

PRODUCTION OF THE MYXOSPOREAN PARASITE, *CERATOMYXA SHASTA*, IN
FALL-RUN CHINOOK SALMON CARCASSES IN THE KLAMATH RIVER
BASIN

By

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ABSTRACT

PRODUCTION OF THE MYXOSPOREAN PARASITE, *CERATOMYXA SHASTA*, IN FALL-RUN CHINOOK SALMON CARCASSES IN THE KLAMATH RIVER BASIN

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Three experiments were performed to investigate *Ceratomyxa shasta* myxospore production by post-spawned Chinook salmon carcasses in the Klamath River watershed. Post-spawned Chinook salmon carcasses presumably infected with *C. shasta* were placed in flow-through plywood boxes submersed in the main-stem channel of the Klamath River, California. Parasites were molecularly quantified in water samples collected daily from the boxes to determine timing of parasite release into the environment. No parasites were detected in samples at any point in time. Myxospore densities in intestinal contents collected from infected Chinook salmon were monitored through time using phase microscopy to identify timing of myxospore development and proliferation in fish carcasses. No significant changes in parasite density were observed in incubating samples. Myxospore prevalence and abundance was measured in intestinal contents collected from 242 Fall-run, post-spawned Chinook salmon carcasses. Carcasses were sampled in October through December of 2011 from four tributaries of the Klamath River: Salmon River, Shasta River, Scott River and Bogus Creek. The probability of *Ceratomyxa shasta* myxospore detection in post-spawned Chinook salmon carcasses was estimated by fitting candidate binary logistic regressions to myxospore presence-absence data. Myxospore infection severity in post-spawned Chinook salmon carcasses was estimated by fitting candidate linear regressions to myxospore abundance data collected from spore-bearing carcasses. Candidate models were constructed from varying

combinations of five explanatory variables: geographic sampling location, date of sampling, carcass fork length, carcass sex and degree of carcass decomposition at the time of sampling. AICc model selection determined that myxospore prevalence was specific to sampling site and was positively related to level of carcass decomposition. Log-transformed myxospore abundance was positively related to carcass fork length. These relationships in connection with age and site-specific Chinook salmon run size estimates are likely responsible for vast differences in regional myxospore production throughout the Klamath River watershed.

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INTRODUCTION

The Klamath River Basin drains approximately 12,100 square miles of land in southern Oregon and northern California and is classified into two water management regions, the upper and lower basin (Congressional Research Service 2005). The lower basin is drained by 200 miles of main stem channel and 4 major tributaries: the Salmon River, Scott River, Shasta River and the Trinity River. Anadromous fish passage to the upper basin is blocked by Iron Gate Dam which was constructed in 1962. To mitigate for lost fish production previously occurring in spawning grounds and natal habitat upstream of the dam, Iron Gate Hatchery was built in 1966 in Hornbrook, California.

The Klamath River Basin is defined by multiple climactic and geomorphic zones (National Research Council 2004). Landscape variability paired with historic connections to the Great Basin, Columbia River Basin, and the Sacramento River Basin (Moyle 2002) has enabled a diverse community of fish to populate its waterways. Native resident fishes in the Klamath River Basin include members of the families Cyprinidae (chub and dace), Catostomidae (suckers), Cottidae (sculpins) and Gasterosteidae (stickleback). Families of native anadromous fish in the Klamath River Basin include Osmeridae (smelt and eulachon), Petromyzontidae (lamprey), Acipenseridae (sturgeon) and Salmonidae (steelhead and salmon) (National Research Council 2004).

Fish in the basin have supported commercial and recreational fisheries for decades. Tribal subsistence fisheries predate written records and continue today. For many fish species, however, population abundance has varied dramatically over time, and in some cases populations are severely depressed (Brown et al. 1994, Markle and Cooperman 2001). In 1988, the shortnose and Lost River suckers were listed as

endangered under the federal Endangered Species Act and were followed by the Southern Oregon – Northern California Coast coho salmon evolutionary significant unit which was listed as threatened in 1997. According to historical catch records, Chinook salmon abundance in the Klamath River Basin appears to be in overall decline (National Research Council 2004). Contemporary monitoring has shown that Chinook salmon abundance in the Klamath River Basin fluctuates dramatically from year to year (National Oceanic and Atmospheric Administration 2011).

Inconsistent returns of Chinook salmon to the Klamath River Basin each year are apparent across multiple indices of Chinook salmon abundance. Adult natural spawner escapement is a term used to describe fish that return to spawning grounds at ages of 3, 4 and 5 years. Abundance estimates of these fish are used to govern allowable harvest of Klamath River Chinook salmon. In 2004, 2005, and 2006, returns fell below the Pacific Fishery Management Council's conservation objective to preserve 35,000 naturally spawning adult Chinook salmon (Pacific Fishery Management Council 2007). Six years later (2012), natural spawning adult escapement was estimated at 69,331 fish (California Department of Fish and Game 2012).

Total run size is comprised of all Chinook salmon that return to the basin regardless of age, origin or escapement. In 2004, 2005, and 2006, total run size of Chinook salmon was 34,275, 67,523, and 88,309 fish respectively. Six years later (2012), the total run size was estimated at 323,582 fish. This was greater than any estimate recorded over the course of 34 years of population monitoring (California Department of fish and Wildlife 2013). Returns such as those observed in 2004, 2005

and 2006 have significantly impacted commercial, subsistence and recreational fisheries (Congressional Research Service 2005) and have garnered intense interest in Klamath River Basin Chinook salmon research and recovery.

Historically, fish populations in the Klamath River Basin have been depleted by overfishing (Wales 1951, Markle and Cooperman 2001), but are now thought to be impacted most by habitat degradation (Brown et al. 1994, Markle and Cooperman 2001), variable ocean conditions (Taylor 1998, Fujiwara and Mohr 2009) and disease (Foott et al. 2002, Belchik et al. 2004, Fujiwara et al. 2011). Events of the past 20 years show that disease has both acute and chronic effects on migrating salmon in the Klamath River Basin. In 2002, an exceptionally large run of returning fall-type Chinook salmon crowded into the unfavorably warm water of the lower main stem Klamath River. These conditions caused an epizootic involving two pathogens: the ciliate *Ichthyophthirius multifiliis* (ich) and the bacteria *Flavobacter columnare*. This resulted in the deaths of at least 34,000 adult salmon (Belchik et al. 2004). Annually, out-migrating juvenile salmon are infected by the myxozoan parasite *Ceratomyxa shasta*, which has at times been implicated as the leading cause of salmon disease and mortality in the Klamath River Basin (Foott et al. 2002). Prevalence of infection in out-migrating juvenile Chinook salmon captured in Klamath River upriver of the Trinity River confluence has ranged between 19 and 54% in 8 of the 11 years of histological monitoring (True et al. 2011, True et al. 2013). In 2002 and 2004, researchers predicted that the majority of infections observed in juvenile salmon would have eventually resulted in death. The high prevalence and severity of infections observed in out-migrating juvenile salmon has

inspired a great deal of research devoted to understanding *Ceratomyxa shasta* and its interactions with salmon in the Klamath River Basin.

Ceratomyxa shasta is a myxosporean parasite that infects trout and salmon living in various watersheds of the Pacific Northwest (Hoffmaster et al. 1988, Hendrickson et al. 1989, Stocking et al. 2006). *Ceratomyxa shasta* causes disease in fish characterized by necrosis of tissues in the gastrointestinal tract, and particularly in the descending intestine (Bartholomew et al. 1989). It has also been observed in sections of kidney, liver, and the peripancreatic region (Foott et al. 2003).

Ceratomyxa shasta has an indirect life cycle (Figure 1). Infections are found in salmonids and in the obligate polychaete host, *Manayunkia speciosa* (Bartholomew et al. 1997). Two infective, spore life stages can occur in the aquatic environment, myxospores and actinospores. Infection in polychaetes begins when consumed myxospores attach to the gut wall and release sporoplasm between epithelial cells into the tissues of the worm. Multiple proliferative life stages are produced here and sexual reproduction of the parasite ensues (Meaders and Hendrickson 2009). These infections culminate in the release of actinospores from epidermal pores of the polychaete into the external environment. Fish are infected when actinospores attach to the gills and release sporoplasm onto the surface epithelium. Proliferative life stages travel through the bloodstream and arrive at intestinal tissues 5-7 days after first exposure (Yamamoto and Sanders 1979, Bjork and Bartholomew 2010). By 15-16 days post exposure, myxospore development can occur in intestinal tissues (True et al. 2012). A single actinospore can cause lethal infection in susceptible fish (Bjork and Bartholomew 2009).

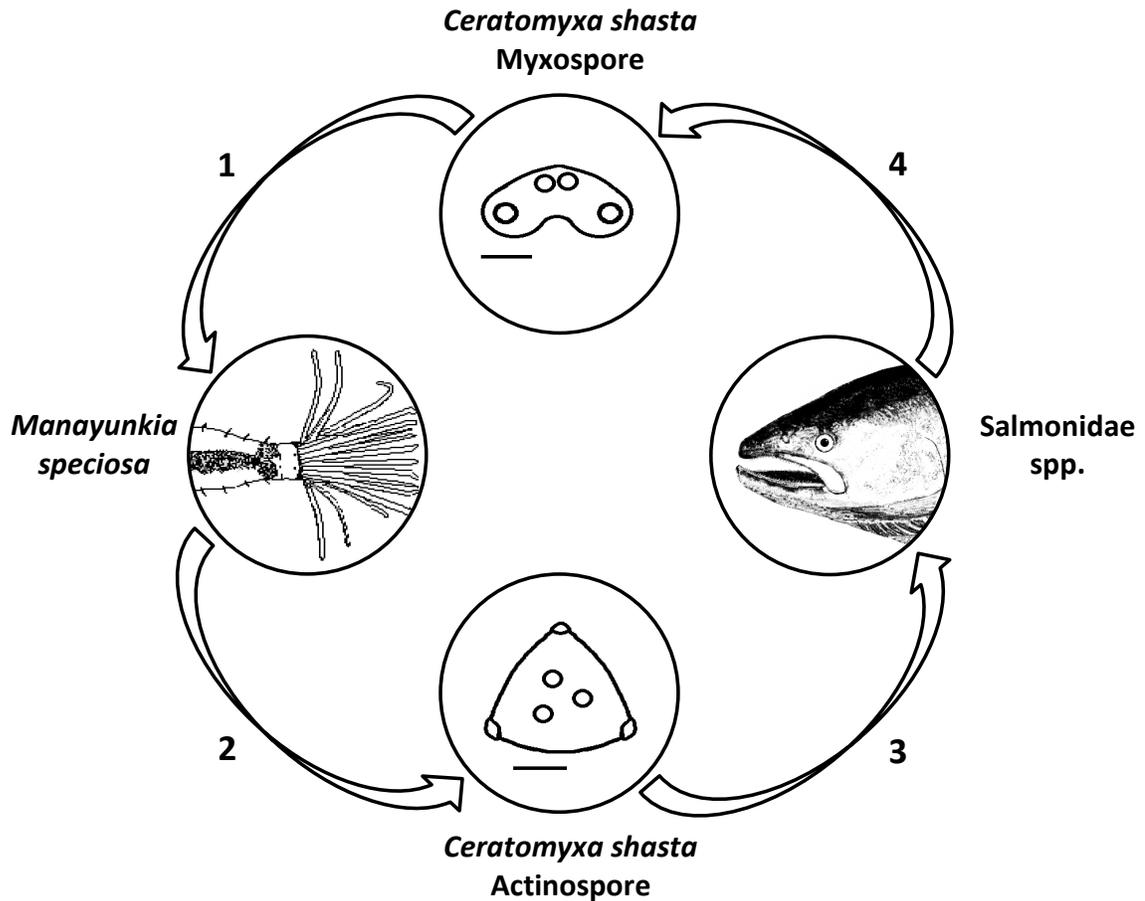


Figure 1. Life cycle of *Ceratomyxa shasta*. Myxospores are consumed by the invertebrate host, *Manayunkia speciosa*. Myxospores attach to the gut wall and initiate infection in the intestinal epithelium (1). Parasites migrate to the epidermis, proliferate, and develop into actinospores. Actinospores are released into the water from the polychaete's skin (2). Actinospores attach to the gills of trout or salmon and transmit infection into the bloodstream (3). Parasites proliferate and migrate to intestinal tissues where myxospores develop. Myxospores are released into the water from the vertebrate host and sink (4).

Much research has been devoted to defining factors that influence *Ceratomyxa shasta* infection in trout and salmon. Water temperature has been shown to affect rate of ceratomyxosis progression and host mortality in infected fish (Udey et al. 1975, Hallett et al. 2012, Ray et al. 2012). The time during which fish are held in infectious water has been related to prevalence of infection and disease induced mortality in sentinel fish. This suggests that effective dose of the infective stage of the parasite can influence ceratomyxosis in fish (Ratliff 1981, Hemmingsen et al. 1986, Ibarra et al. 1992, Foott et al. 2007). Parasite dose may also explain the differential disease induced mortality observed in sentinel fish exposed at varying locations within the basin and times of year (Hendrickson et al. 1989, Stocking et al. 2006). Hallett et al. (2012) determined that water-borne parasite density varied temporally and spatially within the basin. They tied disease related mortality in sentinel fish to density of water-borne parasites present at the time of exposure.

Susceptibility to infection has been shown to vary across host species and genetic strain (Hallett et al. 2012, Ibarra et al. 1992, Hemmingsen et al. 1986). Some previous assertions regarding species-specific susceptibility must be revisited, however, due to the recent discovery of genetically and behaviorally distinct parasite strains within the species *Ceratomyxa shasta*. Atkinson and Bartholomew (2010) described genetic differences between parasites collected from fish hosts of different species and concluded that the species *C. shasta* is a complex of four sympatric, host-specific strains or genotypes.

Numerous studies have examined infection in juvenile fish. This has led to a sophisticated understanding of *Ceratomyxa shasta* as it applies to out-migrant juvenile Chinook salmon. Less is known about interactions between *C. shasta* and adult fish. Infection surveys suggest that adult Chinook salmon play a critical role in *C. shasta* life history in the Klamath River Basin. Post-spawned carcasses sampled from spawning grounds can contain more than 1 million myxospores in their descending intestines (Foott et al. 2009b, Foott et al. 2010, Fogerty et al. 2012). Surveys project that returning adult Chinook salmon are capable of delivering billions of myxospores to the Klamath River Basin each year after spawning and death (Foott et al 2009b, Foott et al 2010, Fogerty et al 2012). It is presumed that the infectious river reaches near the upstream boundary of infectivity to fish are provided with myxospores by adult salmon migrating to spawning grounds (Foott et al. 2009b).

Presence of *C. shasta* myxospores in adult Chinook salmon tends to vary according to when infections are observed. Infections are often dominated by pre-spore life stages when they are observed in fish sampled before spawning, such as those captured in hatcheries and in the net harvest. In 2007, prevalence of infection determined by histology was 86% and 75% in adult Chinook salmon sampled at Trinity River Hatchery and Iron Gate Hatchery respectively (Foott et al. 2009a, Slezack 2009). Although prevalence of infection by pre-spore life stages was relatively high, myxospores were only observed in 7% of these fish. Myxospore prevalence is typically greatest in fish allowed to spawn and die naturally and can be greater than 40% in some river reaches (Foott et al. 2009b). Among fish that spawn naturally, density of myxospores

observed in decomposed carcasses tends to be greater than in fresh carcasses (Foott et al. 2009b). The fate of *C. shasta* pre-spore life stages in dead fish hosts is unknown. If pre-spore life stages in carcasses can develop into myxospores, then current estimates of myxospore contribution by carcasses are highly understated, as they are calculated based on numbers of myxospores observed after a short period of incubation.

Previous myxospore surveys have used a frequentist approach (null hypothesis testing using p values) to analyze how *C. shasta* infection severity varies in salmon carcasses. Akaike's information theoretic approach offers an alternative presentation of the information found in data. In this approach, multiple hypotheses are expressed as a candidate set of models (Anderson 2008). AICc model selection assigns ranks to each model in the candidate set according to its estimated Kullback-Leibler distance or AICc (Akaike's information Criterion adjusted for small sample size). Kullback-Leibler distance is rooted in the concept that some unknown amount of information is lost when models are used to approximate true reality. This amount of lost information is called Kullback-Leibler distance and can be imagined as the distance between truth and a model/hypothesis (Anderson 2008).

Expected Kullback-Leibler distances for each model are estimated using Akaike's information theoretic approach (Akaike 1973) which is described in detail in Anderson (2008). Because Kullback-Leibler distance derived from sample data is an estimate, there is a level of uncertainty associated with ranking models in this fashion. This uncertainty is acknowledged by a model's weight which is the probability that each model in the candidate set is the Kullback-Leibler best model among those fit. The

summation of all model weights in a candidate set is equal to 1.0. The model among a candidate set that produces the shortest estimate of Kullback-Leibler distance (Kullback-Leibler best model) minimizes inefficiency (Anderson 2008) and is considered the best approximating model. Variables that are included in the Kullback-Leibler best model are then considered the most informative in predicting the response.

Identifying the temporal and circumstantial details surrounding myxospore production and release into the environment by Chinook salmon carcasses is the focus of this study. The primary objectives of this study are:

- 1) Record relative parasite concentrations as they are released through time from an infected adult carcass.
- 2) Determine if myxospore production continues after death of the fish host
- 3) Identify host and circumstantial characteristics related to prevalence and severity of infection in post-spawned Chinook salmon carcasses.

Currently, an epidemiological model is being constructed by a team at Oregon State University that traces *Ceratomyxa shasta* production in the Klamath River watershed. Reliable estimates of myxospore contribution by carcasses are vital to the model's development. My research focuses on identifying contributing factors to myxospore production by carcasses and aims to further develop methods that will generate accurate and unbiased estimates of myxospore production in the Klamath River Basin.

MATERIALS AND METHODS

Experiment 1: Parasite Dispersal from Post-Spawned Chinook Salmon Carcasses

Two flow-through flume boxes were constructed from marine-grade plywood. Boxes were 2.44 meters with a square cross section of 50.8 x 50.8 cm. Each box was longitudinally divided into four chambers by 2 cm. mesh, plastic screen.

Twenty-five post-spawned Chinook salmon carcasses were collected from 100 m above to 100 m below the Bogus Creek, California Department of Fish and Wildlife (CDFW) fish counting weir by CDFW technicians. The carcass sampling procedures were included in the Institutional Animal Care and Use Committee protocol 10/11.F.19-E. Intestinal material was collected from each carcass by inserting a clean metal inoculation loop into the vent. Gentle, circular motion was applied to the loop before it was removed with a sample of intestinal contents. Contents were smeared onto the center of a glass microscope slide. A drop of phosphate buffered saline was placed on the intestinal contents. A glass cover slip was placed on the drop of saline. Wet mounts were microscopically examined for *Ceratomyxa shasta* myxospores at a total magnification of 400x (bright field). Myxospores were detected in only one carcass from 25 collected. This carcass and 5 of those that screened negative for myxospores were set aside for later experimental treatment.

Flume boxes were submersed in the Klamath River at a site upstream from the confluence of Beaver Creek and the main stem Klamath River (river kilometer 262). They were anchored to the river bottom with rebar. Box floors were covered with river benthic material collected on site. A single Chinook salmon carcass was placed in each

of the three upstream-most chambers of each box. Carcasses were held in place in separated chambers to promote even and consistent water flow through the flume boxes. The fourth and downstream-most chamber served as the site for water sample collection. Boxes were positioned to promote water passage through the fish holding compartments before entering the sampling chamber. Boxes were periodically shifted to accommodate changing current and depth but always remained between 5 and 20 feet from the river bank.

Water sampling occurred from October 31 to November 29, 2010. Once per day, 3 one-liter samples of water were removed from the collection chamber of each box. Three one-liter background water samples were also collected immediately upstream of each box to serve as controls. Water samples were collected by submersing a closed 1 liter plastic bottle through the downstream, open end of the box into the water sampling chamber. The bottle lid was then removed, the bottle would fill, and the lid was replaced to retain sampled water.

Water velocity through the boxes was measured daily with a model 2100 Swiffer flow meter. The meter propeller was placed just inside the open sampling chamber and aimed toward the upstream end of the box. Velocity was measured at 3 vertical transects within the sampling chamber. Average water velocity of the three transects was used as daily water velocity through the box.

Water samples were filtered on site. Water was filtered through 5 um Millipore brand nitrocellulose membrane filters. Sample membranes were then rolled, folded and placed in 2 mL microcentrifuge tubes. Membranes were covered in 95% ethyl alcohol

and stored. At the HSU Fish Pathology Laboratory, sample tubes were centrifuged and the majority of alcohol was pipetted off and discarded. The remaining alcohol was allowed to evaporate from the samples.

Once dry, samples were shipped to Oregon State University's Department of Microbiology, where *C. shasta* DNA was quantified in each sample using quantitative PCR. Quantitative PCRs were performed following the methods of Hallett et al. (2012).

Experiment 2: Myxospore Development after Host Death

Three experimental trials were performed to investigate myxospore development in Chinook salmon carcasses. In the first two experimental trials, sub-yearling Chinook salmon (from Iron Gate Hatchery) were held in the Klamath River for 72 hours to expose them to the infectious stage of *Ceratomyxa shasta*. The juvenile salmon used in this experiment were reported in the Institutional Animal Care and Use Committee protocol 10/11.F.74-A. Fish were held in modified, plastic minnow traps upstream from Beaver Creek (river kilometer 262). This site is known to be highly infectious to sentinel Chinook salmon and hatchery steelhead trout for *C. shasta* (Foott et al. 2003, Stone et al. 2008). The trial 1 exposure period began June 17, 2011 and ended June 20, 2011. The trial 2 exposure period began July 22, 2011 and ended July 25, 2011. Control fish were obtained from Iron Gate Hatchery immediately after each 72 hour exposure. Experimental (exposed) and control fish were transported to the HSU Fish Pathology Laboratory in 3 separate, aerated ice chests filled with Klamath River water. To reduce temperature shock on fish, sealed plastic bags of ice were placed into the water with roughly half of the exposed fish. In this way, water temperature in ice chests was

adjusted to match the temperature of water in holding tanks at Humboldt State University. Approximately 45 min before arrival at HSU, fish were prophylactically treated with 52.6 mg/L Binox® nitrofurazone to suppress incidental bacterial infections. Bacterial prophylaxis was continued during rearing at HSU.

Once at HSU, a sub-set of fish was immediately sampled systematically from each of the three transport tanks (0 days post exposure). A systematic sample was collected by transferring each of the fish from a given transport tank into two 5 gallon buckets containing water from the transport tank. Every K^{th} fish captured was included in the systematic sample and was placed in a bucket with tank water. K was equal to $\frac{N}{n}$ where N is the total number of fish in the group from which the sample was drawn and n is the desired sample size. This was done to prevent sampling bias related to the capture of sampled fish. The remaining fish were distributed among 3 identical 114 L circular tanks filled with HSU Hatchery water (free of *C. shasta*). Each tank was equipped with a 38 L water tempering sump and a temperature controlling device. Water flow between tank and sump was set at a rate of 15 L per minute. A Marine Land Stealth Pro® 300 watt aquarium heater (18 - 21°C) or the chilling coil of a ¼ hp, Aqua Logic Cyclone® Chiller (8 - 10°C) was placed in each sump to achieve treatment temperatures (Table 1). Two treatment temperatures were used to determine how seasonal differences in temperature may affect the progression of infection in fish and subsequently, at which point in the infection (developmentally) can a host die and eventually produce myxospores. Achieving precise temperature control of water in tanks was difficult, because the experiment was performed outdoors, and because each tank was tempered by

Table 1. Allocation of juvenile Chinook salmon across two temperature treatments. Intestinal contents were collected from fish after 0, 5, 10, and in trial 2, 15 days post exposure. Units expressed as number of fish held / number of pooled gut content samples produced.

		Mean Rearing Temperature	Mean Incubation Temperature	fish / pools			
				0 days	5 days	10 days	15 days
Trial 1	Warm	19°C	21°C	10 / 2	16 / 2	16 / 2	0 / 0
	Cool	8.5°C	7.5°C	10 / 2	16 / 2	16 / 2	0 / 0
	Control	20°C	21°C	5 / 1	5 / 1	5 / 1	0 / 0
Trial 2	Warm	19°C	20.5°C	14 / 2	14 / 2	14 / 2	14 / 2
	Cool	10°C	7.5°C	*14 / 1	14 / 2	14 / 2	14 / 2
	Control	20°C	20.5°C	7 / 1	7 / 1	7 / 1	7 / 1

* One seven-fish pool of intestinal contents was spilled and discarded before observations could be made.

independent devices and thermostats. Experimental fish were divided between temperature treatments. Non-exposed fish (control fish) were held separately in heated water (20 - 20.5°C). Aeration was provided to each tank with an airline and diffuser stone. Forty-five liters of water from each tank was replaced daily with fresh HSU hatchery water. Fish were fed a commercial diet once per day.

Sub-sets of fish were systematically sampled from the control group and from each temperature treatment group at intervals of 0, 5, 10, and in trial 2, 15 days post exposure (dpe). These intervals will be referred to as sampling occasions. Fish from each sampling occasion were euthanized with an overdose of tricaine methanesulfonate. Intestines (from the rectum to pyloric caecae) were removed with scissors and forceps. To obtain intestinal contents, an intestine was placed on a glass slide and held at one end with forceps. Contents were pushed through the gut and onto the glass slide by scraping the backside of a #21 scalpel blade along the outside of the intestine (Foott et al. 2009a, Foott et al. 2009b, Fogerty et al. 2012). Contents were transferred to a 2 mL microcentrifuge tube with a disposable, single-edged razor blade. Individual intestinal contents samples were combined so that two large pooled samples were accumulated from exposed fish in each temperature treatment and one large pooled sample was accumulated from control fish (Table 1). The control group primarily functioned to determine that HSU hatchery water is free of the infective stage of *C. shasta*. Replication in this group was thus deemed unnecessary. Consequently, only a single pooled sample was collected from the control group on each sampling occasion. A large pooled sample volume was required to provide enough material for sub sampling through time.

Numbers of fish contributing to each pooled gut contents sample differed between trials (Table 1). In trial 1, no fish were sampled at 15 dpe. In trial 2, one sample was spilled and discarded before observations could be made. This sample was collected from fish held 0 days after exposure under the cool water treatment.

Pooled intestinal contents in both experimental trials were diluted with sterilized HSU hatchery water and vortex mixed. Dilution concentrations differed between trials. In trial 1, samples were diluted with less than 1 mL sterilized hatchery water per pooled sample. In trial 2, the volume of pooled samples was measured with sterilized, graduated pipettes. Samples were then diluted 9x by volume (10x final dilution) with sterilized hatchery water. In trial 2 only, one hundred μL of diluted suspension was collected from each sample and frozen for QPCR analyses. Samples were then incubated according to temperature treatment (warm = 19.0 - 23.5° C; cool = 3.0 – 12.5° C) (Table 1). Incubation temperatures in the cold treatment varied considerably. This may have been due to the frequent opening of the incubator's door (laboratory refrigerator) or to the general lack of sensitivity of the thermostat in the device itself.

In trial 1, when sufficient volume of sample material was available, myxospore densities in incubating samples were estimated on 9 occasions between 0 and 56 days after sample collection. In two samples, no material was remaining after 20 days of incubation. Both samples were collected from fish held for 0 days after exposure under the cold water treatment.

In trial 2, myxospore densities in incubating samples were estimated at 1, 2, 4, 8, 14, 20, 26, 42 and 56 days after extraction from fish. Myxospore densities in samples

from fish held 5, 10 and 15 days were also calculated the day of extraction (0 days). Myxospore densities were estimated following methods of Foott et al. (2009a, 2009b) and Fogerty et al. (2012). A sample was vortex mixed and allowed to settle for less than 3 min. A short settling period excludes large particulates from wet mounts and the hemocytometer chamber. In addition, it is thought to have a negligible effect on a sample's suspended myxospore density. Myxospore density in a sample was estimated from four replicate hemocytometer counts of the suspension under 400x total magnification using phase contrast microscopy. The remainder of the sample was returned to the incubator for further incubation and examination. Genetic samples were shipped to Oregon State University's Department of Microbiology where samples containing *C. shasta* DNA were identified using qPCR. QPCRs were performed following the protocol of Hallett et al. (2012). QPCR level of detection was estimated from 3 serially diluted samples known to contain *C. shasta* DNA.

In the third experimental trial, descending intestines were removed from post-spawned Chinook salmon recovered from Shasta River and Bogus Creek. Carcass sampling protocol was included in the International Animal Use and Care Convention permit 10/11.F.19-E. Using clean, disposable, single-edged razor blades, intestines were removed from the pyloric caecae to the vent. Each intestine was stored in a zip top plastic bag with an identification tag made of water proof paper. Intestines were stored cool (ice packs or refrigeration) for 24-48 hours. Intestines were then cut into 2-6 cm sections. Sections were placed on a sterilized glass plate and were held in place with forceps. Intestinal contents were pushed through each section onto the glass plate by scraping the

backside of a #21 scalpel blade along the outside of each section. Contents were transferred to 15 mL graduated centrifuge tubes with a disposable razor blade. Contents were diluted 3x with either hatchery water or phosphate buffered saline (4x total dilution). I assumed that, to some unknown degree, water from the surrounding environment infiltrates into the tissues of a fish after death and continues throughout decomposition. Hydration of the carcass, including the lumen and contents of the descending intestine, should change the ion concentrations of this environment. Two incubation media were applied as experimental treatments to gut content samples to determine how ion content of the environment may be related (if at all) to parasite persistence and proliferation in a dead host. The suspension was vortex mixed and allowed to settle 1-3 min. Two replicate wet mounts were made from the mixed suspension. To make a wet mount, 10 μ L of mixed suspension was placed on a glass slide and was covered with a glass cover slip. Fifty fields from each wet mount were examined for myxospores using phase contrast microscopy at a total magnification of 400x. For most samples, screening was terminated if a myxospore was observed. Ten samples containing myxospores were further diluted to a total of 10x the original volume of intestinal contents. Samples were stored at mean temperature 4° C for 17 days. Myxospore densities in these samples were estimated at 5 points in time within the 17 day incubation period. To estimate myxospore densities at a given point in time, a sample was vortex mixed and allowed to settle 1-3 min. Myxospore densities were estimated from four replicate hemocytometer counts performed on 10 μ L of sample

supernatant. Hemocytometer counts were performed under 400x total magnification using phase contrast microscopy.

Experiment 3: Modeling Infection Prevalence and Severity in Post-Spawned Chinook Salmon Carcasses

I collected intestines (from vent to pyloric caecae) from post-spawned Chinook salmon carcasses collected at four sampling sites in the Klamath River Basin. Carcass sampling protocol was included in the International Animal Use and Care Convention permit 10/11.F.19-E. Sampling occurred from Oct 21 to Nov 29, 2011. Forty-five carcasses were recovered from Salmon River (California Department of Fish and Wildlife reach 4, RM 17-19.5), 58 carcasses from Shasta River (California Department of Fish and Wildlife fish counting weir, 700 ft above mouth), 48 carcasses from Bogus Creek (California Department of Fish and Wildlife fish counting weir, reach 2, RM 0.3-0.6) and 91 carcasses from Scott River (California Department of Fish and Wildlife reach 8, RM 21-24.0).

Fork length, sex and decomposition status of each carcass was recorded. Decomposition was assessed from condition of eyes and gills (Foott et al. 2009b, Chesney and Knechtle 2011) and tissue firmness (Chesney and Knechtle 2011). Eye clarity was ranked as 0 (both eyes clear and free of fungus), 1 (one eye cloudy or covered in fungus) or 2 (both eyes cloudy or covered in fungus). Gill color was ranked 0 (red), 1 (pink), or 2 (white). Tissue firmness was ranked by assessing the degree to which tissue resisted gentle pressure applied to the outside of the carcass with two or three fingers. Three ranks were given. Rank 0 was assigned to the freshest carcasses sampled and it

was associated with firm flesh like that of an unpeeled orange. Rank 1 was given to carcasses in which gentle finger pressure would result in fingers recessing slightly into the flesh. Musculature was solid but tissues were obviously softening. In the abdominal cavity, organs and muscles remained clearly defined and intact. Rank 2 was given to carcasses in the most advanced state of decomposition and was associated with tissue liquefaction and paste-like musculature. In some rank 2 carcasses, abdomens were filled with a slurry of tissues and fluid. Decomposition rank was calculated as the mean of ranks in eye clarity, gill color and tissue firmness. To minimize sampler bias, decomposition in all carcasses was assessed by a single observer.

I used clean, disposable, single-edged razor blades to remove intestines from the pyloric caecae to the vent. Each intestine was stored in a zip top plastic bag with an identification tag made of water proof paper. Intestines were stored cool (ice packs or refrigeration) for 24-48 hours or were frozen before contents were extracted. Intestines were cut into 2-6 cm sections. Sections were placed on a sterilized glass plate and were held in place with a forceps. Intestinal contents were pushed through each section onto the glass plate by scraping the backside of a #21 scalpel blade along the outside of each section. Contents were transferred to 15 mL graduated centrifuge tubes with a disposable razor blade. Contents were diluted 3x with either hatchery water or phosphate buffered saline (4x total dilution). The suspension was vortex mixed and allowed to settle 1-3 min. Two replicate wet mounts were made of the mixed suspension. To make a wet mount, 10 μ L of mixed suspension was placed on a glass slide and was covered with a glass cover slip. Fifty fields from each wet mount were examined for myxospores using

phase contrast microscopy at a total magnification of 400x. For most samples, screening was terminated if a myxospore was observed.

Positive screening samples were further diluted to a total volume of 10x or 20x that of the extracted contents. Myxospore densities were estimated in positive samples from four replicate hemocytometer counts performed on 10 μL of sample supernatant. Hemocytometer counts were performed under 400x total magnification (phase contrast microscopy). The number of myxospores in a sample (MPS) was estimated as spore density (spores / μL) multiplied by volume of sample (μL).

To assess screening sensitivity, I made five samples of varying myxospore densities by diluting myxospore positive samples of known spore densities with samples classified as negative. Ten wet mounts of each sample were made following the previously described wet mount screening methods. Fifty fields from each wet mount were examined for myxospores using phase contrast microscopy at a total magnification of 400x. Number of myxospore positive wet mounts was recorded to summarize screening sensitivity for the various spore densities.

The relationships between myxospore prevalence and abundance and five carcass variables were evaluated using two types of models. Variables assessed were carcass fork length (cm), sex, decomposition rank, collection site, and collection date. Thirty-nine candidate logistic regression models were fit to binary myxospore prevalence data ($n=242$, binary data = spore positive or spore negative). Binary logistic regression models were used to predict the probability of myxospore detection ($\text{Pr}(\text{Detection})$) in a given carcass using maximum likelihood estimates of each predictor variable effect (β_i).

$$\mathit{Pr}(\mathit{Detection}) = \frac{1}{1 + e^{-(\beta_0 + \beta_1 x_1 \dots)}}$$

Twenty-four candidate linear models were fit to the natural logarithm of myxospores per sample data collected from spore-bearing carcasses. Linear models used maximum likelihood estimates of each predictor variable effect (β_i) to predict the natural logarithm of number of myxospores expected in a given, spore-bearing carcass ($\log(\text{MPS} \mid \text{Detection})$).

$$\log(\text{MPS} \mid \text{Detection}) = \beta_0 + \beta_1 x_1 \dots$$

Both types of models were generated using varying combinations of carcass variables and interaction terms between continuous (length, decomposition, date) and discrete (sex, site) variables. Models representing plausible hypotheses were included in pre-defined candidate sets. Binary logistic models included in the candidate set were created from all possible combinations of carcass variables (excluding interaction terms). In contrast, only models containing the explanatory variable carcass fork length were included in the linear model candidate set. In this case, I believed fork length was strongly tied to myxospore abundance and needed to be controlled for in all models. Models were fit using maximum likelihood parameter estimation and were compared by their estimated Akaike's information criterion corrected for sample size (AICc). Twelve logistic regression models and six linear models were duplicates of better performing models with the exception of one additional, uninformative parameter ("pretending variables", Anderson 2008). These models were removed from the candidate sets and AICc values were recalculated. No interaction parameters between sex and Shasta River

collection site were included in any models, because no female carcasses were sampled at this site.

Unconditional expected myxospores per sample ($E[MPS]$) was calculated as the product of the probability of myxospore detection and number of myxospores per spore-positive sample.

$$E[MPS] = (MPS | Detection) * Pr(Detection)$$

Unconditional Expected myxospores per sample was estimated for 16 carcass groups of interest. Groups were delimited by the estimated average fork length of carcasses born in the same year (age cohort) and by collection site. Estimated site-specific, cohort fork lengths were generated from a data set provided by the Yurok Tribe's Fisheries Department (Williams 2011, unpublished). This data set consisted of fork length measurements and age estimates taken on 2917 fish classified as two-year olds, 1031 three-year olds, 824 four-year olds and 7 five-year olds. Fish age was estimated based on scale analysis. Data was collected from returning adults in 2011. Estimates assumed a maximum decomposition rank of 2.0 so that inference can be made about a carcass' maximum spore production potential rather than about its spore contents at the time of collection.

Ninety-five percent confidence intervals were calculated for each estimate from simulated group-specific sampling distributions. Distributions were approximated from 2000 Monte Carlo simulations of group-specific myxospores per sample using parametric bootstrapping. All models, estimates and simulations were generated using R statistical software (The R Project for Statistical Computing 2010).

RESULTS

Experiment 1: Parasite Dispersal from Post-Spawned Chinook Salmon

Carcasses

A subset of water samples was molecularly assessed for presence and quantity of *C. shasta* DNA. No *C. shasta* DNA was detected in any water sample from any group (Table. 2). Therefore, no further samples were examined.

Experiment 2: Myxospore Development after Host Death

In trial 1, no myxospores were observed in any sample of pooled intestinal contents at any point in time. No molecular diagnostics were performed on this sample set. In trial 2, *C. shasta* DNA was detected in 13 experimental samples (Table 3). In only one of these samples were myxospores observed at any point in time. Myxospore densities in this sample changed little through time (Figure 2). No significant linear ($p=0.7496$) or quadratic ($p=0.5806$) trends were observed in myxospore density data. A relatively small amount of *C. shasta* DNA was also observed in 1 control sample (average $C_q = 36.60$) likely caused by a sample labeling error or cross contamination. In trial 3, myxospore densities in the 10 samples monitored changed little through time (Figure 3). No significant linear ($p=0.9046$) or quadratic (0.4024) trends were observed in myxospore density data collected from samples diluted with water. No significant linear ($p=0.8228$) or quadratic (0.9059) trends were observed in myxospore density data collected from samples diluted with phosphate buffered saline. No evidence for *C. shasta* persistence or development in samples from fish carcasses was observed.

Table 2. Cq values of qPCRs performed on experimental and background water samples collected from carcass boxes on three sampling occasions. Average Cq and standard deviation are from three replicate one-liter water samples.

Collection Date	Sample Type	Average Cq	Standard Deviation	Water Velocity (ft/sec)	Water Temperature (°C)
31-Oct	Background	42.00	0.00	-	12
	Box	39.29	2.34	0.08	12
7-Nov	Background	40.54	2.53	-	11
	Box	40.75	2.17	0.13	11
19-Nov	Background	41.04	1.67	-	8
	Box	42.00	0.00	0.09	8

Table 3. Cq values of qPCRs performed on juvenile gut contents diluted with hatchery water. Samples were collected 0, 5, 10 and 15 dpe (days post exposure). Juveniles were held live at two temperature treatments. Cq < 38.00 indicates presence of *C. shasta* in the sample (bold).

	Treatment	Mean Rearing Temperature	Cq			
			0 days	5 days	10 days	15 days
Trial 2	Warm	19°C	39.87	30.39	28.34	*21.79
			39.81	32.61	28.94	35.22
	Cold	10°C	35.58	31.20	35.56	42.00
	Control	20°C	40.19	39.81	42.00	36.60

* Myxospores microscopically detected

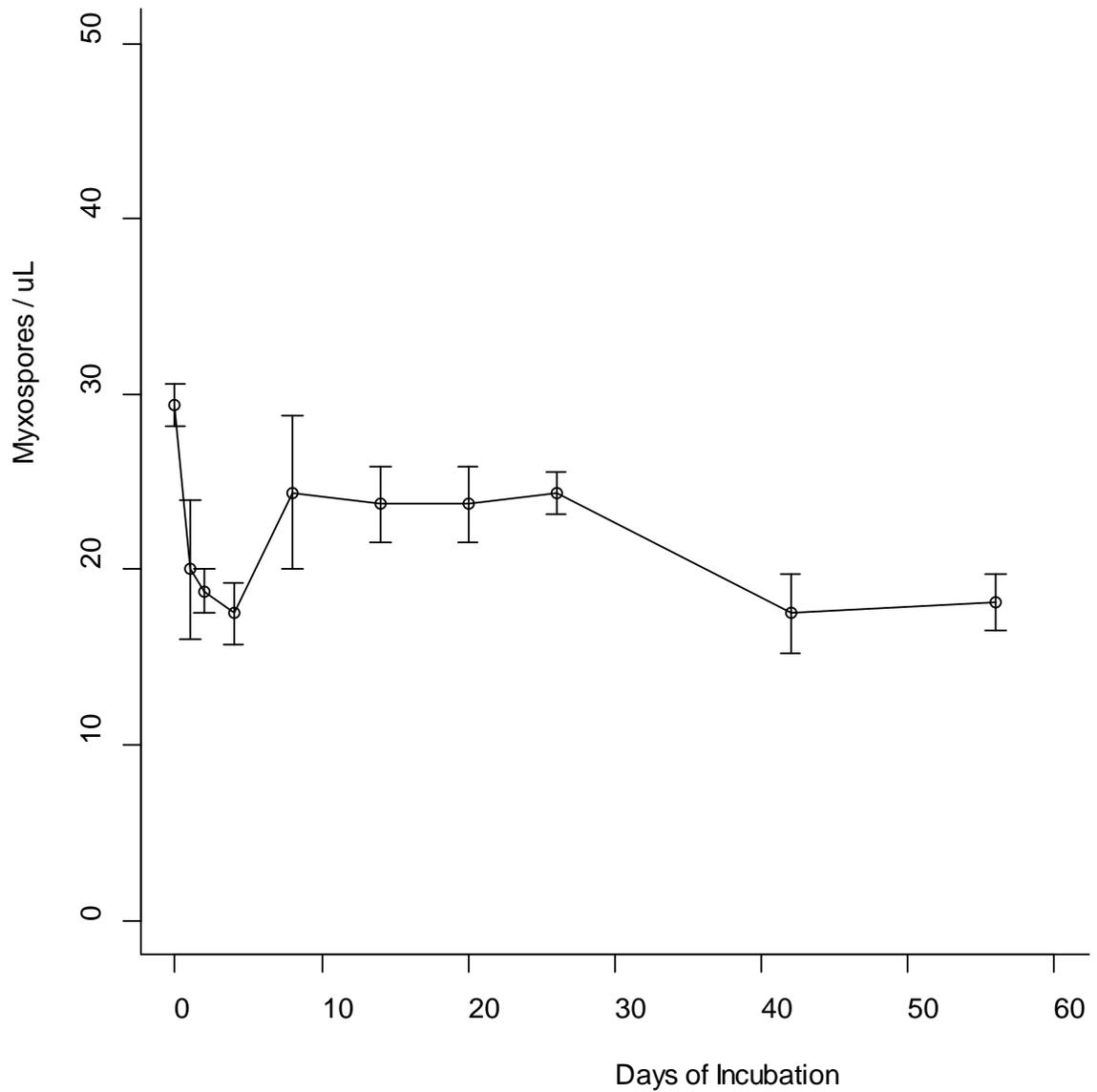


Figure 2. Myxospore density through time in pooled, diluted gut contents from juvenile Chinook salmon reared 15 dpe at mean temperature 19°C. Contents were diluted 9x with sterilized hatchery water and incubated at mean temperature 20.5°C. Error bars are ± 1 SE of the mean estimate.

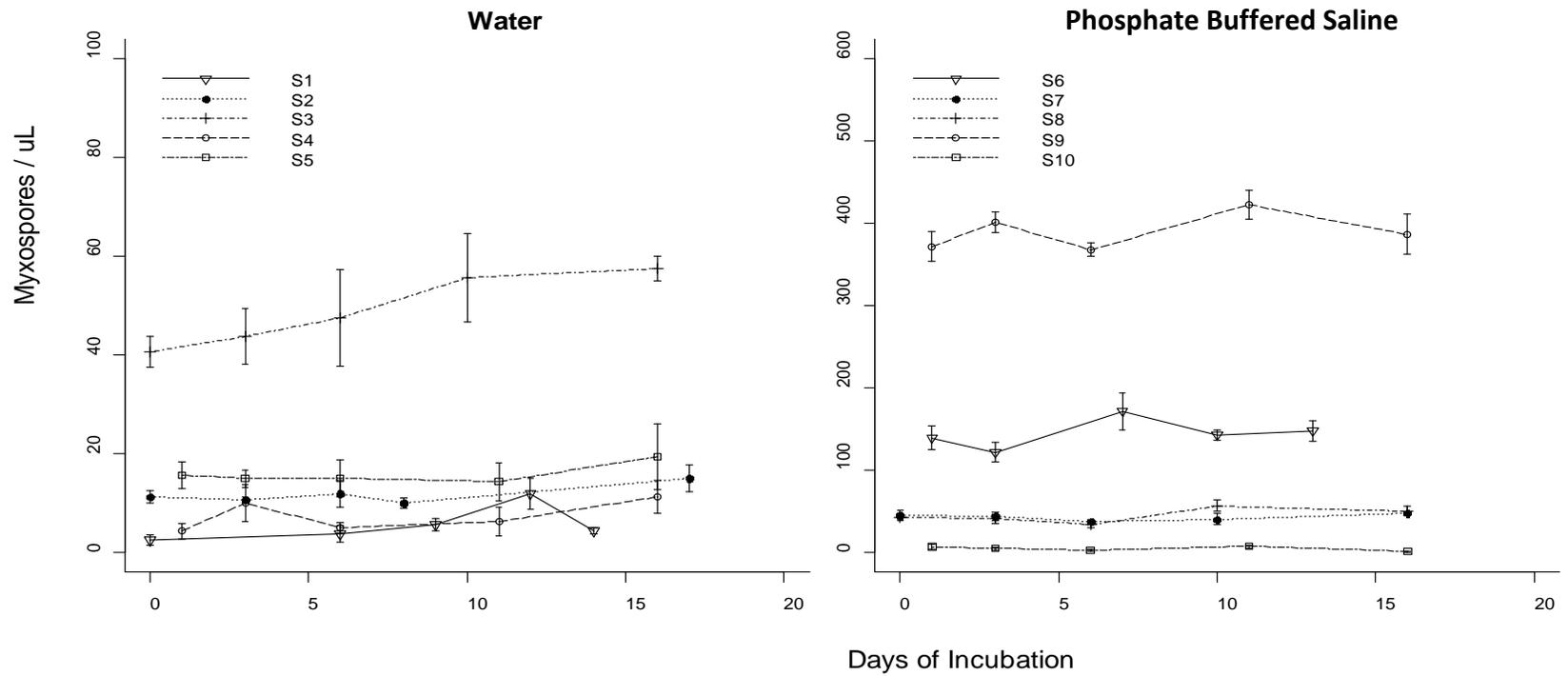


Figure 3. Myxospore densities through time in 10 samples (S1-10) of diluted intestinal contents collected from post-spawned Chinook salmon carcasses. Contents were diluted 9x with hatchery water or phosphate buffered saline. Error bars are ± 1 SE of the mean estimate.

Experiment 3: Modeling Infection Prevalence and Severity in Post-Spawned Chinook Salmon Carcasses

On average, undiluted gut contents accounted for 33% (SE=0.71) of the weight of intestines before contents were removed. Mean volume of contents per intestine was 0.62 mL (SE=0.03). Mean weight of contents per intestine was 0.61 g (SE=0.03). Length of intestine was, on average, 31% (SE=0.21) of carcass fork length. Spore densities in myxospore positive samples ranged from 5,682 (SE=5,682) to 52,542,857 (SE=1,962,991) myxospores per gram undiluted intestinal contents. Estimates of total myxospore abundance in individual spore positive samples ranged from 1,875 (SE=1,875) to 41,543,750 (SE=696,079) myxospores. Estimates of myxospores per sample were greater than 1 million spores in 33 of the 242 samples observed (14%). Spore abundance in these heavily infected samples accounted for 95% of the myxospores predicted in all samples combined.

Among the 5 samples designated for screening sensitivity trials, sensitivity ranged from 10% (myxospores observed in 1 out of 10 positive wet mounts) in a sample containing 6,250 myxospores per mL undiluted contents to 100% (myxospores observed in 10 out of 10 wet mounts) in a sample containing 150,000 myxospores per mL. Six survey samples containing low densities of myxospores (6,250-106,250 spores per mL; mean=36,458 spores per mL) screened negative on the first wet mount and positive on second. This data suggested that myxospore positive samples containing spore densities less than 6,250 myxospores per mL undiluted contents had less than 19% chance of being classified as myxospore positive in the initial screening process $(1.0 - (1.0 - 0.1)^2)$. If

low densities of myxospores (106,250 myxospores per mL undiluted contents) were assumed present in the 125 negative screening samples, these samples are projected to contain less than 3% of the total myxospores surveyed based on mean volume of intestinal content samples. Given that most negative screening samples likely contained less than 106,250 myxospores per mL, the relative spore contribution of positive samples missed in the screening process is probably quite low.

The Kullback-Leibler best logistic regression used sampling site and carcass decomposition level to predict the probability of myxospore detection in carcass intestinal contents and had Akaike weight 0.247 (Tables 4, 5, Figure 4). The top 4 models included these predictors and produced a cumulative Akaike weight of 0.594. All models including these predictors produced a cumulative Akaike weight of 0.742. There was 2.87 times more evidence (“evidence ratio”, Anderson 2008) for models containing both site and decomposition than for models that lacked one or both of these variables. There was 6.05×10^8 times more evidence for the best logistic regression than there was for the null model.

The best linear model predicted the natural logarithm of number of myxospores in spore-positive samples with carcass fork length and had an Akaike weight of 0.357 (Table 6, Figure 5). There was 5.61×10^5 times more evidence for the best linear regression than there was for the null model.

Sixteen ecologically important estimates of expected myxospores per sample were generated by the best binary logistic and linear regression models and are reported in Table 7. Simulated sampling distributions of these estimates are shown in Figure 6.

Table 4. Maximum likelihood estimates of model parameters identified in the Kullback-Leibler best binary logistic and linear regressions. Binary Logistic Regressions predicted Log(Odds(Detection)). Linear models predicted Log(MPS|Detection). P is the significance of each parameter. For Log(Odds(Detection)), (n) is the number of sample measurements fit by the logistic regression. For Log(MPS|Detection), (n) is the number of sample measurements fit by the linear regression. For Bogus Creek, Salmon River, Shasta River and Scott River, (n) is the number of myxospore-positive samples / total number of samples screened from each sampling location.

Parameter	n	Estimate	Standard Error	Test Statistic	P
Log(Odds(Detection))	242				
Intercept (Bogus Creek)	6 / 48	-2.528	0.532	Wald z = -4.75	< 0.001
Decomposition	-	0.499	0.250	Wald z = 2.00	0.046
Salmon River	22 / 45	1.791	0.534	Wald z = 3.35	< 0.001
Scott River	62 / 91	2.802	0.498	Wald z = 5.63	< 0.001
Shasta River	27 / 58	2.100	0.536	Wald z = 3.92	< 0.001
Log(MPS Detection)	117				
Intercept	-	7.353	0.884	t = 8.32	< 0.001
Fork Length	-	0.074	0.013	t = 5.64	< 0.001

Table 5. Candidate logistic regression models fit to myxospore detection prevalence data. Models are ranked by probability (Akaike weight). Models use sex, decomposition status (Decomp), fork length (FL) and collection site and date to predict the probability of myxospore detection in Chinook salmon carcass intestinal contents. Open circles (\circ) indicate the predictors fit by each model. Closed circles (\bullet) indicate the predictors and their associated interaction terms fit by each model. K = number of parameters fit; $\log(\mathcal{L})$ = log-likelihood; AIC_c = Akaike's second-order information criterion; ΔAIC_c = differences in AIC_c ; w_i = Akaike weights; Cw_i = cumulative Akaike weights. No interaction term between sex and Shasta River site were fit, because no female carcasses were encountered at this site.

Rank	FL	Sex	Site	Decomp	Date	K	$\log(\mathcal{L})$	AIC_c	ΔAIC_c	w_i	Cw_i
1			\circ	\circ		5	-144.23	299.01	0.00	0.247	0.247
2			\circ	\circ	\circ	6	-143.58	299.93	0.93	0.156	0.403
3		\circ	\circ	\circ		6	-144.06	300.89	1.88	0.096	0.500
4	\circ		\circ	\circ		6	-144.08	300.92	1.92	0.095	0.594
5			\circ			4	-146.28	300.93	1.92	0.095	0.689
6		\bullet	\bullet			7	-142.98	300.98	1.97	0.092	0.781
7	\circ		\circ	\circ	\circ	7	-143.31	301.64	2.64	0.066	0.848
8		\circ	\circ	\circ	\circ	7	-143.36	301.74	2.73	0.063	0.911
9			\circ		\circ	5	-146.14	302.81	3.81	0.037	0.948
10			\bullet	\bullet		8	-143.46	304.25	5.25	0.018	0.966
11			\bullet		\bullet	8	-143.63	304.60	5.59	0.015	0.981
12	\circ		\circ		\circ	6	-146.00	304.76	5.75	0.014	0.995
13	\bullet		\bullet			8	-144.65	306.63	7.62	0.006	1.000
14		\bullet		\bullet		4	-162.17	332.70	33.69	0.000	1.000
15		\bullet			\bullet	4	-163.13	334.62	35.61	0.000	1.000
16			Null Model			1	-167.61	337.25	38.25	0.000	1.000
17				\circ		2	-166.62	337.35	38.35	0.000	1.000
18	\circ					2	-167.26	338.63	39.62	0.000	1.000
19	\circ			\circ		3	-166.39	339.00	39.99	0.000	1.000
20				\circ	\circ	3	-166.45	339.12	40.11	0.000	1.000
21					\circ	2	-167.60	339.30	40.29	0.000	1.000
22		\circ				2	-167.61	339.32	40.32	0.000	1.000
23	\bullet	\bullet				4	-165.59	339.53	40.53	0.000	1.000
24	\circ	\circ				3	-167.15	340.52	41.51	0.000	1.000
25	\circ	\circ		\circ		4	-166.10	340.56	41.56	0.000	1.000
26	\circ			\circ	\circ	4	-166.25	340.85	41.84	0.000	1.000
27	\circ	\circ		\circ	\circ	5	-165.96	342.46	43.46	0.000	1.000

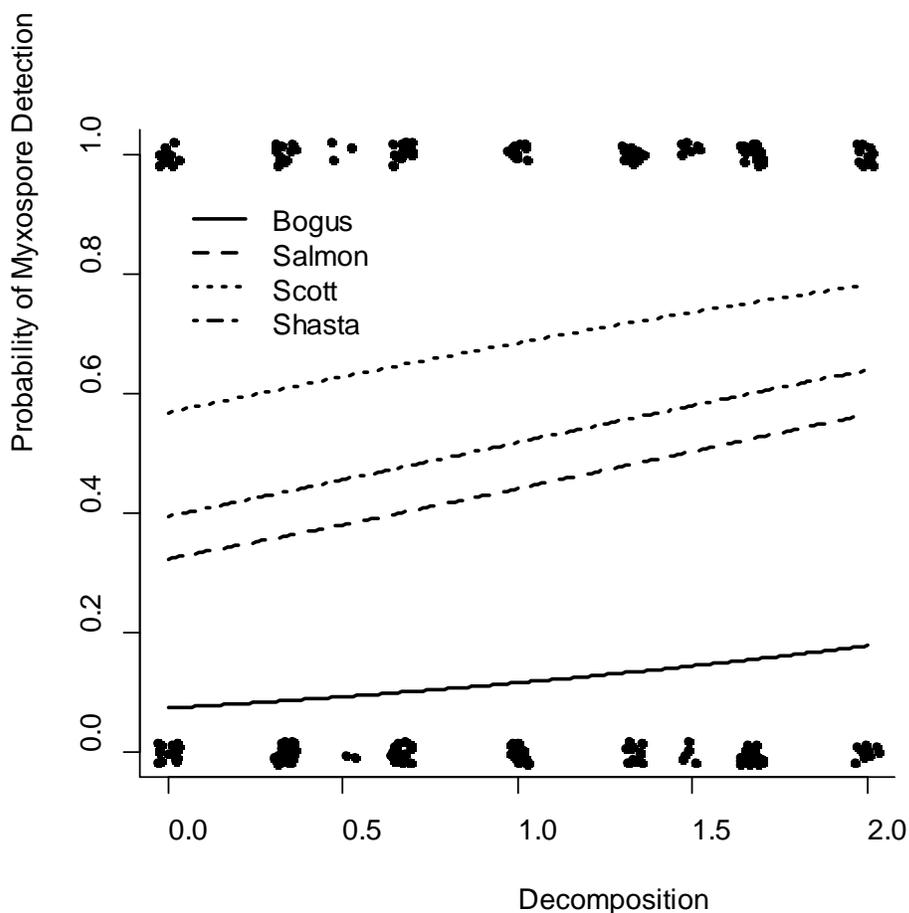


Figure 4. Probability of myxospore detection in gut content samples from post-spawned Chinook salmon carcasses by level of carcass decomposition and collection site (Bogus Creek, Salmon River, Scott River, Shasta River). Black dots represent individual samples screened for myxospores and are plotted in clusters at probability 0.0 (no myxospores detected, n=125) or at probability 1.0 (myxospores detected, n=117). Carcasses ranged in level of decomposition from 0.0 (no decomposition) to 2.0 (advanced decomposition).

Table 6. Candidate linear regression models fit to the natural logarithm of myxospores per sample data and ranked by model probability (Akaike weight). Models use sex, decomposition rank (Decomp), fork length (FL) and collection site and date to predict the natural logarithm of number of myxospores per sample of intestinal contents given that myxospores have been detected in the screening process. Open circles (○) indicate the predictors used in each model. Closed circles (●) indicate the predictors and their associated interaction terms included in each model. K = number of parameters fit; $\log(\mathcal{L})$ = log-likelihood; AIC_c = Akaike's second-order information criterion; ΔAIC_c = differences in AIC_c ; w_i = Akaike weights; Cw_i = cumulative Akaike weights.

Rank	FL	Sex	Site	Decomp	Date	K	$\log(\mathcal{L})$	AIC_c	ΔAIC_c	w_i	Cw_i
1	○					3	-254.49	515.20	0.00	0.357	0.357
2	○				○	4	-253.89	516.13	0.93	0.224	0.582
3	○			○		4	-254.33	517.02	1.82	0.144	0.725
4	○	○			○	5	-253.79	518.11	2.92	0.083	0.808
5	○	○		○		5	-254.27	519.08	3.88	0.051	0.860
6	●	●				5	-254.32	519.18	3.99	0.049	0.908
7	○	○		○	○	6	-253.77	520.31	5.12	0.028	0.936
8	○		○			6	-254.01	520.78	5.59	0.022	0.958
9	○		○		○	7	-253.38	521.79	6.59	0.013	0.971
10	○	○	○			7	-253.91	522.85	7.66	0.008	0.979
11	○		●		●	10	-250.54	523.16	7.97	0.007	0.985
12	○	○	○		○	8	-253.21	523.76	8.56	0.005	0.990
13	○	●	●			8	-253.46	524.25	9.06	0.004	0.994
14	○	○	○	○		8	-253.89	525.10	9.91	0.003	0.997
15	○	○	○	○	○	9	-253.20	526.08	10.89	0.002	0.998
16	●		●			9	-253.25	526.18	10.99	0.002	1.000
17	○		●	●		10	-253.94	529.96	14.76	0.000	1.000
18			Null Model			2	-268.78	541.67	26.48	0.000	1.000

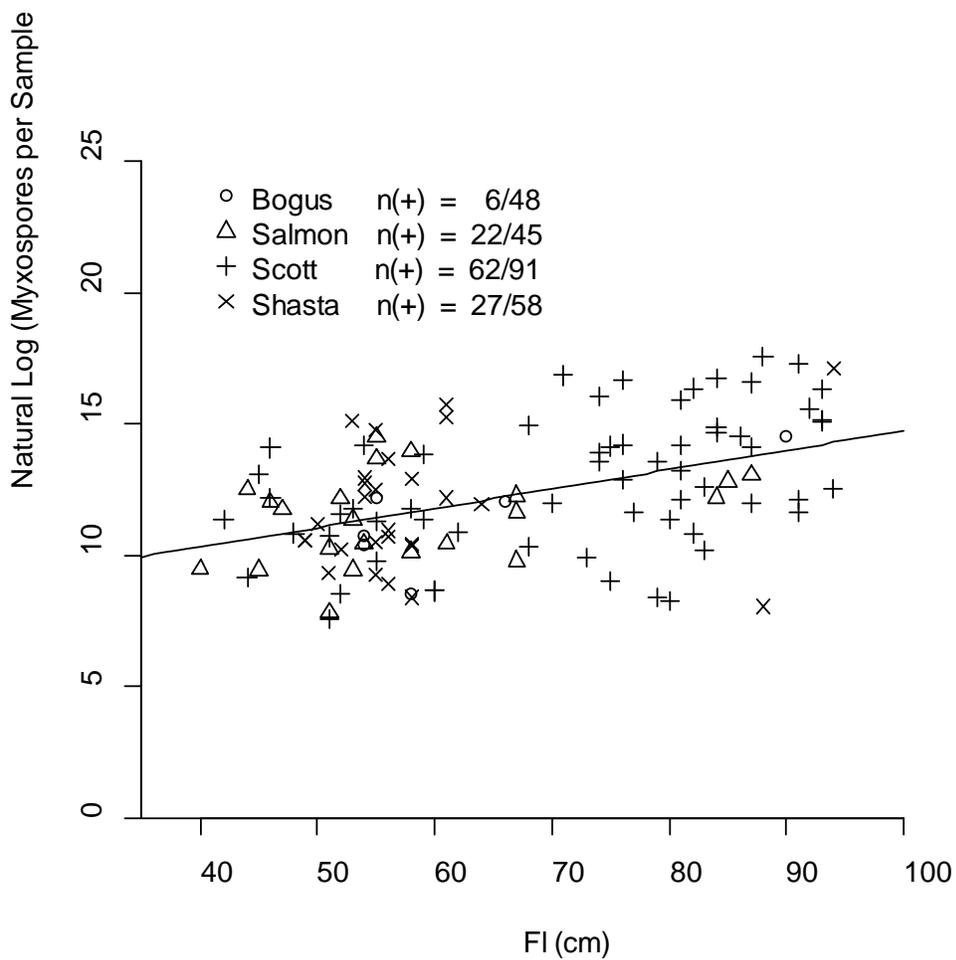


Figure 5. Natural log of myxospores per spore-positive sample by carcass fork length (FL). Number of myxospore-positive samples out of total number of samples from each of the four collection sites (Bogus Creek, Salmon River, Scott River, Shasta River) is indicated by n(+).

Table 7. Estimates and 95% confidence intervals of expected myxospores per sample (E(MPS)) among 16 carcass demographic groups, calculated from Monte Carlo simulations. Groups are classified by location of carcass and by fork lengths used to represent 4 age cohorts (2, 3, 4 and 5 year olds). Confidence intervals (95% CI) do not account for uncertainty in age-at-fork length estimates. Number of returns is the number of fish in each age cohort expected to have returned to each sub-basin in 2011 (Klamath River Technical Team 2012).

	Age / FL (cm)	E(MPS)	95% CI		Number of Returns
			Lower	Upper	
Bogus Creek	2 / 57	18,667	7,509	43,988	2,303
	3 / 72	56,478	22,075	125,203	2,046
	4 / 83	127,195	44,446	311,557	869
	5 / 95	308,399	92,985	927,464	5
Salmon River	2 / 54	47,453	25,851	84,442	1,819
	3 / 79	300,333	162,094	525,074	1,885
	4 / 85	467,655	229,086	892,906	1,789
	5 / 92	783,973	335,074	1,730,202	0
Shasta River	2 / 55	57,776	31,425	100,688	11,187
	3 / 71	188,193	105,451	296,779	23
	4 / 82	423,831	214,059	768,143	190
	* / 92	886,604	383,977	1,894,111	0
Scott River	2 / 53	60,951	35,535	103,314	2,499
	3 / 71	230,114	146,631	351,576	978
	4 / 83	557,940	305,772	1,004,669	2,038
	5 / 88	806,967	406,070	1,601,805	0

* No data available for age-at-fork length estimate. Fork length used is mean of estimates calculated for the 3 remaining sub-basins.

** Age-at-fork length data provided by Desma Williams, Yurok Tribe Fisheries Department.

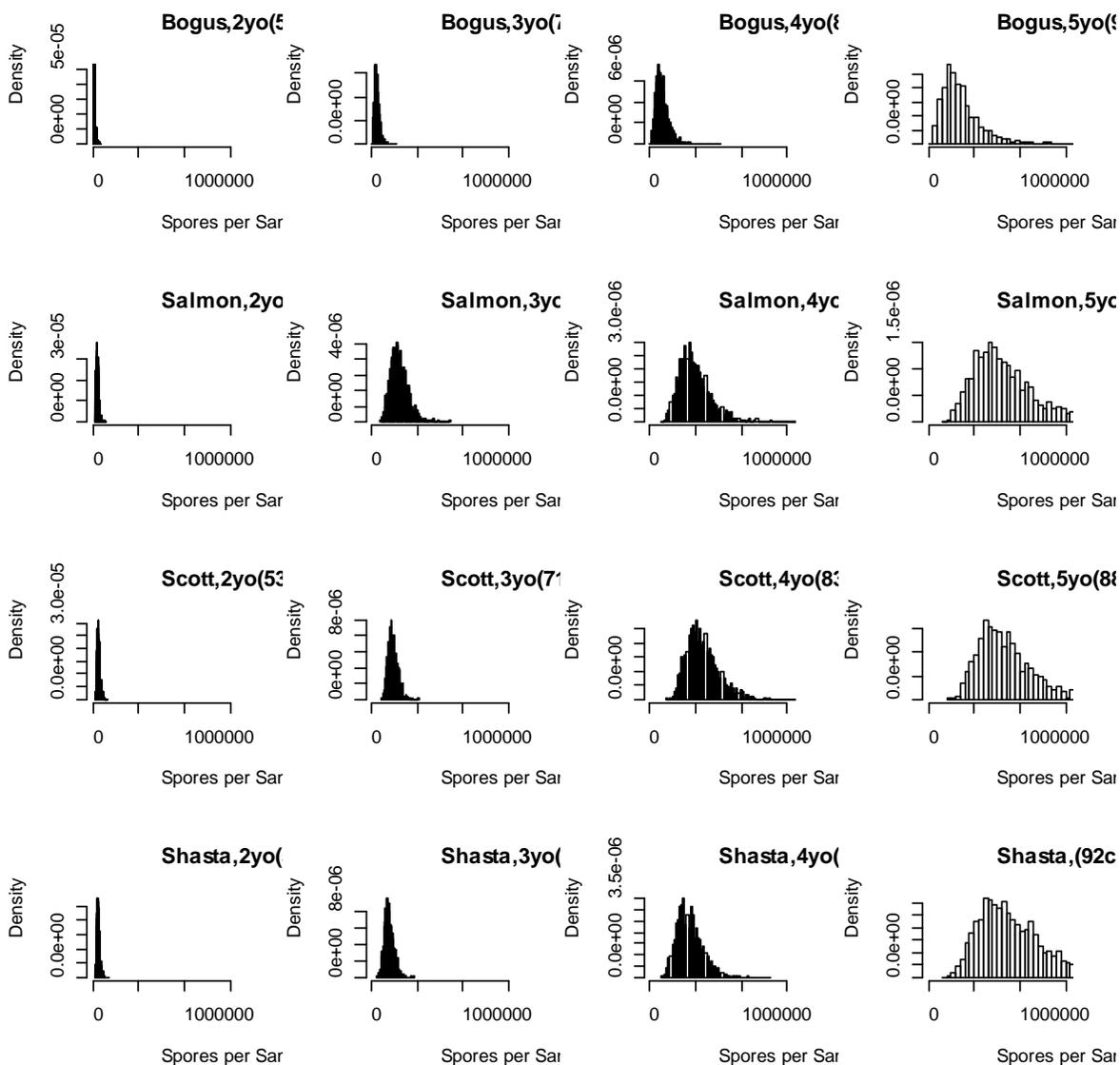


Figure 6. Simulated distributions of expected myxospores per sample for each of 16 demographic groups. Each distribution was approximated by 2000 Monte Carlo simulations of myxospores per sample. Simulations were generated from model-based parameter and parameter variance estimates calculated by the Kullback-Leibler best logistic and linear regression models. Each distribution is specific to collection site and estimate of mean carcass fork length-at-age. Mean fork length-at-age was calculated from measurements and age assessments made on returning adult Chinook salmon in 2011 by the Yurok Tribe's Fisheries Department. No data was available for the estimate of age-at-fork length of 5 year old Shasta River fish, so the mean of the available estimates was used.

Although estimates of expected myxospores per carcass likely represent the majority of spores produced in a given carcass, they do not account for spores present in tissues outside of the descending intestine or for spores present in negatively screening samples. Therefore, expected myxospores per sample is an estimate of the minimum number of spores that are within a carcass at the time of sampling (Foott et al. 2009b, Foott et al. 2010, Fogerty et al. 2012).

DISCUSSION

Experimental attempts to identify temporal patterns in *Ceratomyxa shasta* production by Chinook salmon carcasses were inconclusive. No parasites were observed at any point in time in water samples collected from submersed experimental boxes. Carcasses may not have been sufficiently degraded to release parasites into the water. More time or agitation may have been required to facilitate this process. High prevalence of infection in returning adult Chinook salmon by early, pre-spore life stages of *C. shasta* has been demonstrated in previous myxospore surveys (Foott et al. 2009a, Slezack 2009). Thus some if not all boxed carcasses were likely infected by pre-spore life stages of *C. shasta*. These early life stages, however, may be fragile and susceptible to breaking down in the process of carcass decomposition. Absence of myxospores in experimental carcasses could explain why no *C. shasta* DNA was observed in water samples. Myxospores were observed in laboratory samples of carcass intestinal contents, however, no temporal trends in parasite densities were identified. Observed myxospores most likely developed before the observation period, since spore densities never deviated far beyond those observed on the first day of monitoring. I expected to observe an increase in myxospore densities through time. Experimental laboratory cultures may not have provided the environmental conditions necessary for pre-spore life stages of *C. shasta* to persist and develop into myxospores.

Estimates of myxospore production by carcasses varied by carcass size and level of decomposition and between geographic sampling sites. These estimates exemplified how local myxospore prevalence, average size of fish, and abundance and age

composition of returning adult salmon can create vast differences in regional myxospore production within Klamath Basin.

In three of the four sampling sites, expected myxospore production by heavily decomposed carcasses (rank 2) was more than 20% greater than that expected in fresh carcasses (rank 0) (Figure 4). This increased production was due to increased prevalence of myxospores in these fish. It is unclear why myxospore prevalence is usually greatest in decomposed carcasses (Foott et al. 2009b).

The a priori hypothesis proposed that pre-spore life stages of the parasite may continue to develop into myxospores for some time after host death. As a carcass decomposes, myxospore densities would continue to increase toward a critical detection threshold. Heavily decomposed carcasses, having had more time for myxospores to develop, would have the greatest myxospore prevalence and abundance. Results from my studies were unable to strongly support or refute this hypothesis. However, these results raised some relevant considerations.

I expected carcass level of decomposition (an index of time after host death) to affect spore prevalence and abundance similarly. Survey results did not indicate a relationship between carcass decomposition and myxospore abundance in spore-bearing carcasses. If parasite development continues after the death of the fish host, both spore prevalence and abundance should increase with level of decomposition.

Level of carcass decomposition may be related to myxospore screening sensitivity. Thus, densities of myxospores would not differ across varying levels of carcass decomposition. Rather, spores collected from heavily decomposed carcasses

would be more visually evident in the screening process or more readily retrieved when intestinal contents were collected. This possible sampling bias could underestimate myxospore prevalence in fresh carcasses.

Myxospore production by carcasses varied considerably between the four geographic sampling sites. Contrary to expectations, myxospore production was lowest in carcasses sampled from Bogus Creek (Figure 3, Table 7). Compared with Bogus Creek carcasses of equal size, expected myxospore production was 39% greater in Salmon river carcasses, 46% greater in Shasta River carcasses, and 60% greater in Scott River Carcasses (assuming heavy decomposition, rank = 2)(Figure 4). This is surprising, because critical comparisons of the site-specific migration histories unique to each group of fish suggest that Bogus Creek fish are most at risk of infection among those sampled.

Host migration distance should impact severity of *Ceratomyxa shasta* infection by influencing the time between initial infection with *C. shasta* and death. Time post-infection has been implicated in influencing infection severity in adult salmon (Foott et al. 2013). Since parasite development accelerates according to a predictable timeframe (Yamamoto and Sanders 1979, Bjork and Bartholomew 2010), prolonging survival with infection naturally extends the duration of parasite development and proliferation in the host. I expected that fish traveling the greatest distance to spawning grounds would host the oldest and most severe infections. This hypothesis assumed that distance traveled to spawning grounds is a reliable index of time spent in-river. It is plausible that the spawning migrations of these fish are more complex and in-river residence time is related to other variables such as water temperature (Strange 2010).

Host spawning migration history should also impact severity of *Ceratomyxa shasta* infection by influencing the magnitude of infectious challenge, or parasite dose, imposed on migrating fish. Infectious challenge by *Ceratomyxa shasta* has been linked to severity of infection in fish (Ratliff 1981, Hemmingsen et al. 1986, Ibarra et al. 1992, Foott et al. 2007) and is, in part, a function of the duration of exposure to the infective life stage of the parasite. Fish that travel great distances through the main stem Klamath River to reach spawning tributaries are subject to greater infectious challenge, because infection transmittance is largely restricted to the main stem river (Hendrickson et al. 1989, Stocking et al. 2006, Foott et al. 2009b).

Infectious challenge also varies within the main stem Klamath River (Hallett et al. 2012). Fujiwara et al. (2011) determined that juvenile survival rates were reduced in fish that migrated through a highly infectious river reach located near Seaid Valley, California. Specifically, survival rates of fish born in Bogus Creek and Shasta River (upriver of infectious zone) differed from survival rates observed in fish born in the Scott River and Shasta River (downriver of infectious zone).

I expected Bogus Creek and Shasta River fish to exhibit higher infection prevalence and more severe infections, because they migrate further through the main stem Klamath River than fish that returned to the Salmon and Scott rivers. Furthermore, Bogus Creek and Shasta River fish are exposed to the highly infectious region of the river in Seaid Valley as they migrate through to their spawning tributaries. Contrary to this reasoning, myxospore prevalence in my study was lowest among Bogus Creek carcasses and very similar between Salmon River fish and Shasta River fish. Furthermore,

myxospore abundance in spore-bearing carcasses was very similar between fish sampled from different locations. It is unclear why infection severity varied geographically in these ways.

Susceptibility to infection with *Ceratomyxa shasta* can vary across host species and genetic strain (Hallett et al. 2012, Ibarra et al. 1992, Hemmingsen et al. 1986). It seems plausible that differences in infection susceptibility mitigated by host genetic strain could explain observed site-specific differences in myxospore prevalence. In fact, many of the adult Chinook salmon that return to Bogus Creek are presumably of Iron Gate Hatchery origin (Knechtle and Chesney 2011) suggesting some degree of genetic homogeneity among these fish. Stone et al. (2008) tested this theory, however, and found that susceptibility to *C. shasta* infection was essentially equal across multiple strains of hatchery and naturally produced Chinook salmon.

Large carcasses, on average, contributed more myxospores per fish to the basin than smaller carcasses. Estimates show that four year old carcasses (average fork length = 83 cm) infected with myxospores were expected to produce more than twice the number of myxospores than three year old fish (average fork length = 73 cm) at 3 of the 4 sampling sites. Four year old carcasses were expected to produce more than six times the number of myxospores produced by 2 year old fish (average fork length = 55 cm). Five year old fish probably do not contribute meaningful numbers of myxospores to the Klamath Basin, because so few return to the system each year (California Department of Fish and Wildlife 2013). I expected that myxospore production potential would be positively related to the size of fish, because larger fish have larger organ systems and

subsequently more tissue that is susceptible to infection. Larger fish likely host more localized sites of infection and parasite proliferation which ultimately result in more myxospore production.

Level of carcass decomposition has previously been related to number of myxospores present in salmon carcass intestinal contents. Foott et al. (2009b) observed greater spore loads in samples from decomposed carcasses compared to samples collected from fresh carcasses. Fogerty et al. (2012) also referenced differences in infection due to level of carcass decomposition.

Infection prevalence and severity in juvenile Chinook salmon varies spatially in the Klamath River Basin. (Hendrickson et al. 1989, Hallett et al. 2006, Stocking et al. 2006, Atkinson and Bartholomew 2010, Hallett et al. 2012, True et al. 2013). Similarly, prevalence of *C. shasta* myxospores varies spatially in post spawned Chinook salmon carcasses. Myxospore surveys often find that, across three regularly sampled locations, spore prevalence is greatest in samples collected from the Shasta River, followed by the main stem Klamath River, while the lowest prevalence is observed in Bogus Creek samples (Slezack 2009, Foott et al. 2010, Fogerty et al. 2012). I also observed the lowest myxospore prevalence among Bogus Creek carcasses. These results mirrored myxospore prevalence comparisons reported in previous myxospore surveys suggesting that regional differences in prevalence may not be an artifact of year but are related to some unknown, persistent mechanism in host-parasite interactions.

Myxospore densities and total spores per sample varied considerably among carcasses sampled in 2011. Estimates of myxospores per carcass were greater than 1

million spores in 33 of the 242 fish observed (14%). Spore abundance in these heavily infected fish accounted for 95% of the myxospores predicted in all samples combined. This pattern is similar to that observed in previous myxospore surveys which have concluded that the majority of spores released into the basin (90-91%) are likely derived from a small number of carcasses (2-12%) (Foott et al. 2010, Fogerty et al. 2012).

Previous myxospore surveys on post spawned Chinook salmon carcasses have investigated temporal trends in myxospore production by post spawned carcasses. Results from these studies are inconsistent. Foott et al. (2009b) and Fogerty et al. (2012) found no significant correlation between date of sample collection and myxospore abundance. Foott et al. (2010) found no obvious temporal trend in myxospore prevalence data. Slezak (2009) visually investigated seasonal differences in *C. shasta* pre-spore densities present in Chinook salmon intestinal tissues. He observed a significant difference in parasite densities in samples collected before and after November 1. He also noted a peak in prevalence of infection on Oct 25 in samples collected from Iron Gate Hatchery that was not evident in Trinity River Hatchery fish. It is important to note that samples exhibiting these temporal relationships were collected from pre-spawned mortalities. Temporal trends in infection severity may be more obvious in early infections observed in live, pre-spawned fish.

Previous authors have not found a direct relationship between ceratomyxosis and host sex to date. Foott et al. (2009b) found no significant differences in infection status between sexes of carcasses sampled from Bogus Creek. Fogerty et al. (2012) observed that prevalence of myxospores was greater in Bogus Creek female carcasses, but greater

in male carcasses sampled from the main stem Klamath River. Foott et al. (2013) found greater prevalence of myxospores in males from both the main stem Klamath River and in Shasta River. Reproductive activity has been previously implicated in the sex-specific suppression of immune function and increase in susceptibility to some parasites in salmonids (Skarstein et al. 2001).

Prevalence of myxospores and average size of fish vary geographically (Tables 4, 7). This pattern likely has profound effects on regional myxospore production by fish in a given age class. For example, 3 year old carcasses from Bogus Creek (ave. Fl 72 cm) were expected to produce, on average, 70% fewer myxospores per carcass than 3 year old carcasses from the Shasta River (ave. Fl 71 cm), 75% fewer myxospores per carcass than Scott River carcasses (ave. Fl 71 cm), and 81% fewer myxospores per carcass than those from the Salmon River (ave. Fl 79 cm) (Table 7). Because carcass size is strongly tied to potential myxospore production, the abundance and age composition of returning adults to the system will likely affect overall myxospore production in the basin.

This is further substantiated by regional differences in the size and age composition of fish. In 2011, the Shasta River Fall Chinook salmon run was composed primarily of precocious male jack salmon with an average fork length of 55 cm (Table 7, California Department of Fish and Wildlife 2012). In contrast, more than 35% of the Scott River run was comprised of large (ave. Fl 83 cm) 4 year old fish. If myxospore prevalence and abundance estimates are representative of basin-wide patterns, myxospore production in the Scott River would be approximately twice that of the Shasta River despite having a Fall Chinook salmon run of half the size.

When formulating estimates of basin-wide myxospore production, it is important to consider the degree of decomposition in sampled carcasses. Significant numbers of fresh carcasses included in a myxospore survey may bias estimates of total myxospore production low, due to the insufficient decomposition period necessary for accurate detection of infection. Low bias in myxospore production estimates can be prevented by selectively sampling decomposed carcasses. Alternately, bias in random samples can be removed through the application of a decomposition correction factor to estimates using modeling techniques.

Results of the 2011 myxospore survey on post spawned Chinook salmon have reconfirmed the importance of characterizing site specific myxospore prevalence when estimating basin-wide myxospore production. Furthermore, the study has introduced carcass size as a valid predictor of spore production potential in post spawned carcasses. In formulating projections of myxospore production for a reach in a given year, it is advisable to consider the specific myxospore prevalence for that region and the age/size composition of returning adult fish, because age composition of Fall-run Chinook salmon that return to the Klamath River Basin varies dramatically from year to year (California Department of Fish and Wildlife 2012). This can have dramatic consequences on regional and overall myxospore production in the basin.

The removal of fresh, myxospore-bearing carcasses from the channel by scavengers (Cederholm et al 1999) could result in a reduction of the number of myxospores that would otherwise be released into the system after sufficient carcass breakdown. If significant numbers of carcasses were removed from the system before

releasing their maximum potential spore load, basin-wide estimates of myxospore production based on spawner escapement would be biased high. The proportion of carcasses that release their maximum potential myxospore load into the system is unknown. An estimate of this figure could be used to correct the bias in basin-wide myxospore production estimates due to early carcass removal from the system.

Identifying the most significant sources of myxospores to the basin remains an intriguing objective for future research. Investigations into the timing of spawning migrations of adult Chinook salmon could shed light on how in-river residence time is related to infection severity. This information could be used to predict which salmon stocks are most at risk of infection and should be further investigated. In addition, future myxospore surveys will continue to reveal the most significant myxospore producing regions in the basin. This knowledge could be used to direct management activity (such as carcass removal) aimed at reducing overall infection rates in the Klamath River Basin.

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