Production of Myxospores of *Ceratomyxa shasta* in Chinook Salmon Carcasses

Final Project Report

February 11, 2013

Humboldt State University, Sponsored Programs Foundation

Sub-award No. NA231A-A

Prepared for:

Oregon State University

By

Nick Campise

Department of Fisheries Biology

Humboldt State University

and

Gary Hendrickson

Department of Fisheries Biology

Humboldt State University

Rationale: The number of myxospores released by post-spawned Chinook salmon carcasses likely influences incidence of infection in polychaetes. Incidence of infection in polychaetes may also relate to incidence of infection and magnitude of disease related mortality in out-migrant juvenile fish. Determining factors that are related to myxospore abundance observed in carcasses and monitoring the subsequent dispersal of *Ceratomyxa shasta* into the environment would help to describe the role carcasses play in *Ceratomyxa shasta* life history. We conducted three experiments:

1. Quantity of parasites shed from in-river carcasses was monitored through time using molecular techniques.
2. Myxospore densities were monitored over time in incubating samples of infected intestinal contents to determine *Ceratomyxa shasta* persistence and development in a dead fish host.
3. Myxospores were surveyed in carcasses collected at four sites in the Klamath River basin to determine how infection status (myxospore abundance in intestinal contents) is related to carcass size, sex, and level of decomposition. Spatial and temporal relationships were also investigated.

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

Methods: Twenty-five post-spawned Chinook salmon carcasses were collected within 100 m above or below the Bogus Creek, California Department of Fish and Game (CDFG) fish counting weir by CDFG technicians. Carcass infection status was determined on site from microscopic observations made on wet mounts of intestinal material. This material was collected by inserting a metal inoculation loop into the vent of each carcass. Gentle, circular motion was applied to the loop before it was removed with intestinal contents. Myxospores were detected in only one carcass from 25 collected. This carcass and 5 that contained presumptive pre-sporogonic life stages of *C. shasta* were set aside for later experimental treatment.

Two flow-through flume boxes were constructed from marine-grade plywood. Boxes were eight feet long and had a square cross section of 50.8 cm x 50.8 cm. Each box was divided into four chambers by large mesh, plastic screen. 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) and were anchored to the river bottom with rebar. Box floors were covered with river benthic material collected on site. A single post-spawned Chinook salmon carcass was placed in each of the three upstream-most chambers of each box. 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 over a 30 day period (October 31, 2010 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 with water and the lid was replaced. Water velocity through the boxes was measured once daily with a model 2100 Swoffer 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 three 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 through Millipore brand nitrocellulose membrane filters of 5 um pore size. 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 qPCR. Materials and methods of the molecular assays are described by Hallett et al (2012).

Results and Discussion: A subset of water samples was molecularly assessed to quantify *C. shasta*. We hoped that this method would allow for detection and quantification of parasite DNA as it was released

from carcasses in experimental boxes. No *C. shasta* DNA was detected in any individual water sample from any group (Table. 1). Therefore, no further samples were examined.

These results were unexpected. It is logical to assume that myxospores are at some time released from decomposing carcasses. There are several explanations for our inability to detect *Ceratomyxa shasta* in water samples: (1) Perhaps we did not allow enough time for carcass decomposition and myxospore development. During the experiment, water temperatures were fairly cold (mean temperature = 8.6ºC, SE = 0.40). Decomposition of carcasses was slow and carcasses were still recognizable at the end of the experiment. Likewise, myxospore development was likely slow. Udey et al. (1975) showed that ceratomyxosis progression, as measured by host time to death, accelerated when temperature increased. If given more time, perhaps carcasses would have decomposed sufficiently to release myxospores and (or) myxospores would have reached an appropriate state of development for release. (2) Perhaps infections in boxed carcasses were too light to be detectable by this study design, or perhaps infections in boxed carcasses had not developed far enough to continue after death of the fish host. Microscopic examination suggested that parasite infections were dominated by low densities of myxospores or presumptive pre-sporogonic stages of the parasite. It is unlikely that carcasses were "misdiagnosed" and were uninfected. Prevalence of infection in Bogus Creek carcasses was estimated at 86% in 2009 and over 90% in 2011(Foott et al. 2010, Fogerty et al. 2012).

Table 1**.** Cq values of qPCRs performed on experimental and background water samples collected from carcass boxes on three sampling occasions. Average Cq and standard deviation is from three replicate one-liter water samples.

Collection Date	Sample Type	Average Cq	Standard Deviation	Water Velocity (ft/sec)	Water Temperature (ºC)
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**

Experiment 2. Myxospore Development in Chinook Salmon Carcasses

Methods: *Trial 1 and 2.* In 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*. Fish were held in modified, plastic minnow traps above Beaver Creek (river kilometer 262), a site known to be highly infectious to sentinel Chinook salmon and steelhead trout (Foott et al. 2003, Stone et al. 2008). Control fish were obtained from Iron Gate Hatchery immediately after each 72 hour exposure. 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. Experimental (exposed) and control fish were transported to HSU Fish Pathology Laboratory in separate, aerated ice chests filled with Klamath River water. 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. Fish exposures and transport to HSU were very similar between the two experimental trials.

Once at HSU, a sub-set of fish was immediately sampled (0 days post exposure). These fish were held separately from the remainder of fish which were distributed among three identical 114 L circular tanks filled with HSU Hatchery water (free of *C. shasta*). Water would circulate between the tank and a 38 L

water tempering sump at a rate of 15 L per minute. We placed either 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) in each sump to achieve treatment temperatures (Table 2). Experimental fish were divided between temperature treatments. 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. Fish rearing conditions were very similar between the two experimental trials. 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 MS222. Intestines (from the vent to pyloric cecae) were removed with scissors and forceps. To extract intestinal contents, an intestine was placed on a glass slide and held at one end with a forceps. Contents were pushed through the gut and onto the glass slide by scraping the backside of a number 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 content samples were combined so that two pooled samples were accumulated from fish in each temperature and control treatment. This process was repeated for fish from each sampling occasion. Numbers of fish contributing to each pooled gut contents sample differed between trials (see Table 2 for details) and no fish were sampled at 15 dpe in trial one. With these exceptions, fish sampling and gut contents collection was very similar between the two experimental trials.

Table 2. Allocation of juvenile Chinook salmon across two temperature treatments. Intestinal contents were collected from fish sampled 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	14 / 2

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

Pooled intestinal contents in both experimental trials were diluted with sterilized HSU hatchery water and vortex mixed. Dilution concentrations differed between trials. Trial 1 samples were diluted with less than 1 mL sterilized hatchery water per pooled sample. In trial 2, volume of pooled samples was measured with sterilized, graduated pipettes then samples were diluted 9x by volume (10x final dilution) with sterilized hatchery water. In trial 2 only, 100 uL of diluted suspension was collected from each sample and frozen for qPCR infection diagnostics. Samples were then incubated according to temperature treatment (warm = 19.0 - 23.5° C; cool = 3.0 – 12.5° C) (Table 1). Incubation of diluted samples was very similar between experimental trials.

Myxospore densities were estimated in incubating contents 1, 2, 4, 8, 14, 20, 26 or 27, 42 and 56 days after extraction from fish. In addition, spore densities were calculated the day of collection (0 days after extraction) in trial 2 samples from fish held live 5, 10 and 15 days after exposure. 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 a period of time ranging from 1 to 3 min. Myxospore density was estimated from four replicate hemocytometer counts of the suspension using phase microscopy (400x total magnification). The remainder of the sample was returned to the incubator for future incubation. 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 detailed in Hallett et al (2012). A separate level of detection analysis was conducted by staff in Oregon State University's department of microbiology by performing replicate qPCRs on three serially diluted samples known to contain *C. shasta* DNA. This analysis determined that a sample of Cq value < 38 likely contained *C. shasta* DNA. Results are shown in Table 2.

Trial 3: Ten samples of myxospore positive intestinal contents were collected from post-spawned Chinook salmon carcasses found in Shasta River and in Bogus Creek. Of the 10 carcasses, eight were sampled at CDFG Shasta River fish counting weir, one was sampled at the CDFG Upper Shasta spawning survey reach, and one was sampled from the CDFG Bogus Creek fish counting weir. Spore abundance data from these samples were included in experiment 3: Myxospore Survey of Post-Spawned Carcasses Found in the Klamath River Basin. To collect a contents sample, an intestine was cut into 2-6 cm sections. Sections were placed on a sterilized glass plate and held in place with forceps. Contents were pushed through each intestinal 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 and were diluted 9x with either sterilized HSU hatchery water or phosphate buffered saline (10x total dilution). Myxospore densities were estimated in samples at five points in time within a 17 day period after collection. Myxospore densities were estimated from four replicate hemocytomer counts using phase microscopy (400x total magnification). Samples were stored at mean temperature 4° C during this 17 day incubation period.

Results and Discussion: In trial 1, no myxospores were observed in any of the pooled intestinal contents samples 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 changed little through time in this sample (Figure 1). A relatively small amount of *C. shasta* DNA was also observed in 1 control sample (average Cq = 36.60). In trial 3, myxospore densities in the 10 samples monitored changed little through time (Figure 2). No evidence for *Ceratomyxa shasta* persistence or development in fish carcasses was observed.

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). Juveniles were held live at two temperature treatments. Cq< 38.00 indicates presence of *C. shasta* in the sample.

	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**
	Cold	10°C	39.81	**32.61**	**28.94**	**35.22**
			35.58	**31.20**	**35.56**	42.00
Trial 2	Warm	19°C	39.87	**30.39**	**28.34**	***21.79**
	Control	20°C	39.81	**32.61**	**28.94**	**35.22**
	Cold	10°C	40.19	39.81	42.00	**36.60**
			35.58	**31.20**	**35.56**	42.00
			36.26	**33.94**	**36.15**	**26.16**
	Control	20°C	40.19	39.81	42.00	**36.6**

* Myxospores detected microscopically

Foott et al. (2009b) determined that decomposed carcasses, on average, contained greater densities of myxospores than fresh carcasses. In our laboratory setting, however, densities remained rather constant through time. This is consistent with a scenario in which myxospore production in fish terminates near the time of host death. Thus, myxospores are released at a rather constant rate for only a short time thereafter?) One possible explanation for these differing results might be that pre-sporogonic life stages may require an array of environmental conditions to develop into myxospores, some of which were not provided in our experimental cultures.

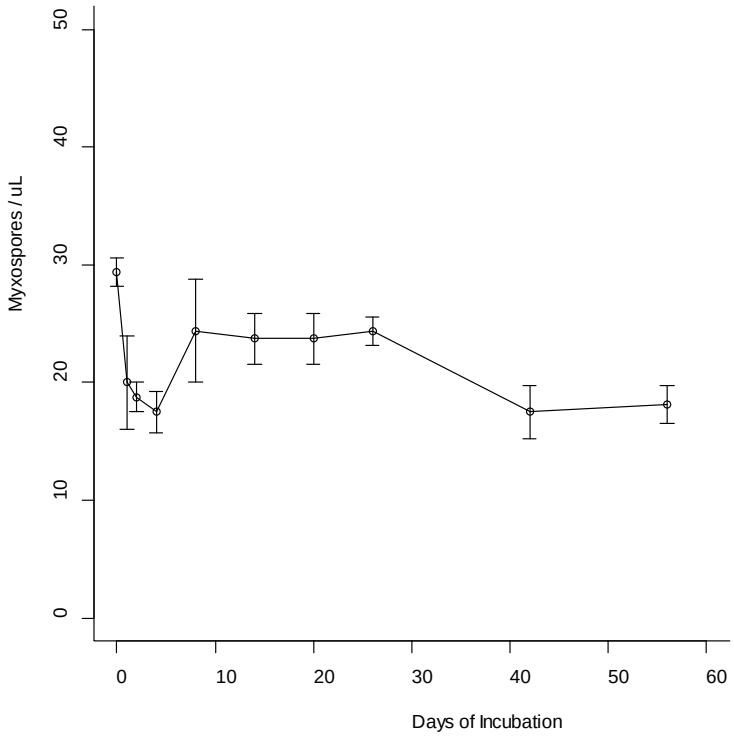

Figure 1. Myxospore density through time in pooled, diluted gut contents from juvenile Chinook salmon reared 15 dpe at mean temperature 19°C. Contents were diluted 9x with sterilized hatchery water and incubated at mean temperature 20.5°C. Error bars are ± 1 SE of the mean estimate.

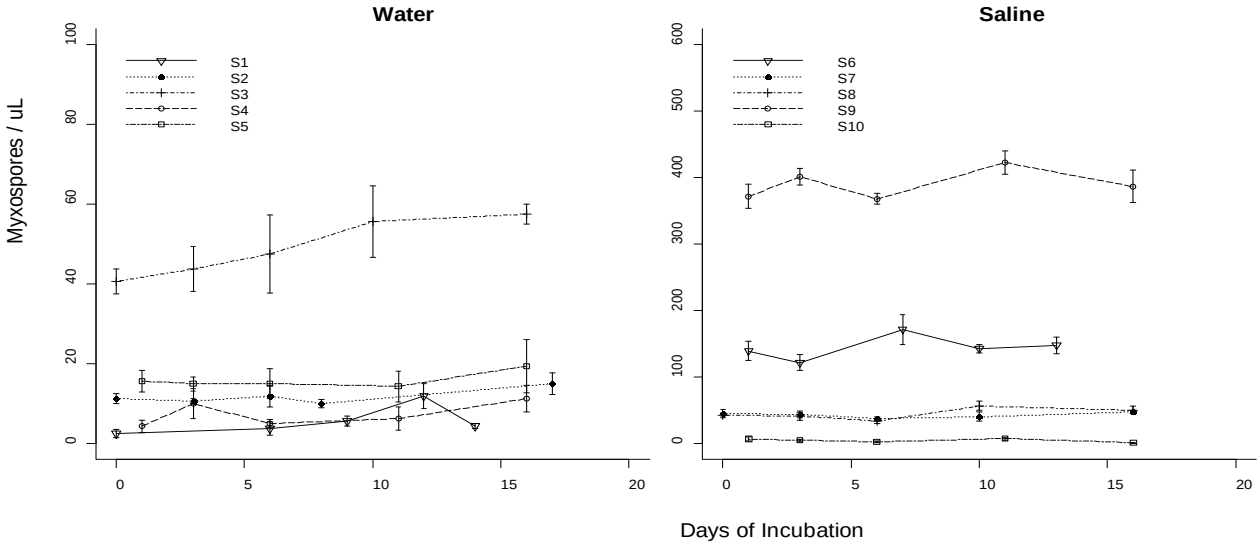

Figure 2. Myxospore densities through time in 10 samples (S1-10) of diluted intestinal contents collected from post-spawned Chinook salmon carcasses. Contents were diluted 9x with hatchery water or phosphate buffered saline. Error bars are ± 1 SE of the mean estimate.

Experiment 3. Myxospore Survey of Post-Spawned Carcasses found in the Klamath River Basin

Methods: Intestines (from vent to pyloric cecae) were collected from post-spawned Chinook salmon carcasses encountered at four sampling sites in the Klamath River Basin. Sampling occurred from Oct 21, 2011 to Nov 29, 2011. Forty-five samples were collected from Salmon River (CDFG reach 4, RM ~17.0-19.5), 58 samples from Shasta River (CDFG fish counting weir, 700 ft above mouth), 48 samples from Bogus Creek (CDFG fish counting weir, reach 2, RM 0.3-0.6) and 91 samples from Scott River (CDFG reach 8, RM 21.0-24.0). Escapement, here, refers to the number of Chinook salmon that returned to their natal watershed for spawning in 2011. This sample set accounts for 0.81% of the estimated Chinook salmon escapement in the Salmon River, 1.65% of that in the Scott River, 0.51% of that in the Shasta River and 0.92% of that in Bogus Creek (Klamath River Technical Team, 2012).

All post-spawned Chinook salmon carcasses encountered were eligible for sampling regardless of condition, so long as fork length was measurable and the abdominal cavity remained visibly closed to the environment. Decomposition status was assessed based on condition of eyes and gills (Foott et al. 2009b, Chesney and Knechtley, 2011) and tissue firmness (Chesney and Knechtley, 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 as 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 2 or 3 fingers. Three ranks were used. Rank 0 was assigned to the freshest of carcasses sampled and was associated with firm flesh like that of an unpeeled orange. Rank 1 was given to carcasses in which gentle digital pressure would result in fingers recessing lightly 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 extreme state of decomposition and was associated with tissue liquefaction and a 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.

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. Intestinal contents were collected following methods previously described and 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. Samples were screened for myxospores by examining 50 fields in each of two replicate wet mounts made from 10 uL of suspension. Positive screening samples were further diluted (10-20x total dilution) to promote visibility of myxospores counted within the hemocytometer chamber. Myxospore densities were estimated in positive samples from 4 replicate hemocytometer counts. Myxospores per sample (MPS) was calculated as spore density of diluted contents sample (spores / mL) multiplied by volume of diluted sample (mL). All wet mount screening and hemocytometer counts were performed using phase microscopy at 400x total magnification.

To measure screening sensitivity, myxospore densities were estimated in 5 samples of intestinal contents containing varying concentrations of myxospores. Each sample was then screened by examining 10 wet mounts of suspension following methods previously described. Sensitivity for a specified spore concentration was calculated as the proportion of wet mounts in which at least 1 myxospore was observed.

The relationships between myxospores per sample and 5 potential carcass demographic variables were evaluated using two types of models. Variables assessed were carcass fork length (cm), sex and decomposition rank, and collection site and date. Thirty-nine candidate binary logistic regression models (BLR) were fit to binary myxospore prevalence data (positive or negative). These BLR models predict the probability of myxospore detection in a given sample, as given by the equation

$$\boldsymbol{Pr(Detection)} = \frac{\mathbf{1}}{\mathbf{1}+e^{-(\beta_0+\beta_1 x_1 \cdots)}} \quad (1)$$

where $\boldsymbol{x_1, x_2}$ ... are potential predictor variables and $\boldsymbol{\beta_0 + \beta_1}$ ... are maximum likelihood estimates of model coefficients.

Twenty-four candidate linear models (LL) were fit to the natural logarithm of MPS data collected from myxospore positive carcasses. LL models predict expected number of myxospores per sample given that myxospores have been detected in the screening process, as given by the equation

$$\boldsymbol{log(MPS \mid Detection) = \beta_0 + \beta_1 x_1 \ldots} \quad (2)$$

where $\boldsymbol{x_1, x_{2\ldots}}$ are potential predictor variables and $\boldsymbol{\beta_0 + \beta_1 \ldots}$ are maximum likelihood estimates of model coefficients.

Both types of models were generated using varying combinations of carcass demographic variables and interaction terms between continuous (length, decomposition, date) and discrete (sex, site) variables. Models were fit by maximum likelihood parameter estimation and were compared by their Akaike's information criterion corrected for sample size (AICc). Twelve BLR models and six LL 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 model, because no female carcasses were sampled at this site.

Unconditional expected MPS was calculated as the product of number of myxospores per spore-positive sample and the probability of myxospore detection, using the two models described above and the formula

$$\boldsymbol{E[MPS] = (MPS \mid Detection) * [Pr(Detection) + (Pr(No\ Detection) * 0)]} \quad (3)$$

Expected MPS was predicted for 16 carcass demographic groups of interest. Groups were classified by location of carcass (4 sample sites) and by fork lengths used to represent four ages of fish (2, 3, 4 and 5 year olds). Cohort fork lengths were estimated from age and site-specific fork length data collected from adults returning to the Klamath Basin in 2011 by the Yurok Tribe Fisheries Department (Desma Williams, unpublished). Cohort and site-specific fork length estimates were calculated as the average fork length measured in 2917 fish classified as two-year olds, 1031 three-year olds, 824 four-year olds and 7 five-year olds. Fish age assignment was based on scale analysis (Klamath River Technical Team, 2012). Estimates of expected MPS assume 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. Expected MPS estimates were calculated using equation (3) and parameter estimates produced from the AICc-best LL and BLR models (Table 4). Monte Carlo simulation was used to calculate the 95% confidence intervals around expected MPS values. For each carcass demographic group, 2000 independent vectors of parameters were randomly selected from a multivariate normal distribution. Each demographic-specific distribution was defined by a mean parameter vector and a covariance matrix constructed from parameter and parameter variance estimates in the AICc-best LL and BLR models (Table 4). Each parameter vector was then used in equation (3) to calculate a demographic-specific simulation MPS value. The 2.5th and 97.5th percentiles of the 2000 MPS simulations form the ninety-five percent confidence intervals around the demographic-specific expected MPS value. Model fitting, parameter estimation, expected MPS estimation and sampling distribution simulation was performed using R statistical software (R Development Core Team 2012).

Results and Discussion: On average, undiluted gut contents accounted for 33% (SE=0.71) of the weight of the intestine before contents removal and had mean volume 0.62 mL (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 ± 11,364 to 52,542,857 ± 3,925,982 myxospores per mL undiluted intestinal contents. Estimates of total myxospore abundance in individual spore positive samples ranged from 1,875 ± 3,750 to 41,543,750 ± 1,392,159 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. 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 minority of carcasses (2 -12%) (Foott et al. 2010, Fogerty et al. 2012). Although estimates of MPS likely represent the majority of spores produced in a given carcass, they do not account for spores present outside of the descending intestine. Therefore, MPS 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).

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 containing150,000 myxospores per mL. Screening failure (negative on first wet mount, positive on second) was observed in 6 survey samples containing low densities of myxospores (≤ 106,250 spores per mL). This data suggests that myxospore positive samples containing spore densities less than 6,250 myxospores per mL undiluted contents had less than 20% chance of being classified as myxospore positive in the initial screening process (2 wet mounts per sample X 10%). 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 contents 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 best logistic regression model uses watershed and carcass decomposition level to predict the probability of myxospore detection in carcass intestinal contents samples and had Akaike weight 0.247 (Table 5). 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 0.741. Relative importance of variables, calculated as the sum of Akaike weights associated with models containing a particular variable (Anderson et al. 2001), was 1.00 for site and 0.76 for decomposition rank. Under the standard frequentist interpretation of repeated sampling, models containing site and decomposition as predictors are 2.86 times more likely to be ranked as the best model than models in the candidate set that do not include these predictors (“evidence ratio”, (Anderson 2008)). This analysis provides strong evidence that prevalence of myxospore detection varied depending on the location from which a carcass was recovered and moderate evidence that spore prevalence was related to level of decomposition.

The best linear model predicted the natural logarithm of number of myxospores per spore-positive sample with carcass fork length and had Akaike weight 0.357 (Table 6). The probability that the best model in the candidate set will fit a fork length effect in an identical experiment is 1.00. This is evidence that among those tested, carcass fork length was the best predictor of total myxospore abundance in spore-positive post-spawned Chinook salmon carcasses.

Table 4. Maximum likelihood estimates of the effect of site and carcass decomposition level on the log (odds(Myxospore Detection)) in gut contents from adult Chinook salmon carcasses and maximum likelihood estimates of the effects of carcass fork length on the natural logarithm of number of myxospores per spore-positive gut contents sample (Log(MPS|Detection). P is the significance of each individual parameter. Sample size (n) in Pr(Detection) is total number of samples used in the logistic regression model. For site effects, n is expressed as # myxospore-positive samples / total # samples screened in each group. Sample size in Log(MPS|Detection) is number of myxospore-positive spore count observations used in the regression model.

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 and ranked by model probability (Akaike weight). Models use sex, decomposition status (Dec), fork length (FL) and collection site and date to predict the probability of myxospore detection in Chinook salmon carcass intestinal contents. Open circles (○) indicate the predictors fit by each model. Closed circles (●) 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 AICc; 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	Dec	Date	K	$\log(\mathcal{L})$	AIC_c	ΔAIC_c	w_i	Cw_i
1			○	○		5	-144.23	299.01	0.00	0.247	0.247
2			○	○	○	6	-143.58	299.93	0.93	0.156	0.403
3		○	○	○		6	-144.06	300.89	1.88	0.096	0.500
4	○		○	○		6	-144.08	300.92	1.92	0.095	0.594
5			○			4	-146.28	300.93	1.92	0.095	0.689
6		●	●			7	-142.98	300.98	1.97	0.092	0.781
7	○		○	○	○	7	-143.31	301.64	2.64	0.066	0.848
8		○	○	○	○	7	-143.36	301.74	2.73	0.063	0.911
9			○		○	5	-146.14	302.81	3.81	0.037	0.948
10			●	●		8	-143.46	304.25	5.25	0.018	0.966
11			●		●	8	-143.63	304.60	5.59	0.015	0.981
12	○		○		○	6	-146.00	304.76	5.75	0.014	0.995
13	●		●			8	-144.65	306.63	7.62	0.006	1.000
14		●		●		4	-162.17	332.70	33.69	0.000	1.000
15		●			●	4	-163.13	334.62	35.61	0.000	1.000
16			Null Hypothesis			1	-167.61	337.25	38.25	0.000	1.000
17				○		2	-166.62	337.35	38.35	0.000	1.000
18	○					2	-167.26	338.63	39.62	0.000	1.000
19	○			○		3	-166.39	339.00	39.99	0.000	1.000
20				○	○	3	-166.45	339.12	40.11	0.000	1.000
21					○	2	-167.60	339.30	40.29	0.000	1.000
22		○				2	-167.61	339.32	40.32	0.000	1.000
23	●	●				4	-165.59	339.53	40.53	0.000	1.000
24	○	○				3	-167.15	340.52	41.51	0.000	1.000
25	○	○		○		4	-166.10	340.56	41.56	0.000	1.000
26	○			○	○	4	-166.25	340.85	41.84	0.000	1.000
27	○	○		○	○	5	-165.96	342.46	43.46	0.000	1.000
Relative Importance	**0.18**	**0.25**	**1.00**	**0.76**	**0.337**						

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 (Dec), 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 AICc; w_i = Akaike weights; Cw_i = cumulative Akaike weights.

Rank	FL	Sex	Site	Dec	Date	K	$\log(\mathcal{L})$	AICc	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
Relative Importance	**1.00**	**0.23**	**0.06**	**0.23**	**0.36**						

Location of a carcass is likely important in predicting its expected myxospore load due to the strong relationship between site and prevalence of myxospores. Abundance of spores in spore-bearing carcasses did not vary between sites. Carcasses from Bogus Creek are expected to produce fewer myxospores than carcasses from any other site (Table 7, Figure 3). For example, 3 year old fish from Bogus Creek are expected to produce, on average, 70% fewer myxospores than Shasta River carcasses, 75% fewer myxospores than Scott River carcasses, and 81% fewer myxospores than Salmon River carcasses. Myxospore prevalence in carcasses from Bogus Creek has historically been low compared to that observed at other sites (Foott et al. 2010, Fogerty et al. 2012). Differences in myxospore abundance estimates are less pronounced among carcasses found in the Salmon River, Scott River and Shasta River (Figure 5).

On average, greater numbers of myxospores are expected in decomposed carcasses. This is an artifact of greater myxospore prevalence (relative importance = 0.76 in best logistic model) rather than abundance (relative importance = 0.23 in best linear model). These results are in agreement with Foott et al. (2009b) who determined that myxospore density is greatest in decomposed carcasses. The significance of the relationship between a high level of decomposition in a carcass and a heightened probability of myxospore detection is unknown. Experiments monitoring myxospore development in incubating intestinal contents have been inconclusive (Experiment 2 of this document). There is no documentation of myxospore development outside of a living fish.

Carcass size was most related to myxospore abundance in spore-positive carcasses among the predictors assessed. Larger fish have greater spore producing potential (Table 7, Figure 4). For example, estimates show that 5 year old salmon, on average, will produce more than 13X the number of myxospores expected from 2 year old grilse.

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.

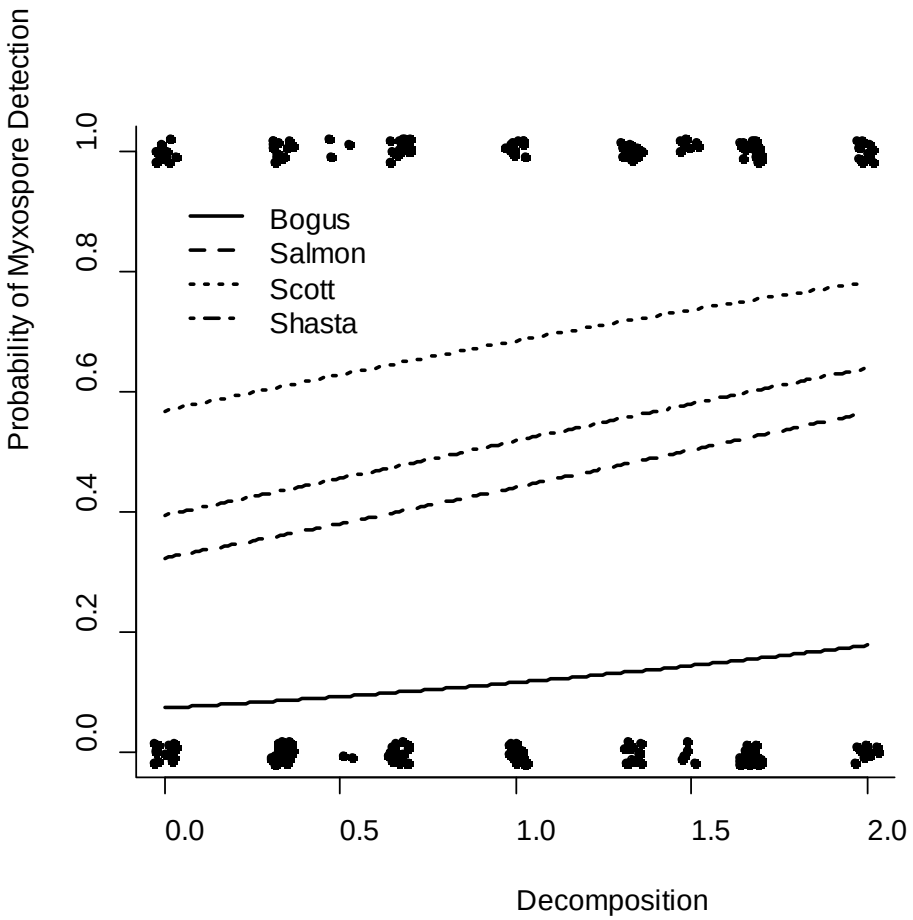

Figure 3. Probability of myxospore detection in gut content samples from post-spawned Chinook salmon carcasses by level of carcass decomposition and collection site (Bogus Creek, Salmon River, Scott River, Shasta River). Black dots represent individual samples screened for myxospores and are plotted 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).

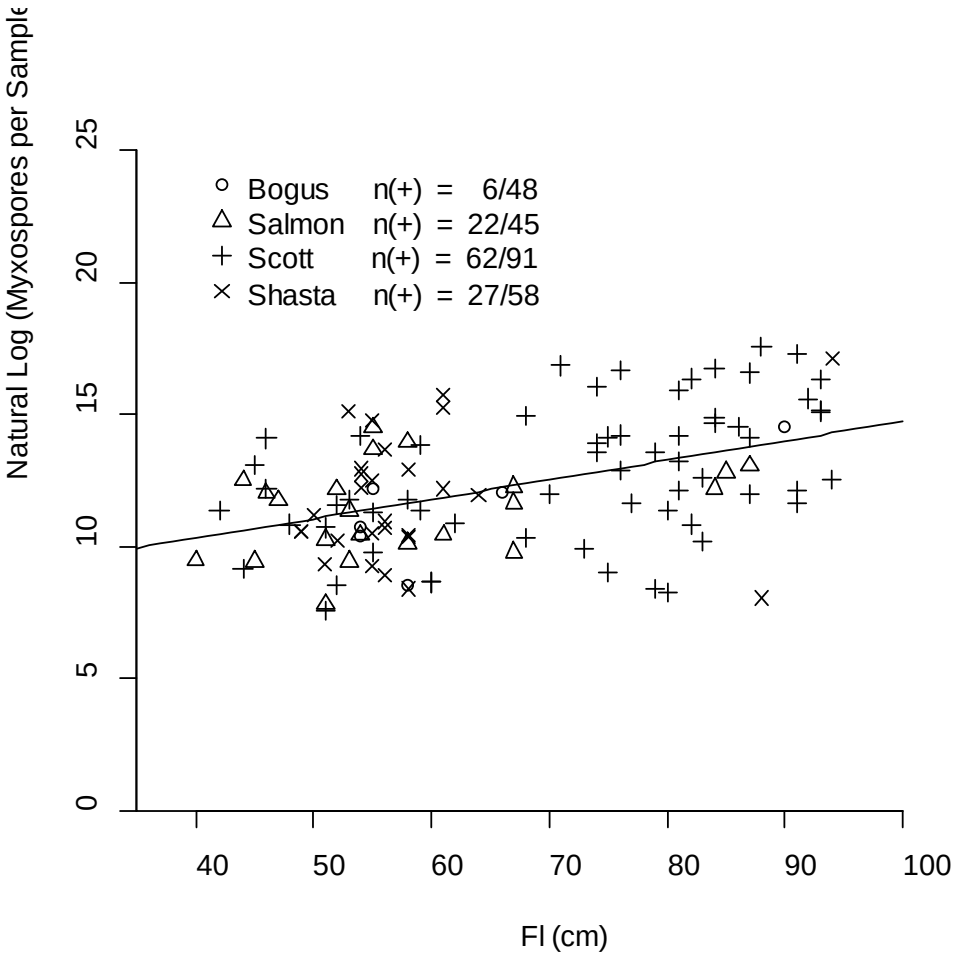

Figure 4. Natural log of myxospores per spore-positive sample by carcass fork length (FL). Number of myxospore-positive samples out of total number of samples from each of the four collection sites (Bogus Creek, Salmon River, Scott River, Shasta River) is indicated by n(+).

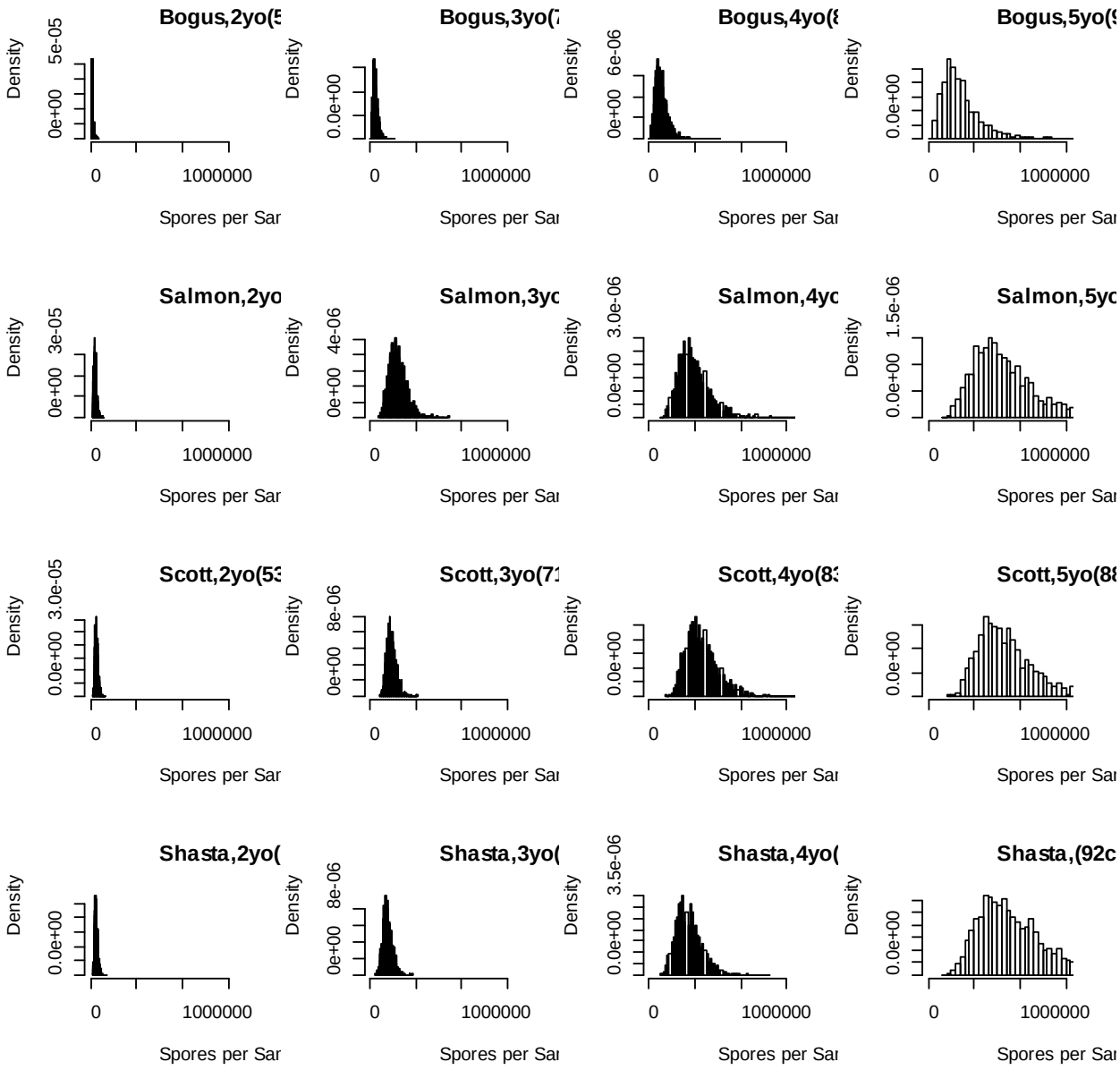

Figure 5. Simulated distributions of expected myxospores per sample for each of 16 demographic groups. Each distribution was approximated by 2000 Monte Carlo simulations of myxospores per sample. Simulations were generated from model-based parameter and variance estimates from the logistic regression model (detection probability) and the linear model (logarithm of spore count given detection). Each distribution is specific to the estimate of carcass average fork length-at-age and collection site. Average 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.

A number of questions remain related to myxospore output from adult Chinook carcasses. Prevalence of myxospores in Bogus Creek carcasses was clearly lower than that observed at other sites within the basin. However, it is unclear what processes drive this pattern. Future surveys might include sampling in other portions of Bogus Creek to determine if low myxospore prevalence is ubiquitous to the sub-basin or is spatially segregated to a small region around the counting weir. These results further substantiate previous observations that connect decomposition and myxospore load in carcasses. Experiment 2 of this document tested the effects of time on myxospore densities in incubating intestinal contents. Despite finding no substantial effect of time on spore densities in these laboratory cultures, greater myxospore prevalence is consistently tied to carcasses that are advanced in decomposition. We were unable to define the mechanism(s) responsible for this pattern. This work has identified relationships that will allow researchers to better predict the myxospore output by adult Chinook carcasses. However, there is still a great deal of uncertainty surrounding the fate of myxospores once released into the environment (Foott et al, 2010). Particularly, do myxospores released by tributary carcasses contribute to incidence of infection in the main stem Klamath River?

Our work suggests that myxospore production in the Klamath River Basin is most related to the number of large, myxospore bearing carcasses present and less related to carcass sex and date. Prevalence of myxospores in a given carcass is strongly tied to location. Thus, overall myxospore production will vary considerably between stretches of river.

Acknowledgments: We thank California Department of Fish and Game for allowing us to collect samples with their carcass survey crew (Sara Borok and Morgan Knechtley). We thank Dr. Scott Foott (California-Nevada Fish Health Center, Anderson, California) for advising in lab procedures and for his myxospore survey work which provided the foundation for our own myxospore studies. We thank Gerri Buckles and Dr. Sascha Hallett (Oregon State University) for advising in genetic techniques and for performing qPCRs on water and gut contents samples. We thank Dr. Rob Vankirk for modeling support. We thank Anthony Desch, Eric Laudenslager and Marty Reed for industrial support. We thank Desma Williams and the Yurok Tribe Fisheries Department for 2011 FL-age data from returning adults. We thank Raven Knickerbocker, Scott Benson, Adam Robinson, Sara, and Hallie Heath for lab and field assistance. This work was funded with federal appropriations for disease reduction in the Klamath Basin.

LITERATURE CITED

Anderson,D.R. 2008. Model based inference in the life sciences: A primer on evidence. Springer Science Business Media, New York, New York.

Anderson, D.R., W. Link, D. Johnson and K. Burnham. 2001. Suggestions for presenting the results of data analyses. Journal of Wildlife Management 65:373-378

Chesney, D. and M. Knechtley, 2011. Shasta River Chinook and coho salmon observations in 2010-2011Siskiyou County, CA. California Department of Fish and Game Draft Report. 26 pages

Fogerty, R., S. Foott, R. Stone, A. Bolick, and K. True. 2012. FY2011 Technical Report: *Ceratomyxa shasta* myxospore survey of fall-run Chinook salmon carcasses in the Klamath and Shasta Rivers, and Bogus Creek, 2011. U.S. Fish and Wildlife Service, California – Nevada Fish Health Center, Anderson, CA.

Foot, J.S., R. Fogerty, and R. Stone.2010. FY2009 Technical Report: Ceratomyxa shasta myxospore survey of fall-run Chinook salmon carcasses in Bogus Creek, Shasta River and Klamath River: Component of joint OSU-Yurok Fisheries-CDFG pilot project testing the effect of carcass removal on C. shasta myxospore levels in Bogus Creek, 2009-2010. United States Fish and Wildlife Service, California-Nevada Fish Health Center, Anderson, CA.

Foott, J.S., R. Harmon, and R. Stone. 2003. FY2002 investigational report: Ceratomyxosis resistance in juvenile Chinook salmon and steelhead trout from the Klamath River. United States Fish and Wildlife Service, California-Nevada Fish Health Center, Anderson, CA.

Foot, J.S., R. Stone, R. Fogerty, and K. True. 2009b. FY2009 Technical Report: Ceratomyxa shasta myxospore survey of fall-run Chinook salmon carcasses and sentinel trout trials in Bogus Creek: Component of joint OSU-Yurok Fisheries-CDFG pilot project testing the effect of carcass removal on C. shasta myxospore levels in Bogus Creek, 2008. United States Fish and Wildlife Service, California-Nevada Fish Health Center, Anderson, CA.

Foot, J.S., J. Strange, and R. Slezack. 2009a. FY2007 technical report: *Ceratomyxa shasta* myxospore survey of adult Rainbow trout / Steelhead, Chinook and Coho salmon in the Klamath River basin in 2007-2008: Cooperative Humboldt State University – Yurok Fisheries-CA-NV FHC project. United States Fish and Wildlife Service, California-Nevada Fish Health Center, Anderson, CA.

Hallett, S. L., R. A. Ray, C.N. Hurst, R. A. Holt, G. R. Buckles, S. D. Atkinson and J. L. Bartholomew. 2012. Density of the waterborne parasite *Ceratomyxa shasta* and its biological effects on salmon. Applied and Environmental Microbiology 78:3724-3731

Klamath River Technical Team. 2012. Klamath River fall Chinook salmon age-specific escapement, river harvest, and run size estimates, 2011 run. Annual Report.19 pages. Available from the Pacific Fishery Management Council, 7700 NE Ambassador Place, Suite 101, Portland, OR 97220-1384. http://www.pcouncil.org/wp-content/uploads/age_comp_2011_final_feb21.pdf

Stone, R., J.S. Foott, and R. Fogerty. 2008. FY2006 investigational report: Comparative susceptibility to infection and disease from *Ceratomyxa shasta* and *Parvicapsula minibicornis* in Klamath River basin juvenile Chinook, coho and steelhead populations. United States Fish and Wildlife Service, California-Nevada Fish Health Center, Anderson, CA.

The R Project for Statistical Computing, Development Core Team. 2010. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at: http://www.R-project.org.

Udey, L. R., J. L. Fryer, and K. S. Pilcher. 1975. Relation of water temperature to ceratomyxosis in rainbow trout (*Salmo gairdneri*) and coho Salmon (*Oncorhynchus kisutch*) Journal of the Fisheries Research Board of Canada 32: 1545-1551.

Williams, D. 2011. Unpublished Data: Age and fork length data from Klamath Basin adult Chinook salmon Returns in 2011. Yurok Tribe Fisheries Department,190 Klamath Boulevard, P.O. Box 1027 Klamath, CA 95548.