluence with the Trinity River during the months of May-June over the years 2006-2011. Minimum, mean, and maximum infection prevalence is presented for Iron Gate Hatchery coded wire tagged fish alone, and for pooled wild and hatchery fish. Total myxospore contribution is based on infection prevalence in 5,100,000 juvenile Chinook salmon (Iron Gate Hatchery release goal) and the estimated number of spores released per infected fish as found by this study.

Fish Group	Infection Prevalence	Total Myxospore Contribution
Iron Gate Hatchery CWT Chinook	min: 11%	79,647,975,000
	mean: 25%	181,018,125,000
	max: 69%	499,610,025,000
Wild and Iron Gate Chinook, Pooled	min: 17%	123,092,325,000
	mean: 32%	231,703,200,000
	max: 49%	354,795,525,000

Table 6. Estimate of *Ceratomyxa shasta* myxospore production from an individual adult Chinook salmon carcass. Estimates were generated by using intestine weight to scale up myxospore production estimates from juvenile fish in this study. Intestines from 141 juvenile Chinook from Iron Gate Hatchery were weighed to the nearest 0.0001 gram. Intestines from 22 post-spawn adult Chinook carcasses from tributaries in the lower Klamath basin were weighed to the nearest 0.01 gram.

Tank	Estimated Total Myxospores	Juvenile Intestine Weight (g)	Myxospores/g Juvenile Intestine	Adult Intestine Weight (g)	Estimated Total Adult Myxospores
FP-5	197,724	--	3,802,385	--	7,110,460
FP-1	86,226	--	1,658,192	--	3,100,819
Mean (Std Dev)	141,975 (78,841)	0.052 (0.0362)	2,730,289 (1,516,173)	1.87 (0.86)	5,105,639 (2,835,244)

adult Chinook salmon carcass may be capable of producing about 5.1 million myxospores. Table 7 shows estimates of total myxospores released from adult carcasses below Iron Gate Dam and from carcasses just in Bogus Creek. The minimum, average, and maximum numbers of spawners were obtained from Chesney and Knechtle (2011) and represent the period from 1978-2010. Carcass myxospore surveys since 2007 have shown that about 25 percent of Bogus Creek carcasses have myxospores. Across all samples drawn from Bogus Creek, Shasta River, and sites on the Klamath main stem, about 33 percent of carcasses have myxospores. Iron Gate Hatchery returns are not included because their carcasses do not decompose in the river, so they are not likely a source of myxospores.

Table 7. Estimated *Ceratomyxa shasta* myxospore production from adult Chinook salmon carcasses in the lower Klamath River and Bogus Creek. Prevalence of myxospores in carcasses is shown as a percentage in parentheses under the corresponding region. Numbers of spawners were obtained from data covering 1978-2010. Number of spawners in the lower Klamath does not include hatchery returns because their carcasses do not decompose in the river.

Region (Carcass Myxospore Prevalence)	# Spawners	Total Spore Contribution
Lower Klamath (33.53%)	min: 15,054	25,771,255,071
	mean: 68,836	117,841,777,208
	max: 194,372	332,749,461,321
Bogus Creek (25.77%)	min: 785	1,032,842,689
	mean: 8,654	11,386,268,316
	max: 46,432	61,091,658,243

DISCUSSION

Timing of *Ceratomyxa shasta* DNA release from juvenile Chinook salmon corresponded to timing of death of the fish. In the 2010 experiments, mean time to death (17-21 dpe) corresponded to peak *C. shasta* DNA concentration in water samples. Between both 2010 exposure groups, myxospores were seen in gut contents from 79 percent (53/67) of clinically infected fish at the time of death. Observations from the July 2011 exposure group show *Ceratomyxa shasta* DNA concentration increasing rapidly just before death, and peaking at the time of death. Concentration decreased for 4-6 days after death, then a second peak was observed. Times to death were 15 and 21 days post exposure, so most *C. shasta* was released about 2 to 4 weeks post-infection. Myxospores were seen in gut contents from one clinically infected fish (FP-5), but not the other (FP-1).

The June 2011 exposure group, and one fish (FP-4) from the July 2011 group, experienced subclinical infections. These fish were all positive for *C. shasta* DNA by QPCR. Parasite development may have been slowed or halted by physiological mechanisms in the fish such as formation of granulomas to isolate the pathogens (Ibarra et al. 1992, Bartholomew 1998). Alternatively, if a low number of actinospores infected the fish, more time may have been required for early developmental stages to continue replicating in the blood stream and create a higher level of infection in the intestine.

A single juvenile Chinook salmon in the July 2011 group produced an average of about 141,975 myxospores. This equates to about 2.7 million spores per gram of

intestine. A cohort (5.1 million) of juvenile Iron Gate Hatchery Chinook salmon released into the Klamath River would release about 7.96×10^{10} to 5.00×10^{11} myxospores, based on varying prevalence of infection published by Bolick et al. (2012). Applying the rate of myxospore production per gram of intestine to adult Chinook salmon yields an estimate of about 3.1 million to 7.1 million myxospores per fish. Based on the range of spawner escapement of adult Chinook salmon from 1978-2010 (Chesney and Knechtle 2011), and assuming about 33 percent of carcasses contain myxospores (Foott et al. 2009a, Foott et al. 2009b, Foott et al. 2010, Fogerty et al. 2012), I estimated about 2.58×10^{10} to 3.33×10^{11} myxospores are released into the lower Klamath River by returning adult Chinook salmon.

Between the 2010 and 2011 experiments, a significant confounding factor was discovered. In October of 2010 an informal procedure was conducted at Oregon State University's Salmon Disease Laboratory (Corvallis, Oregon). Ascites fluid from the abdominal cavities of moribund rainbow trout with severe ceratomyxosis was collected and analyzed. The fluid contained pre-spore stages of *C. shasta*, but very few myxospores. Aliquots were mixed with molecular grade water and filtered the same way water samples were filtered in this study, and assayed with QPCR. DNA from pre-spore stages of *C. shasta* showed up in the assay. The previous assumption was that pre-spore stages had a weak cell membrane which broke up during filtration, and mature spores were the only significant source of *C. shasta* DNA detected by the assay (personal communication, J. Bartholomew 2010. Department of Microbiology, Nash Hall 524, Oregon State University, Corvallis, OR 97331). This finding needs further investigation

to be substantiated, but it means there is uncertainty about which *C. shasta* life stages contributed the DNA detected in water samples in this study.

A major source of uncertainty in the results of this study is the fact that pre-spore stages of *C. shasta* may have been present in water samples. Timing of *C. shasta* DNA release was observed, but without knowing the life stages that contributed DNA it is impossible to know when myxospores in particular were released. During this study most wet mounts of gut material taken from clinically infected fish at the time of death contained myxospores. Myxospores were not seen in fish FP-1 of the July 2011 exposure group, but Figure 10 shows *C. shasta* DNA being released from the time of death through decomposition. Bjork and Bartholomew (2010) stated that myxospores are formed at a time when significant damage to intestinal tissue of the fish has already occurred. Because the fish in FP-1 died of a clinical infection, significant intestinal damage probably occurred. It may be reasonable to assume that myxospores were produced, but were not seen at time of death. Foott et al. (2009b) mentioned unpublished data from a 2008 exposure study in which they did not see myxospores in gut samples from clinically infected juvenile Chinook salmon at the time of death. After removing the intestines and incubating them at 18°C (the temperature at which fish had been held) for 48 hours, samples contained 6 to 9 million myxospores per gram of intestine. When ascites fluid containing pre-spore stages was stored in a refrigerator at the Humboldt State University Fish Pathology Laboratory, a casual observation revealed the fluid was full of myxospores after sitting untouched for two months. The ascites fluid had been drawn

from rainbow trout carcasses at Oregon State University's Salmon Disease Laboratory. Prior to euthanasia, the trout were moribund from ceratomyxosis.

Uncertainty in estimates of total myxospore production comes from the question of *C. shasta* DNA source in samples and the small sample size. If pre-spore stages showed up in assays, then the total counts of myxospores per juvenile Chinook salmon would be overstated unless pre-spores released into the water can develop without a host. Overestimating myxospore production from individual fish would inflate the rest of the *C. shasta* production estimates calculated in this study. Sample size is also problematic because total production of *C. shasta* was estimated from only 2 fish. Subclinical infections in both 2011 groups and losses to bacteria in the July 2011 group unexpectedly reduced sample size. The 2010 experiments were designed to test the exposure and rearing protocols, and to gather initial data on the timing of *C. shasta* release relative to exposure and time of death/decomposition. The results lead to refinement of procedures in the 2011 experiments, but the 2010 protocol was not conducive to precise numerical estimates.

A factor that may affect estimates about adult Chinook salmon in particular is that parasite production in this study occurred at a higher temperature and stress level than what most adults returning to the Klamath encounter. Adult Chinook salmon do not typically die of ceratomyxosis like juveniles do during summer outmigration. The fish in my study were completely overwhelmed by *C. shasta* and reared at a high temperature, so the parasite was able to fully exploit the host and proliferate to the greatest extent possible. Fish were also handled daily during water changes, leading to increased stress.

Parasite production per gram of intestine under these conditions probably does not represent what happens in adult carcasses.

The estimate of about 141,975 myxospores released per juvenile Chinook salmon (2.7 million spores per gram of intestine) is low compared to other studies. Foott et al. (2009b) observed 6 to 9 million myxospores per gram of intestine from juvenile Chinook salmon. They were examining fixed smears microscopically, a method which provides more precise counts than genetic analysis alone. Ray (2010) counted 499,000 to 908,000 myxospores per juvenile Chinook salmon while observing intestine and kidney tissue on a hemocytometer.

The water sampling protocol used in this study was a good way to document timing of *C. shasta* release from fish. However, it is not as precise as methods used by Foott et al. (2009b) and Ray (2010) for counting myxospores. Parasite distribution in holding tanks must be totally homogenous before sampling, and as fungus consumes the carcasses it may also destroy parasites in the tissue. The discovery that pre-spore stages of *C. shasta* show up in QPCR assays suggests myxospore production from fish might be overestimated, but results do not show this relative to other studies. An improvement on the procedures of this study would be pairing genetic analysis of filtered water samples with visual analysis of centrifuged water samples to determine which stages of *C. shasta* are detected by QPCR. Microscopic examination of homogenized tissues would probably yield the best estimates of parasite numbers at a fixed point in time, but carcasses could not be left to decompose undisturbed.

The estimate of about 7.96 x 10^{10} to 5.00 x 10^{11} myxospores being released from a cohort of 5.1 million Iron Gate Hatchery juvenile Chinook salmon falls within the range proposed by Ray (2010). He estimated between 9.38 x 10^{8} and 1.77 x 10^{13} myxospores could be produced by an emigrating Iron Gate Hatchery cohort. Both estimates only account for hatchery fish, not naturally produced fish entering the Klamath River from its tributaries. Infection rate among juvenile Chinook salmon varies from year to year (Bolick et al. 2012), so the true number is difficult to estimate with precision.

Observations by Bjork and Bartholomew (2009) and Ray (2010) indicated that *C. shasta* proliferation is unlimited, meaning that total parasite production is proportional to the amount of available tissue in the host and not limited by initial number of infecting parasites. Therefore, estimating how many myxospores might be produced per gram of intestinal tissue in juvenile Chinook salmon and scaling up by intestine weight is a logical approach to estimating myxospore production from adults. The resulting estimates range from about 3 to 7.4 million myxospores, which is much higher than other studies have shown. Fogerty et al. (2012) summarized several years of myxospore surveys of carcasses in the Klamath main-stem, Shasta River, and Bogus Creek. Myxospores are not detected in about 62 - 78 percent of carcasses, 20 – 32 percent of carcasses contain about 1,000 to 499,999 spores, and 2 – 12 percent contain 500,000 or more spores. Fogerty et al. have observed numbers of myxospores per gut scraping ranging from 1,131 to 13.2 million (an outlier). Foott et al. (2010) reported the average myxospore count from the small number of high producers (defined as carcasses that produce over 1

million myxospores) is about 1.8 million. Estimates generated from this study fall in the upper range of myxospore production from the highest-producing carcasses.

I estimated total myxospore contribution to the Klamath River by returning adult Chinook salmon at about 2.58×10^{10} to 3.33×10^{11}. This is based on an average number of spawning adults returning to the lower Klamath River, not including fish that return to Iron Gate Hatchery. Foott et al. (2009b) estimated that 1.08×10^{7} to 5.28×10^{10} myxospores were present in the Klamath River below Iron Gate Dam in 2008 due to adult carcasses. My estimate is higher than estimates by Foott et al. (2009b, 2010) and Fogerty et al. (2012) because I estimated greater production of myxospores per adult in this study.

The findings of this study suggest that an emigrating group of juvenile Iron Gate Hatchery Chinook salmon can produce more myxospores than a group of returning adults. Although the ranges overlap a little, my estimate for juveniles exceeds my estimate for adults as well as those published by Foott et al. (2009b, 2010) and Fogerty et al. (2012). Ray's (2010) estimate for juveniles is also higher. In terms of perpetuation of the infectious cycle in the Klamath River, though, myxospores released by juvenile Chinook salmon may hold less biological significance than those released from adult carcasses. The total number of myxospores is not irrelevant, but timing of myxospore release in the river is strongly related to where spores will end up and whether or not they are likely to contact polychaetes. If they do not contact polychaetes, they will not contribute to future disease in Chinook salmon. Infected adults carry *C. shasta* upstream on their spawning migration, so myxospores released from their carcasses are much more

likely to drift down through areas of high polychaete density. Fish returning to tributaries above Seiad Valley (Scott River, Beaver Creek, Shasta River, and Bogus Creek) all have the potential to infect polychaete populations which will affect out-migrating juveniles the following spring/summer.

Based on observations in this study, timing of *C. shasta* release from juvenile Chinook salmon appears to be linked to physical processes. There is typically sloughing of intestinal tissue at or just prior to mortality from ceratomyxosis (personal communication, K. True 2012. California-Nevada Fish Health Center, 2411 Coleman Fish Hatchery Road, Anderson, CA 96007). This sloughing appears to release *C. shasta* from the fish because I observed a sharp increase in *C. shasta* DNA concentration in water samples, leading to a peak at the time of death. The second peak in DNA concentration coincided with the point in decomposition when carcasses began splitting open at the abdomen, exposing the intestines to open water and gentle turbulence from air stones. This appears to have washed *C. shasta* out of the carcasses. The absence of other peaks in *C. shasta* release suggests that the parasite is only being shed passively. Therefore, the three main factors that would affect distribution of myxospores in a river are where a fish is initially infected (assuming infection leads to myxospore production and release, i.e. death), how fast myxospores develop, and movements of the fish during this time.

Hendrickson et al. (1989) and Stocking et al. (2006) state that *C. shasta* is confined to the main-stem Klamath River, so the point of initial infection for juvenile Chinook salmon can vary quite a lot based on fish origin (Iron Gate Hatchery or one of

the tributary streams). Stocking et al. identified the reach from the Shasta River confluence downstream to Seiad Valley as the most infective part of the lower Klamath River. Fish entering the Klamath River from tributaries below Seiad Valley are still at risk of infection because *C. shasta* actinospores remain infective long enough to affect fish tens of kilometers downstream of the polychaete from which they are produced (Foott et al. 2007).

Rate of myxospore development appears to be controlled by water temperature because *C. shasta* progresses through its life cycle faster as temperature increases (Udey et al. 1975). Water temperature in the main-stem Klamath River routinely reaches 20-22.5°C between June and August, with maximum temperatures up to 26.6°C (Bartholomew 2005). Foott et al. (2012) stated that juvenile Chinook salmon in the Klamath River rear and migrate in water temperatures of at least 21°C for up to 3-6 weeks. The temperature at which fish were reared in this study (20°C) is comparable to summer temperatures in the Klamath River, so the observed rate of *C. shasta* production was probably comparable to that which occurs the Klamath River.

Iron Gate Hatchery releases sub-yearling fall-run Chinook salmon between mid-May and early June (yearlings, released in the fall, are not considered in this study). These fish migrate out to sea rather quickly. Median travel time to the estuary is 30-34 days (Wallace 2004). Wallace and Collins (1997) noted that juvenile Chinook salmon in the Klamath River move to the estuary between late June and August. Wild fall-run Chinook salmon typically emerge from the gravel in spring and move to the main-stem

river within a few months, where they rear prior to smoltification and ocean entry (Kjelson et al. 1982).

Water temperature and migration behavior are not independent, because high temperature causes juvenile salmonids to make extensive use of thermal refugia in the Klamath River (Belchik 1997, Belchik 2003, Sutton et al. 2007). These are areas of reduced water temperature in the main stem below tributary confluences. Sutton et al. (2007) found that fish mainly leave thermal refugia just before dawn (the time of lowest water temperature), probably to feed in the surrounding area, and return again to spend the daylight hours in the cooler water. Fish used thermal refugia when main-stem temperature reached 22-23°C. This behavior allows fish to reduce time spent at stressful or potentially lethal temperatures (the upper lethal temperature limit of juvenile Chinook salmon is about 25°C (Brett 1952, Orsi 1971)). Time spent at lower temperatures in thermal refugia also has the potential to slow development of *C. shasta*. Therefore, the rate of *C. shasta* development in juvenile Chinook salmon is probably not constant.

The potential interactions between point of infection, water temperature, and migration behavior demonstrate that production and release of *C. shasta* myxospores from juvenile Chinook salmon in the Klamath River are not necessarily straightforward processes. However, given the observation that *C. shasta* is released from juvenile Chinook salmon about 2-4 weeks post-infection at 20°C, and assuming a median migration rate of 30-34 days for Iron Gate Hatchery fish, it is not likely that juveniles would be releasing myxospores high enough in the river to infect polychaete populations

in the Shasta River-Seiad Valley infectious zone identified by Stocking et al. (2006). This is in agreement with Foott et al. (2009b), who stated that most clinically infected juvenile Chinook salmon were collected in reaches of the Klamath below Seiad Valley. Juvenile fish may be infecting the sparse polychaete populations further downstream, which in turn may infect returning adults in the fall. This suggests that despite estimates showing juvenile Chinook salmon may be producing more myxospores than adults, adult carcasses are driving the cycle of disease in the lower Klamath River.

This study investigated the timing of *C. shasta* myxospore release from juvenile Iron Gate Hatchery Chinook salmon. Based on the results of the study, it appears that returning adults play a more important role in perpetuating high *C. shasta* concentration in the lower Klamath River. The timing of release from juveniles suggests that most of their myxospores do not pass through areas of the river with high polychaete density, whereas adults carry the parasite upstream before shedding myxospores. The spore load from adult Chinook salmon is much more likely to contact polychaetes, and subsequently cause high mortality among emigrating juveniles the following summer.

There are several pieces of information about *C. shasta* myxospore production from adult carcasses that are still needed in order to advance understanding of the disease cycle in the Klamath River. A good question to investigate is how long *C. shasta* survives and remains infective after a fish dies, and whether the parasite can continue advancing through developmental stages (producing more myxospores) in a dead host. Samples of gut material from juvenile Chinook salmon in this study, which died with clinical signs of ceratomyxosis, did not always contain myxospores. Adult fall-run

Chinook salmon do not typically die of ceratomyxosis, and adult myxospore surveys have only shown a small percentage of carcasses to contain high spore loads. A long temporal lag between death of a fish after spawning and myxospore formation/release would mean current myxospore estimates from adult carcasses might be low. It would also mean that spores may be entering the river in a continual trickle through late fall and early winter as the last carcasses break down. Cold temperatures this time of year might prolong spore viability (Ratliff 1983) as well as delaying their formation and release. This information is needed to further inform disease models of *C. shasta* in the Klamath River, which may then lead to steps which can be taken to manage the mortality rate among emigrating juvenile Chinook salmon.

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