

## U.S. Fish & Wildlife Service

California Nevada Fish Health Center 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.

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**Summary:**

In fall and winter of 2009-2010, adult Chinook carcass removal from a portion of Bogus creek and its relationship to the concentration of *Ceratomyxa shasta* DNA in the creek and Klamath River was studied. Study cooperators included Oregon State University, Yurok Tribal Fisheries, California Department of Fish and Game, and the California Nevada Fish Health Center (FHC). The FHC surveyed adult Fall-run Chinook carcasses in Bogus creek, Shasta River, and Klamath River for *C.shasta* myxospores. Myxospores were detected in 22 – 53% of the sample groups. QPCR analysis of “undetected” scraping samples suggests that 73-86% of the carcasses were infected with some stage of the parasite. The range of myxospore per scraping varied from 575 to 6.6 million. Only 5-9 % of the samples are classified as high spore contributors (>1 million spores). These carcasses likely input 90% of the myxospores into the river.

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### **Introduction:**

Severe infection, of juvenile Klamath River Chinook salmon and coho salmon, by the myxozoan parasite *Ceratomyxa shasta* may be a contributing factor to declining adult returns in the basin. The incidence of *C. shasta* infection, observed in histological sections of juvenile Chinook collected in the Klamath River above the confluence with the Trinity River between May and July, has ranged from 21 – 38% (Nichols et al. 2009). This incidence is 10 – 27% higher in samples assayed by the more sensitive quantitative polymerase chain reaction assay (QPCR). Approximately 70% of the positive histology samples demonstrated pathology due to the infection. The high prevalence and severity of infection, in native fish that should have high resistance to an endemic disease, indicates this parasite is a key factor limiting salmon recovery in Klamath River.

*Ceratomyxa shasta* has a complex life cycle, involving an invertebrate (polychaete worm) host as well as salmon (Bartholomew et al. 1997). The Klamath river between the confluence with Shasta river and Seaid creek has been identified to be highly infectious to salmon (Stocking et al. 2006) and is the focus for management actions to disrupt the parasite's life cycle. In August 2007, a multidisciplinary panel of fish disease experts and fishery managers met to develop a research plan focused on management actions to reduce disease levels (ceratomyxosis) in natural juvenile salmon of the Klamath R. One of the proposed management actions was removal of adult salmon carcasses in order to reduce myxospores input into the system. The hypothesized effect of this action would be reduced polychaete infection and subsequent reduction of infectious actinospores causing juvenile fish mortality the following spring. The 2009-2010 Bogus creek carcass removal study examines the relationship between carcass removal and parasite DNA quantity in creek and river.

The FHC's role in the 2009 carcass removal project was to:

1. Determine the incidence and concentration of *Ceratomyxa shasta* myxospores in the intestine of carcasses from the main stem Klamath R., Shasta R., and lower Bogus creek.
2. Estimate the incidence of infection for all stages of *C.shasta* in the sample set.
3. Provide Dr. Jerri Bartholomew (OSU) with *C.shasta* positive samples from Coho salmon carcasses in the Klamath R. basin.

## **Methods:**

***Carcass myxospore*** – Fall-run Chinook salmon (carcass) intestine sampling occurred over 6 weeks (10/18-24, 10/25-31, 11/1-7, 11/8-14, 11/15-21, and 11/22-28) in 2009. Sixty-five samples collected from Bogus creek (confluence upstream 0.6 mi) by Yurok Fisheries staff, 56 samples from Klamath River carcasses (Iron Gate dam to confluence of Shasta R.) by USFWS Arcata FWO staff, and 61 samples from the Shasta R. by CA-NV FHC staff. Samples from the Klamath and Shasta River consisted of decomposed carcasses (stage 2 and 3, Baumsteiger and Kerby 2009). Due to the removal schedule, Bogus creek samples tended to be from salmon that were less than 48h old. Intestine (small intestine to rectum) was dissected from the carcass, placed into individually numbered plastic bags, and either refrigerated for 24-48h (Shasta R.) or frozen prior to processing.

The sample was weighed, cut into 8 – 12 cm pieces, and an intestinal content sample (scraping) obtained by grasping the intestine with forceps and pushing the backside of a #21 scalpel blade, held at 45° angle, along the outside of the intestine. The process was repeated several times until only the serosa to stratum compactum layers remained. The scraping subsample was weighed, diluted 3x with PBS (4x final dilution), poured into tubes, vortex mixed, and allowed to settle for 1 -3 min. A 100 µL aliquot was frozen for later QPCR analysis. Duplicate 10 µL samples of this suspension examined for the presence of *C.shasta* myxospores by 20x phase microscopy. Four hemocytometer counts on wet mount positive samples quantified myxospore concentration per gram of sample. This value multiplied by the scraping weight (g) provided the ***myxospore per scraping*** estimate. Given our limited detection sensitivity and other potentially infected tissues within a fish, we consider the myxospore / scraping value to represent the minimum spore load for a given fish.

In order to estimate the incidence of infection by all parasite stages in the undetected samples, 5 or 6 scrapings, classified as myxospore “undetected”, from each sample week per site were assayed for *C.shasta* DNA. Scrapings were digested in 1ml NucPrep Digest Buffer containing 1.25 mg/ml proteinase K (Applied Biosystems, Foster City, CA) at 55°C for 2 hours with constant shaking. A subsample of digested tissue homogenate diluted 1:33 in molecular grade water and extracted in a 96 well filter plate system (Applied Biosystems Model 6100 Nucleic Acid Prep Station). Extracted DNA was stored at -20°C. The combined tissues tested for *C. shasta* 18S rDNA using TaqMan Fam-Tamra probe and primers in an Applied Biosystems 7300 Sequence Detection System (Hallett and Bartholomew 2006). Reaction volumes of 30uL, containing 5uL DNA template, were used under the following conditions: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s and 60°C for 1 min. Standards, extraction control and no template control wells (MGW) were included on each assay plate (True et al. 2008; Nichols and True 2007). Fluorescent Unit change ( $\Delta Rn$ ) was measured at each cycle and positive test results are reported as Cycle Threshold (Ct), the point when fluorescent signal surpasses normalized background levels. Detection thresholds are set at  $\Delta Rn$  of 1000 fluorescent units (FU) which occur at (Ct) values of 38-39.

## **Results and Discussion:**

One coho carcass and 182 Chinook intestine samples were collected between October 10 and November 23, 2009 (Table 1). This sample set represents approximately 1 percent of the total Fall Chinook carcasses in Bogus creek (65 of 5926 salmon recorded at the weir {1.10%}), Shasta River (61 of 6296 {0.97%}), and the Klamath river (56 / 4400 {1.27%}) estimated abundance (M Knechtle CDFG pers. comm., S. Gough 2009). Extremely low coho returns to the Klamath (estimated <10 adults in both Bogus creek and Shasta R) precluded carcass sampling opportunities (M Knechtle CDFG pers. comm.). One female carcass was obtained from the Bogus weir on 09DEC by CDFG and stored at Iron Gate Hatchery.

Scraping weight averaged 25.6% (SE 1.0%) of intestine weight. Screening method sensitivity was examined by both serial dilution and average spore count of low count samples (identified positive during screening as 1 spore / 100 microscopic fields). A high concentration sample (Bogus sample#80, 3540 spores/  $\mu\text{L}$ ) was diluted 10x, 15x, and 30x with an undetected status suspension (Bogus#26). Using this sample set, a sensitivity of approximately 118 spores/  $\mu\text{L}$  of scraping was determined for wet mount screening (1 spore seen in 100 fields). In contrast, average number of spores in low count samples was 12 spores /  $\mu\text{L}$  (range 2.5 – 45). This 10-fold difference between spore concentration of the dilution experiment and low spore samples suggests that intestines containing less than 100 myxospores /  $\mu\text{L}$  of scraping had relatively low probability of detection during the initial screening. When applied to the average scraping weight of 0.92 g, sensitivity of wet mount screening is estimated at 11,040 to 92,000 myxospores per intestine ( $(12 \text{ or } 100 \text{ spores/ } \mu\text{L})(1 \mu\text{L} / 1\text{mg})(1000\text{mg/g}) \cdot 0.92\text{g}$ ).

The incidence of *C.shasta* myxospore detection ranged from 22 – 53% for the 3 collection sites (Table 1) similar to the 30% incidence observed in 2008 Bogus creek carcasses (Foott et al. 2009). No obvious temporal trend was observed in the data. It is noteworthy that Shasta R. carcasses had almost double the incidence as Klamath R. or Bogus creek salmon. Similar to the 2008 data, a wide range of myxospores per scraping (575 – 6,567,600) was seen in the positive samples precluding parametric statistical evaluation of the sites. Scattergram examination of all positive sample myxospore counts suggested 5 bins for examining spore loads: 1) undetected, 2) 575 to 50,000, 3) 50,001 – 500,000, 4) 500,001 – 1,000,000, and 5) greater than 1,000,000. The incidence of high spore samples (>1M) was 5 – 9% in carcasses from the 3 sites yet they contributed between 86% and 91% of the total myxospores calculated from each sample set (Figure1). This observation suggests that less than 10% of the adult carcasses will contribute approximately 90% of the myxospores into the system. The estimated high spore ( $\geq 1$  million spores) contributors in the main stem Klamath above or within the infectious zone is 440 carcasses (0.10X 4400 fish). This estimate excludes all fish spawned at Iron Gate Hatchery as carcasses are not returned to the river. Average myxospore concentration of high spore (>1M) samples was  $1,893,983 \pm 214,600$ . Estimated myxospore input from high spore contributors is  $8.3 \times 10^8$  spores (440 x 1.89M). Input from tributary carcasses will

add to this spore load such that some billion myxospores ( $10^9$ ) likely entered the Klamath River in the winter of 2009-2010.

QPCR analysis of a samples classified as “undetected” demonstrated 61% – 83% incidence of *C.shasta* DNA (Table 2). DNA could be from different parasite stages (trophozoite, pansporoblast, and low numbers of myxospores) in the scraping. In 2008, the incidence of *C.shasta* DNA in QPCR assay of myxospore “undetected” samples was 45% (Foott et al. 2009). When applied to the “undetected” sample numbers, the estimated incidence of *C.shasta* infection in the adult population ranged from 73 to 86% (Table 3). This overall incidence was similar to the 60-80% observed in histological sections and by QPCR assay of adult Chinook salmon intestine from IGH in 2005 and 2006 (Foott et al. 2009). Degraded DNA or inhibitors may be associated with Ct values above the 38 threshold seen in 3 “low” myxospore positive samples (Table2).

Table 1. Incidence of *C.shasta* myxospore detection in intestine samples from Fall-run Chinook Salmon carcasses collected over the 6 week study period in Bogus creek, Shasta River, and the Klamath River (rkm 176.88 – 190.21). Data recorded as number positive / total samples examined.

week	1 Oct18-24	2 Oct25-31	3 Nov1-7	4 Nov8-14	5 Nov15-21	6 Nov22-28	Incidence
Bogus	2 / 10	2 / 20	5 / 14	NC	5 / 21	NC	14 / 65 (22%)
Shasta	NC	16 / 30	10 / 18	6 / 13	NC	NC	32 / 61 (53%)
Klamath	3 / 11	2 / 6	8 / 20	NC	1 / 16	0 / 3	14 / 56 (25%)

NC No collected carcasses

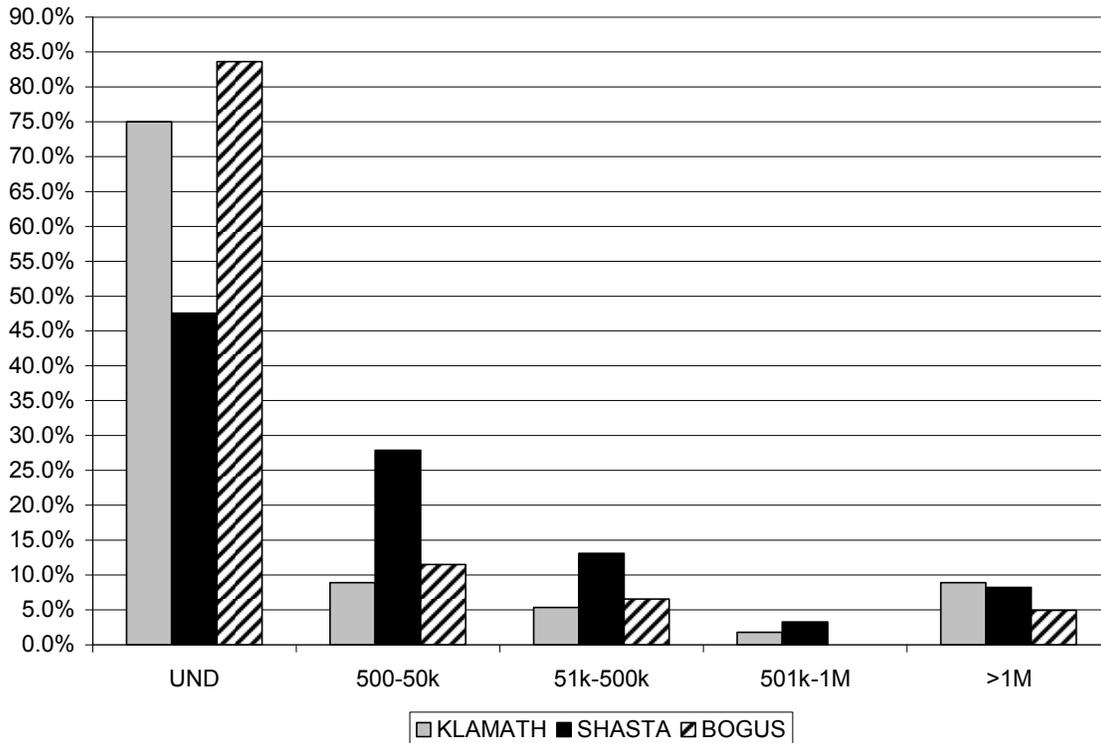
Table 2. Incidence of *C.shasta* DNA detection in gut scrapings classified as myxospore undetected. Cycle Threshold values and myxospore / scraping (sp) of gut scrapings classified as low and high.

	Undetected	Low	High
Bogus	20 / 24 (83%)	1000sp=35.3 7625sp= 30.1	6.6M sp= 20.2
Shasta	10 / 15 (67%)	2703sp = <b>41.99</b> 1000sp = 34.66 2950 sp= 27.2	Not done
Klamath	11 / 18 (61%)	575 sp = <b>41.68</b> 4750sp= <b>&lt;42</b>	4.2M sp = 29.0

Table 3. Estimated *C.shasta* infection (Cs+) using incidence of QPCR positive results for “undetected” scraping samples (Und).

	Spore+	Undetected	Incidence Cs+	Incid. X Undetected	Spore(+) + est.Cs+Und	Cs +
Bogus	14	51	0.83	42	14+42 = 56	56 / 65 (86%)
Shasta	32	29	0.64	19	19+32= 51	51 / 61 (84%)
Klamath	14	42	0.64	27	14+27= 41	41 / 56 (73%)

Figure 1. Prevalence of myxospore concentration categories in Klamath R., Shasta R., and Bogus Creek carcass intestines. Categories are: 1) UND= undetected by wet mount, 2) 500 to 50,000 spores, 3) 50,001 to 500,000 spores, 4) 500,001 – 1 million spores, and 5) >1 million spores.



No myxospores were seen in the adult coho sample collected from Bogus creek however it was QPCR positive for both *C.shasta* and *Parvicapsula minibicornis* (kidney) DNA . This sample and ethanol stored 2007 intestines from Klamath R. coho and rainbow / steelhead adults as well as Trinity R. Hatchery Spring-run

Chinook adults was shipped to OSU for genotype analysis (R. Slezak 2007 sample set).

Eleven “myxospore positive” scraping samples, from Shasta R. carcasses mixed 1:1 with 0.25% methylene blue stain, were examined by 20x phase microscopy. These samples were not frozen prior to examination. Examination was tedious due to the dark background. The prevalence of blue spores (indicating nonfunctional permeable spore values) ranged from 0 – 88% (average  $26\% \pm 9\%$ ). An efficient and accurate method to estimate myxospore viable would aid in the development of transmission models.

The ultimate goal of this work is to provide data for a life cycle model that will inform managers in the feasibility of carcass removal to reduce ceratomyxosis in juvenile salmon (Ray et al. in review). As mentioned above, the low incidence of high spore contributors throughout the spawning season suggests that a high proportion of all carcasses would need to be collected in order for this action to be effective. Outstanding questions include:

1. Myxospore transmission efficiency to main stem river polychaetes within the infectious zone?
  - a. Relative contribution of tributary myxospores to this polychaete population compared to main stem carcasses.
  - b. The estimated percent viable myxospore released per carcass to correctly model spore input into the Klamath River.
2. Of more academic interest, determine the mechanism and triggers for myxospore formation (degree days, post-mortem environment?). Why do we typically observe  $\leq 30\%$  myxospore incidence when the overall parasite infection in the returning adults is 80% ?

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