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Prognosis of *Ceratomyxa shasta* and *Parvicapsula minibicornis* Infections in Klamath River Coho and Trinity River Chinook Salmon

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Summary

Ceratomyxa shasta and *Parvicapsula minibicornis* are myxosporean parasites which share the polychaete host *Manayunkia speciosa* and are associated with disease in Pacific salmon during their freshwater life history phase. In the Klamath River, these myxozoan parasites are associated with disease and mortality in juvenile Chinook and coho during summer emigration. River reaches below Iron Gate Dam to the Seiad Valley (river mile [RM] 141-190) comprise the "infectious zone" of management concern and focused research efforts aimed at reducing parasite infectivity. Dual infections with both myxozoan parasites are also common and raise additional concerns for early ocean survival and recovery of salmonid stocks in the Klamath Basin.

This study examined the progression of myxozoan diseases and the survival probability of Klamath River hatchery stocks following 72 hour exposure to river water conditions typically encountered by out-migrating smolts in early June. Two basin stocks of juvenile salmonids, Iron Gate State Fish Hatchery (IGSFH) coho and Trinity SFH Chinook were exposed in the main stem Klamath River, above Beaver Creek (RM 163) from June 9-12, 2008. An automated ISCO water sampler was used to collect 2L water samples at 1 hr intervals during the exposure, to assess parasite challenge dose encountered by the sentinel fish. Exposure groups were held in 18°C pathogen-free water and monitored daily, by Quantitative PCR and histology, for infection status and disease progression over a 6 week period.

Onset of ceratomyxosis at 10-15 days post exposure (dpe) was similar for Chinook and coho; however severity of disease, mean day to death (MDD), and cumulative percent mortality (CPM) differed for the two hatchery stocks. Chinook MDD was 17.3 compared to 20.6 in coho. Higher levels of *C. shasta* DNA (>1.5 log) were observed in Chinook intestinal tissue by QPCR compared to coho for the same post exposure period. Coho experienced a delayed onset of clinical disease of approximately 3 days; however cumulative percent mortality (98.5%) was higher than Chinook (87.1%) at the conclusion of the study (35 dpe).

Parvicapsula minibicornis infection levels increased gradually in both Chinook and coho until 10 dpe, then prevalence of infection leveled off between 60-70% for the remainder of the 6 week study. The majority of exposed fish (>95%) in this study were co-infected with both myxosporean parasites. We observe a similar pattern of concurrent infections in the annual Klamath River Fish Health Monitoring Program (KFHMP) (Nichols and True 2007, Nichols and True 2008, True et al. 2011), where *P. minibicornis* prevalence increases to high levels and remains high throughout the study season of June-August. Study survivors (35 dpe) were found to have *P. minibicornis* infection levels similar to cohorts that died of ceratomyxosis several days or weeks prior, suggesting that *P. minibicornis* infection is not driving the clinical picture of disease progression, or the calculated survival proportions, observed in dual myxozoan infections of juvenile salmonids in the Klamath basin.

C. shasta myxospore development occurred in both Chinook and coho juveniles by 15-16 dpe at rates of 19 and 34% respectively. While spore numbers were not particularly high, especially relative to levels observed in infected adults in nearby Bogus Creek (Bartholomew et al. 2009), this finding indicates that juvenile salmonids do contribute spores and may be a source of re-infection for polychaete populations below Iron Gate Hatchery.

Due to the sheer size of the Fall Chinook salmon (FCS) juvenile program at Iron Gate Hatchery (4.5 – 5 million Fall Chinook released over a 2 week period), compared to relatively low numbers of adults returning to the hatchery in recent years (average of ~12,000/year from 1988-1997, CDFG 2010), juvenile myxospore contribution may be significant in perpetuating the *C. shasta* parasite life cycle in lower reaches of the Klamath River. While myxozoan infections in adult salmonids clearly established the concentration of infected polychaetes below Iron Gate Dam, which resulted in a highly "infectious zone" for juvenile salmonids, it is unknown what contribution adult Chinook salmon provide when adult escapement is low. The reduced abundance of adult Fall Chinook, compared to the relatively high and stable juvenile production numbers at Iron Gate hatchery, may have resulted in a shift in myxospore contributions in the system in recent years. Juvenile Chinook contribution to the myxozoan parasite cycle, if they are exposed to high doses of actinospores and survive approximately 2 weeks post exposure, may be higher than previously believed and warrants additional study.

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INTRODUCTION

Ceratomyxa shasta and *Parvicapsula minibicornis* are myxosporean parasites which share an alternate polychaete host, *Manayunkia speciosa* (Bartholomew et al. 1997, Bartholomew et al. 2006), and are associated with disease in Pacific salmon during their freshwater life history phase (Kent, Whitaker & Dawe 1997) . In the Klamath River, which spans Southern Southwest Oregon and Northern California, these two myxozoan parasites are associated with disease and mortality in juvenile Chinook and coho during summer emigration (Nichols et al. 2008, Bartholomew et al. 2007). Concurrent infections with both myxozoan parasites are common in fish health monitoring and sentinel studies (Nichols and True 2007, Bartholomew 2009) and raise concern for successful emigration and early ocean survival of Klamath basin salmonid stocks.

Historical Background

The Ca-Nv Fish Health Center (FHC) has been involved with fish health issues in the basin since the early 1990s (Foott 1992, Walker & Foott 1993, Foott et al. 1999). From 2004 to present, the FHC has conducted the Klamath River Fish Health Monitoring Program (KRFHMP) which assesses annual myxozoan prevalence of infection levels (POI) in out-migrating Chinook and coho salmon in 5 reaches of the Klamath River, separated by major tributaries including the Trinity River (Nichols & True 2007, Nichols et al 2007, Nichols & Foott 2006)(Table 1).

Table 1. Sample reach locations (reach code), river mile, and cooperating agencies performing fish collections on the Klamath and Trinity rivers under the Klamath Fish Health Monitoring Program (KFHMP).

Sample Reach (code)	River Mile	Primary collector(s)
Klamath River main stem		
IGD to Shasta (K5)	Klamath 190-177	USFWS and Karuk Tribe
Shasta to Scott (K4)	Klamath 177-144	USFWS and Karuk Tribe
Scott to Salmon (K3)	Klamath 66-144	Karuk Tribe
Salmon to Trinity (K2)	Klamath 66-44	Karuk Tribe
Trinity to Estuary (K1)	Klamath 44-4	Yurok Tribe
Klamath Estuary (K0)	Klamath 4-0	Yurok Tribe
Trinity River		
Upper – Pear Tree Rotary Trap (T2)	Trinity 94	Hoopa Tribe
Lower - Willow Creek Rotary Trap (T1)	Trinity 21	USFWS and Yurok Tribe

The KRFHMP utilizes Quantitative Polymerase Chain Reaction (QPCR) and histology to quantitatively assess *C. shasta* infection status and disease severity of migrating salmonids, from May to July in the main stem Klamath River. *C. shasta* and *P. minibicornis* are not found at significant levels in the Klamath River (KR) tributaries, including the Scott, Shasta, Salmon, or Trinity River (Stocking 2006). Historical *C. shasta* incidence of infection by QPCR has ranged from 17 to 49% in out migrant Chinook sampled above the Trinity River confluence from May to July in 2006-2011 (Table 2).

Table 2. Historic *C. shasta* incidence of infection (% positive) as determined by histology and QPCR, in juvenile Chinook salmon collected from the Klamath main stem between Iron Gate Dam and Trinity River confluence during May through July, 1995-2010. Similar data for coded-wire tagged (CWT) fish released from each hatchery: Trinity Hatchery Chinook (positive/total, (percent positive)) collected below the Trinity R. confluence (K1) and estuary (K0), and the same data for Iron Gate Hatchery Chinook captured in reaches above the confluence of the Trinity River (K5, K4 and K2).

Year	Chinook, May-July, Above TR Confluence (Percent Positive by Assay)		Iron Gate CWT-QPCR (Above TR confluence - reach K5, K4, and K2)	Trinity CWT- QPCR (Below TR confluence – reach K1/K0)
	Histology	QPCR		
1995	44	NS[1]	NS	NS
2002	19[2]	NS	NS	NS
2004	34	NS	NS	NS
2005	35	Not Included[3]	NS	NS
2006	21	34	6/18 (33%)	1/67 (1%)
2007	21	31	15/22 (68%)	46/332 (14%)
2008	37	49	9/13 (69%)	8/257 (3%)
2009	54	45	82/228 (36%)	13/100 (13%)
2010	15	17	17/149 (11%)	1/45 (2%)
2011	2	17	22/176 (13%)	4/49 (8%)
Average (SE)	**25% (7)**	**32% (6)**	**25% (10)**	**9% (2)**

[1] NS= Not Sampled.
[2] Only TR CWT Chinook were assayed in 2002 by histology.
[3] 2005 QPCR data is not included: sample incidence was 62% however only portions of lower intestinal tissue were collected and this smaller tissue sample does not compare directly to the standardized tissue protocol used from 2006-2010 which included the entire intestine tested by QPCR.

Small proportions (5-10%) of IGH Chinook were coded wired tagged prior to 2009, when a constant fractional mark rate of 25% was implemented at Iron Gate Hatchery (IGH) (Buttars and Knechtle 2009). Chinook are volitionally released from IGH in late May through mid-June at a target size of 70mm. However river temperatures (>65°F) are also considered and often prompt an early release, regardless of fish size. Therefore the majority of fish tested under the KRFHMP are of unknown or “mixed origin” in terms of whether these fish represent natural or hatchery fish production. Unmarked juvenile salmonids collected after June hatchery releases, from either rotary screw traps or by beach seine, can be hatchery origin or naturally produced in any of several tributaries below Iron Gate Dam (Shasta, Scott, and Salmon River). The duration of time these salmonids spend in the infectious main stem cannot be determined at time of capture, making it difficult to assess the relationship of parasite exposure to infectivity status at time of capture, or disease prognosis.
Therefore, while the KRFHMP provides relative comparisons of inter-annual prevalence of infection for the entire population, the data collected from individual fish and reach locations

cannot always provide a clear temporal pattern for disease pathology. Information regarding exposure dose/period, exposure location(s), and how disease progression relates to these factors is best determined in controlled studies. This type of information is necessary to develop a general prognosis for the ~4-5 million Chinook released each year, and to correlate disease prevalence data to environmental factors (temperature and flows primarily) associated with climatic year type (drought, normal or wet). The limited numbers of coded wire tagged specimens collected in the KRFHMP each year (~200-500 depending on availability for capture) do provide temporal reference for infectivity status regarding the length of time fish have been in the main stem post hatchery release. We use a temporal metric of Weeks at Large (WAL) to describe infections levels observed at time of capture to presumed main stem residency period (release date – capture date). In the KRFHMP, we observe the majority of IGH CWT Chinook reside for 3-5 WAL, but a small group consistently has longer periods of time at large before recapture (6-9 weeks) without increased prevalence of infection or parasite copy number. It is unclear where these fish reside/hold for the additional time period, but likely in tributaries or cold-water refugia where parasite exposure and/or proliferation is expected to be decreased.

Related Studies

Pathology of infection for *C. shasta* and *P. minibicornis* has been well described in juvenile and adult salmonids (Lom & Dykova 2006. Yasutake & Elliott 2003, Raverty et. al 2000, Kent et al 1997, Hendrickson et al. 1987)). Previous susceptibility and sentinel exposure studies, performed under typical river conditions (June – July exposure in the infectious zone) have shown a post-exposure period of 18-25 dpe produces clinical *C. shasta* infections dominated by trophozoite stages in the intestine and subsequent mortality (Foott et al. 2004a, Stone et al. 2008). Several additional studies identified the reach from Iron Gate Dam to the Scott River as a primary management area based on infectivity data collected from invertebrate host surveys (Stocking et. al. 2006) fish health monitoring studies (Nichols et al 2008, Nichols and True 2007), sentinel fish exposures (Bartholomew 2009, Bartholomew 2008, Stone et al. 2008), and environmental water sampling studies (Hallett & Bartholomew 2006, Foott et al. 2007b).

Combined mortality data from sentinel studies and environmental parasite levels determined by QPCR water sampling have demonstrated a water-born infectivity threshold of ~1-10 spores/L as being associated with significant levels of mortality due to ceratomyxosis (Hallett & Bartholomew 2006, Bartholomew 2008). While QPCR water testing often corroborates mortality rates observed in fish from corresponding sentinel exposures sites, “spore concentration” cannot directly describe infection levels in fish. Molecular assays of water samples cannot differentiate between the actinospore life stage that infects fish and the myxospore stage that only infects the secondary polychaete host. Total spore concentrations obtained from water sampling can be highly variable (Foott 2004b, Hallett and Bartholomew 2006) and are only relevant for the immediate location where sampling was performed. Many environmental characteristics (temperature, flow, habitat substrate) can affect the abundance, dispersal and longevity of viable actinospores (Foott et al. 2007b). These environmental factors, as they relate to a given climatic year, make it difficult to quantitatively determine infection levels in fish from mortality data and environmental spore concentration data alone.

Study Objectives

On-going fish health monitoring studies in the Klamath and Trinity rivers assess parasite infection using QPCR to quantify infection level based on number of parasite DNA copies in fish tissue. Complementary histological assessments provide additional information regarding tissue damage to kidney and intestine caused by these parasites. This study, in particular, was designed to begin to develop infection thresholds in fish that may provide prognostic value in terms of predicting the disease risk and overall outcome (mortality) for Klamath basin salmonids. The definition of *prognosis* as used generally and in veterinary medicine is as follows:

1) A forecast of the probable course and outcome of a disorder.
   (Dorland's Medical Dictionary for Health Consumers ©2007. Published by Houghton Mifflin Company)

2) A forecast of the probably course and outcome of an attack of disease and the prospects of recovery as indicated by the nature of the disease and the clinical signs of the case. In keeping with modern day usage of decision making on the basis of statistics, it is now becoming commonplace to give a percentage probability of a successful outcome in terms of survival, and a similar figure for probability for return to full function. (Saunders Comprehensive Veterinary Dictionary, 3rd Ed. © 2007 Elsevier, Inc.)

The objectives of this study are to provide a finer resolution of myxozoan pathology in order to: quantitatively describe infections levels associated with clinical disease and mortality, and provide probability of survival for a given incidence of disease in KRFHMP Chinook populations. Specifically, the study tasks to meet these objectives were to:

1) Estimate *C. shasta* dose experienced by fish during 72-hour in-river exposure above Beaver Creek (infectious zone).
2) Correlate *C. shasta* and *P. minibicornis* infection levels (C_T score) to parasite log DNA copy number (using standardized DNA plasmid controls).
3) Correlate parasite infection level (DNA copy number) with days post exposure (dpe), mean day to death (MDD) and cumulative percent mortality (CPM) for each fish species.
4) Correlate parasite infection level (DNA copy number) with tissue damage (histology), and assess progression from parasite presence to clinical disease, in each species.
5) Determine survival proportions for each species.
6) Determine the prognostic value, of parasite DNA copy number (as C_T values) for predicting subsequent disease severity and mortality rates in each species.

METHODS

Experimental Fish

Approximately six-hundred juvenile fish of each species (Trinity Hatchery (TRH) Fall Chinook and IGH coho) were transported to the Ca-Nv FHC wet laboratory on May 6 and 20 respectively, and acclimated to 18°C pathogen-free water for approximately 2-4 weeks prior to sentinel exposure. Experimental fish were held in 800L circular tank supplied with 7.6 L/min flow of ozonated water and fed a BioMoist #1 diet at 3% body weight pre-exposure and ad lib post exposure. Oxytetracycline medicated feed was prophylactically fed at rate of 5g/100 pounds of fish for 3 days prior to exposure and 7 days post exposure, to preclude *Flavobacterium columnare* infections which are frequently contracted by sentinel fish at the exposure site (Figure 1).

Pre-screening by QPCR for pre-existing myxozoan infections

Approximately thirty fish of each species were tested by QPCR to determine if hatchery stocks had pre-existing exposure or active infections of *C. shasta* or *P. minibicornis*. Entire kidney and intestine were removed, extracted for total DNA, and assayed for each myxozoan by the QPCR methods described below. Organosomatic data including body weight and fork length, were collected and used to calculate condition factor (KTL= [weight (g) / length $(mm)^3 \times 10^5$]), fish per pound and mean weight and fork length. Any abnormalities, or clinical signs of myxozoan infection, were recorded.

Non-Exposed Control Groups

Chinook and coho tanks were inventoried on June 4 for accurate fish counts and distribution to exposure and control groups. Non-Exposed Control (NEC) groups consisting of 50 Chinook and 25 coho were established by randomly netting a subset of fish into two 20-gallon aquaria, where they were maintained, and monitored twice daily for clinical signs and mortality, throughout the 6 week study period. All remaining control fish were necropsied at the conclusion of the study and tested by QPCR.

72-hour Sentinel Exposure

The exposure site lies within the "infectious zone" of the Klamath River, at RM 163, which is approximately 1 mile above the confluence of Beaver Creek (UTM 10 516058E 4634926N) (Figure 1) .

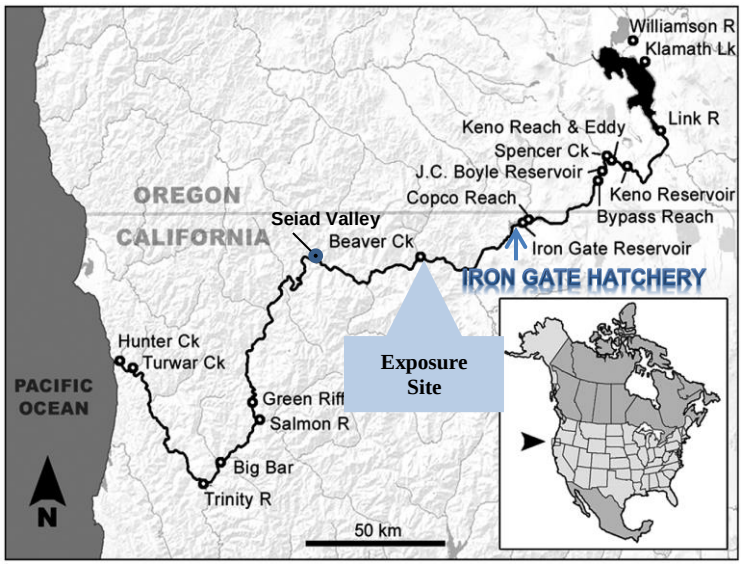

Figure 1. 72-hour exposure site above Beaver Creek (RM 163) on the Klamath River in northern California. The "infectious zone" extends from Iron Gate Dam to Seiad Valley (RM 141 to 190).
Inset: site area in context of North America.

Five hundred fish from each of the two juvenile hatchery stocks were transported to the Klamath River on June 9, 2008. Fish were taken off food 24 hours prior to and during the 72 hour exposure, and given prophylactic Furanase treatments (1mg L^{-1}) during the 1.5 hour transportation time to and from the site. Fish were randomly hand netted into 10ft^3 live cages which were placed approximately 3 feet from the bank at a water depth of 3 feet. Cages were oriented towards the upstream current, and secured to the river bottom with metal rebar stakes. Hobo Data loggers (Onset Corporation, Pocasset Maine) were used to monitor water temperatures in each transport tank, in live cages during the 72-hour exposure, and in the wet lab holding tanks during acclimation and post-exposure periods.

Water Sampling to Estimate Exposure Dose

An ISCO automated water sampler (Teledyne Isco, Inc. Lincoln, NE) was used to collect 2L water samples every 2 hours during the 72-hour exposure period. Two different approaches have been used for water sampling protocols in previous studies; hourly water samples tested individually or in replicate, and composite samples that comprise the same sampling intervals and volumes however samples are either pooled during collection or pooled into a single, or multiple samples prior to filtration and testing (Bartholomew 2008). To further assess the two methods of water sampling, replicate 2L water samples were manually collected during the first 8 hours of the exposure period and tested as individual samples and as an 8-hour "composite" sample. The calculated spore concentrations were compared between methods, as well as to the automated samples collected by the ISCO™ instrument.

Water samples were kept cold during collection by placing crushed ice around the collection bottles in the base of the ISCO sampler every 24 hours. Following collection, water samples were kept on ice in 60 gallon coolers, and immediately vacuum filtered in the field or within 24 hours (samples collected on day 3 of the exposure). Samples were vacuum filtered (300 mm Hg) through a MF-millipore nitrocellulose membrane (5um pore size, 47mm diameter) and stored at -20°C until DNA extraction and QPCR testing were performed.

Mortality Monitoring

At the conclusion of the 72 hour exposure period, all fish were transported to the Ca-Nv FHC wet laboratory and each species was randomly distributed into the following groups: Daily Sample (DS) groups which were to be sampled daily for QPCR and histology testing, and Mortality Control (MC) groups which were not sampled, and fish were allowed to die naturally over the course of the study (Table 3). Feeding resumed in study groups at 24 hours after return to wet lab holding units.

The MC groups provided a replicate for the exposure as well as an accurate means of calculating Cumulative Percent Mortality (CPM) for post exposure fish. Cumulative Percent Mortality equals the sum total of mortality during the 35 days post exposure period, divided by the total mortality in group (x 100). Mean Day to Death (MDD) for each exposure group was calculated as the geometric mean of all days with *C. shasta* mortality within the 35 dpe post exposure holding period.

All experimental groups were checked twice daily for mortality. All mortality that occurred in MC or NEC groups was placed in 6 oz. individual zip-lok™ bags, and frozen at -20°C. At the

conclusion of the study (35 dpe), the surviving fish from the DS, MC and NEC were sampled by QPCR to determine parasite DNA levels. Additionally, a sub-set of the MC fish, which corresponded with the peak mortality period in the DS group, were assayed by QPCR. This provided a comparison of C_T values and parasite DNA levels in the cohort groups for each species.

Table 3. Distribution of experimental Chinook and coho into exposure and control groups.

Experimental Fish **Acclimation and Pre-screening**	**TRH Chinook**	**IGH Coho**
No. Fish acclimated to 18°C (date transferred)	580 (May 6)	555 (May 20)
No Fish Pre-screened by QPCR	30 (May 27)	30 (May 27)
No. Fish assigned to Non-Exposed Control (NEC) Group	50	25
Exposure Fish		
No. of 72-hour exposure fish (June 9-12, 2008)	500	500
Post Exposure Study Groups		
No. Fish in DAILY SAMPLE (DS)	300	300
No. Fish in MORTALITY CONTROL (MC)	200	200

Necropsy for Histology and QPCR Assay

Five fish were randomly netted from the DS group daily and necropsied for QPCR assays and histology examination. No histology samples were collected on weekends (5-6,12-13, and 19-20, and 25-26 dpe) which may have limited the initial observation of parasites in the tissues. To assess parasite distribution in anterior and posterior tissues, each intestine and kidney tissue was quartered into 4 sections using individual sterile scalpel and forceps; two replicate tissues of each type were assessed by histology and two replicates assessed by QPCR. For histology, the two replicate intestine and kidney tissues were placed in 4-section biopsy cassettes, anterior tissues were marked with histological dye (Cancer Diagnostics, Birmingham, MI) for proper orientation. Entire tissue cassettes were placed in Davidson's fixative for 48 hours and stored in 70% ethanol at 48 hours until processed for 5µm paraffin sections stained with hematoxylin and eosin (H&E) (Humason 1979). Each individual fish specimen, containing 2 separate areas of the kidney, lower intestine (mean length 3.8mm

Chinook and 3.4 mm coho), and small intestine (mean length = 3.5 mm Chinook and 2.8mm coho), were examined at 400x for the presence of parasites and lesions. .

A composite pathology score was determined for each fish sampled. Intestine was rated for quantity of *C. shasta* trophozoites (0=none, 1= 1-10 per section, >10 per section) and presence of myxospores. The relative percentage of the epithelium that was hemorrhagic, eroded, or necrotic was recorded for each section. We arbitrarily selected 50% of the section as a threshold value for severe clinical ceratomyxosis: 50% erosion, hemorrhage and necrosis (EHN).

To further assess production of *C. shasta* myxospores in juvenile Chinook, two moribund fish were removed from the MC group during the peak of mortality at 21 dpe. Fish were euthanized, a smear made from the lower intestine fluid (time zero sample), and the small and lower intestine were held for 72h at 18°C. The entire intestinal content was scraped into a tared tube, weighed, diluted 10x with PBS, and myxospores were quantified with a hemocytometer.

Similarly, Chinook and coho kidney was rated for the quantity of *P. minibicornis* trophozoites in the glomeruli and tubules (0=none, 1= 1-10 per glomeruli or within < 25% of the tubules of the section, 2=>10 per glomeruli and/or within >25% of the tubules) and presence of myxospores in the tubules. A kidney disease score (0-12) was assigned to each fish based on the summation of ratings for glomerulonephritis, swollen bowman capsule, interstitial hyperplasia, and protein filtrate within tubules (0 = none, 1 = limited focal, 2 = multifocal, or 3 = diffuse distribution within section). The mean kidney score for each sample day group was calculated from the individual scores. No definition of clinical disease was assigned to kidney sections.

Two additional measurements were performed on Chinook kidney sections to examine the progression of interstitial hyperplasia and dilation of the Bowman's capsule. The number of tubules observed in ten 200x fields of kidney was recorded to provide an inverse measurement of interstitial hyperplasia. No measurement was taken if *C. shasta* trophozoites were observed in the kidney interstitium, as the parasite is associated with a strong inflammatory response. Bowman's capsule dilation, as an indirect measure of nephron hypertension and glomerulonephritis, was estimated from the diameter of 10 Bowman capsules in a minimum of 4 fields. A calibrated micrometer measured the long and short axis of the Bowman capsule to calculate its luminal surface area (ellipse surface area (μm^2) = Pi* Radius 1* radius 2).

For the QPCR assay, five fish were sampled and necropsied from the DS group as described above. Replicate sections of intestine and kidney tissue were combined for a single QPCR sample for the majority of post exposure testing. However on 9 and 16 dpe, intestine and kidney tissues were assayed individually to assess parasite load and distribution in anterior and posterior regions of each tissue. All QPCR tissues were frozen at -20ºC until extraction and specific parasite assays could be performed.

At the conclusion of the six week period, survivors from the DS, MC and NEC groups were tested by QPCR to determine the infection level of fish that did not succumb to disease mortality. Additionally, a sub-set of Chinook MC frozen carcasses (N=20), removed from tanks

during the corresponding peak mortality period of 16-19 dpe in the DS group, was tested by QPCR to determine parasite load in cohort groups undergoing ceratomyxosis. Similarly, frozen carcasses from coho MC group (N=33) were tested from the peak mortality period of 16-22 dpe.

DNA Extraction – Fish Tissues and Water Samples

Fish tissues were extracted with Qiagen DNeasy kits following the manufacturer's protocol for animal tissues. Purified DNA was eluted in a single 200uL volume and stored at -20°C until assayed by QPCR. An Internal Positive Control kit (IPC) was run with all fish tissues tested in the *Parvicapsula minibicornis* (Pm) QPCR to rule out inhibition and false negative test results. In short, the IPC kit provides a second gene of interest at uniform concentration in all test wells. During the QPCR assay, any drop in IPC signal indicates inhibition in the tissue or water sample being tested. Use of an IPC allows for monitoring for inhibition and re-testing samples at additional dilutions, resulting in more accurate quantitation of infectivity levels in fish tissues or environmental samples. Previous testing has shown the *C. shasta* (Cs) QPCR does not perform well when multi-plexed with the IPC target, presumably due to competition between Cs and IPC specific primers (Ca-Nv FHC, unpublished data); therefore an IPC control was not used in Cs QPCR assays.

Water samples tested by QPCR determine the *C. shasta* dose fish were exposed to during the 72-hour in-river challenge (presumed to be primarily actinospores, as the QPCR cannot differentiate between the actinospore and myxospore life stages). DNA was extracted from filter membranes using Qiagen DNeasy™ kits (Qiagen, Valencia CA) following the manufacturer's protocol for animal tissues. Specifically, filter membranes were cut into smaller strips with individual sterile, DNA-RNA-free scissors and placed in 2 mL microcentrifuge tubes. Filters were lysed with double volumes of ATL lysis buffer (360uL) and Proteinase K (40uL) in order to full immerse filters during this step of extraction. Purified DNA was eluted in a single 200uL volume of elution buffer and frozen at -20°C until assayed by QPCR.

A DNA extraction control derived from naturally infected fish tissues, and confirmed by histology, was standardized to a known C_T value and Cs DNA copy number. The extraction control was included for each set of samples processed to monitor extraction efficiency and preclude false negative assay results which can arise from poor quality or low quantity of DNA.

QPCR Assays for *C. shasta* and *P. minibicornis* Infections in Fish

Quantitative PCR for *C. shasta* (Hallett & Bartholomew 2006) and *P. minibicornis* (True et al. 2009) in fish tissues was performed in a final volume of 30μL containing 1x Universal master mix (Applied Biosystems), 900nM concentration of each primer, 250nM concentration Taqman® MGB Probe and 5uL DNA template (approximately 300ng / reaction). Absolute standards (described below), no template control (NTC) wells consisting of molecular grade water, and an extraction control were included on each assay plate. Reactions were analyzed with an Applied Biosystems 7300 Sequence Detection System® (SDS) under the following conditions: 50°C for 2 min, 95°C for 10 min, 40-45 cycles of 95°C for 15s and 60°C for 1 min. Assay thresholds were verified for each run and set manually to 0.20 using the SDS software (v1.3.1, Applied Biosystems) and the component views to evaluate amplification in each sample. Validation studies examining the dynamic range and endpoint of the assays indicate a

C_T of 38 and minimum change in normalized fluorescent signal of at least 10,000 units defines a positive test for the *P. minibicornis* assay (approximately 40 copies, True et al. 2009). Previous assay validation studies, using DNA plasmid controls and naturally infected fish tissue, determined a similar assay threshold for the *C. shasta* assay (True, unpublished data). When abnormal amplification curves, indicative of assay inhibition due to too much DNA present, were observed in the *C. shasta* QPCR assay, extracted DNA was further diluted 1:100 and retested. C_T values were adjusted proportionally based on the amount of inhibition observed in the original sample. QPCR C_T values from fish tissues were transformed to DNA copy number, or log DNA copy number based on the standard curve for each parasite assay.

Plasmid DNA controls and Standard Curves for Fish Tissues

Plasmid DNA control templates, containing the target 18S sequence for *C. shasta* and *P. minibicornis*, were generated by amplifying DNA extracted from infected fish (as confirmed by histology) using the QPCR specific forward and reverse primers for each assay. PCR, cloning and sequencing was performed as previously described (True et al. 2009, Purcell et al 2004). Briefly, the Cs and Pm plasmid DNA was quantified in triplicate using a spectrophotometer and adjusted to a standard concentration. The DNA standards were diluted with molecular grade water in 10-fold increments to produce the standard curve. The Cs standard curve had a slope of -3.1, y intercept of 36.8 and R2 value of 0.997' the Pm standard curve had a slope of -3.7, y intercept of 44.0 and R2 value were 0.998. The amplification efficiencies (E) of the Cs QPCR was calculated to be >100% (instrument manufacturer's formula can exceed a theoretical 100%) using the formula *E = 10–1/s – 1 (Applied Biosystems Guide to Quantitative Gene Expression), and Pm QPCR had an assay efficiency of 86%. C. shasta* and *P. minibicornis* plasmid standards with known parasite copy number, NTC and extraction controls were run on each plate.

Plasmid DNA controls and Standard Curves for Water Samples

C. shasta actinospore and myxospore exposure dose L^{-1} (spore concentration) was calculated using a standard curve developed by Oregon State University (OSU) for sentinel studies conducted at various locations in the Klamath basin (Hallett and Bartholomew 2006). The slope of the *C. shasta* QPCR standard curve is -4.0166 with a y-intercept of 34.7. The amplification efficiency (E) of synthetic template DNA was calculated to be 83% using the formula *E = 10–1/s – 1 (Applied Biosystems Guide to Quantitative Gene Expression). C. shasta* plasmid standards with known parasite copy number, NTC and extraction controls were run on each water sample plate. Water sample C_T values were transformed to spore L-1 concentrations by the method of Hallett and Bartholomew (2006).

To preclude QPCR assay inhibition by environmental samples, water samples were tested at three dilutions (1:50, 1;100, and 1:200) in the Cs QPCR and an IPC was used for the Pm QPCR. Inhibition rates for water samples were determined by comparing all dilutions tested in the Cs QPCR and by analyzing IPC test results for the Pm QPCR (as previously described). An assay inhibition rate for environmental water samples was calculated for each assay.

The average Cs spore L^{-1} was calculated for the 8 hr composite sample (2L every hour pooled), the 8hr individual samples (2L every hour, tested individually), and for each 24-hour period (Day 1,2,3) during the 72-hour exposure period.

Cumulative Percent Mortality (CPM), Mean Day to Death (MDD) and Survival Proportions
Cumulative Percent Mortality equals the sum total of mortality during the 35 days post exposure period, divided by the total mortality in group (x 100). Mean Day to Death (MDD) for each exposure group was calculated as the geometric mean of all days with *C. shasta* mortality within the 35 dpe holding period. Cumulative percent mortality (CPM), and Mean Day to Death (MDD) were calculated for the Daily Sample (DS) and Mortality Control (MC) group.

Survival proportions (Mantel-Cox Log-rank and Gehan-Breslow-Wilcoxan tests) were initially calculated for Chinook and coho DS groups using Prism® 5.0 (Graphpad Inc., La Jolla, CA). Further statistical analysis including survival function modeling and parametric curve fitting was performed by statistician Dr. Nicholas Som, of the Arcata Fish and Wildlife Office.

All survivors from the DS, MC and NEC groups were analyzed by QPCR for parasite infection levels. Additionally, fish tissues from frozen MC mortalities that occurred during the peak mortality period for each species were analyzed to determine parasite load in moribund fish succumbing to ceratomyxosis.

RESULTS

Pre-screening by QPCR for myxozoan infections
Modified organosomatic indices were calculated for TR Chinook and IG coho to determine fork length, weight and condition factor (KTL= formula) as shown in Table 1. Trinity Chinook were smaller than Iron Gate coho at 416 fish/lb compared to 280 fish/lb, and a mean fork length of 47mm compared to 54mm, respectively. Both groups had low variability in the average weight and length, and condition factors greater than 1.0. Fish grew during the acclimation period of approximately 3-4 weeks and no culture problems were noted prior to in-river exposure.

Prescreening QPCR assays were performed 10 days prior to river exposure, on May 27th. TR Chinook and IG coho were found to have no prior exposure to *C. shasta*, however both stocks tested positive for *P. minibicornis* at fairly similar prevalence levels: 18/30 (60%) for Chinook and 14/30 (47%) for coho. The majority of Pm positive Chinook (14/18, 78%) had very low copy numbers, near the assay detection limit, that ranged from 26-47 DNA copies. The median copy number was 80. Three Chinook had copy numbers indicative of active infection, which ranged from 500-142,000, and one fish had high parasite DNA levels at 830,000 copies.

For the coho that tested Pm positive on pre-screening, the median DNA copy number was significantly higher at 39,800 compared to Chinook. The range of copy numbers was 400-348,000 and four fish were observed with copy numbers greater than 100,000.
No clinical signs of infection were noted during necropsy and fish appeared in good health overall. The results indicate coho experienced a significant exposure to *P. minibicornis* at the Iron Gate facility, compared to Chinook juveniles reared at Trinity hatchery. Although Pm POI was higher in TRH Chinook (60%), compared to IGH coho (47%),

the parasite DNA copy number indicates a higher parasite load in coho kidney tissue, likely the result of a higher or longer exposure dose from the water supply at IGH.

At the conclusion of the 6 week study, all surviving NEC fish were tested by QPCR and found negative for *C. shasta* and *P. minibicornis*. Both Chinook and coho were able to resolve initial low level to moderate *P. minibicornis* infections when held for approximately 6 weeks in pathogen-free 18°C water.

Table 4. *C. shasta* (Cs) and *P. minibicornis* (Pm) POI on pre-screening and termination of study for Non-Exposed Control (NEC) groups.

Pre-screening of Experimental Fish (prior to exposure)	**TRH Chinook**	**IGH Coho**
No. Fish acclimated to 18°C at the Ca-Nv FHC wet laboratory (date transferred)	580 (May 6)	555 (May 20)
No. Fish Pre-screened by QPCR (date tested)	30 (May 27)	30 (May 27)
No. Fish/pound	416	280
Median WT (mean, SD)	1.1 (1.1,0.3)	1.6 (1.7,0.4)
Median Fork length (mm)	47.0 (47.3,4.3)	54.0 (53.3, 4.3)
Median KFL (mean, SD)	1.0 (1.0,0.1)	1.1 (1.1, 0.1)
Cs POI	0/30	0/30
Pm POI	**18/30 (60%)**	**14/30 (57%)**
Pm Median Copy Number	80	39,800
Pm Very Low Copy Number (VLCN) Range: (No. positive of total positive)	26-47 (14/18)	NA
Pm Moderate Copy Number (MCN) Range: (No. positive of total positive)	500-142,000 (3/18)	400-348,000 (14/14)
Pm High Copy Number (HCN) (No. positive of total positive)	830,000 (1/18)	NA
Non-Exposed Control Fish		
No. fish in control groups	50	25
CPM (June 8 – July 21)	4% (2/50)	0% (0/25)
Cs POI	**0/48**	**0/25**
Pm POI	**0/48**	**0/25**

<u>Acclimation, Exposure, and post-Exposure Temperature Profile</u>

Pre-exposure temperatures during wet lab acclimation were approximately 5°C lower than in-river mean daily temperatures in May. Temperatures tracked the ascending thermal profile of the Klamath River fairly well during the acclimation period, and prior to the June 9-12 exposure period. During exposure, temperatures of 16.9, 16.8 and 16.6°C measured with Onset® Hobo® data loggers placed in the sentinel live cages, were higher than mean daily river temperatures. However, temperatures in the sentinel cages were in close agreement with maximum daily river temperatures, which is not surprising considering the 3-4 ft depth of water in which cages were placed (Figure 3). Upon return to the wet lab post-exposure, water temperatures were maintained near 18°C (average = 18.5°C, SD± 0.3; temperatures range 17.8 – 19.3°C) throughout the study period. River temperatures continued to climb in late June and early July, as is typically observed in the mid Klamath River reaches, with maximum daily temperatures exceeding 22°C by early July.

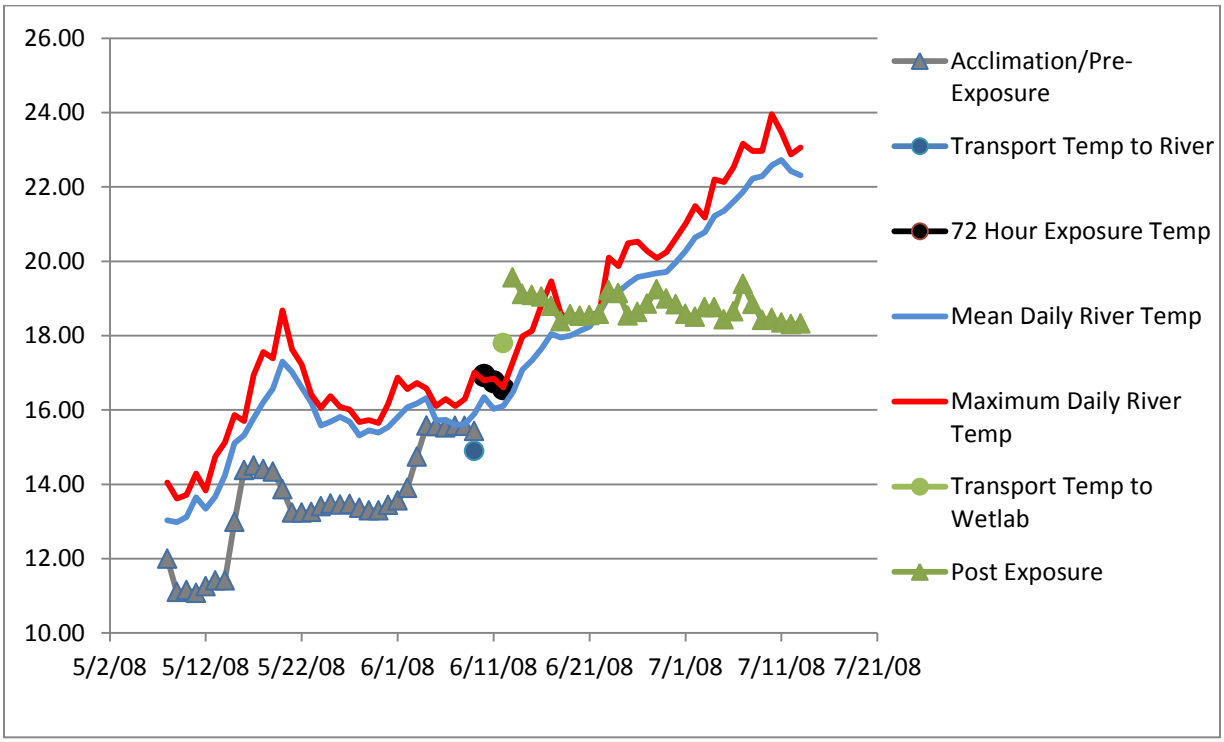

Figure 3. Temperature profiles for: acclimation period, truck transport to and from the exposure site, 72-hour in-river exposure period, and post-exposure period. Mean daily and maximum temperatures in the Klamath River are shown for the same pre and post exposure periods corresponding with fish held at Ca-Nv FHC wet laboratory. (Temperature data from KRIG1, data provided by Anthony Scheif, USFWS, Arcata, CA.)

Exposure Dose

C. shasta actinospore exposure dose was calculated using a standard curve developed by OSU for use in sentinel studies conducted at various locations in the Klamath basin (Hallett et al 2006). Previous studies have shown a spore abundance threshold of 10sproes L^{-1} to cause mortality in relatively resistant Chinook salmon, and 1 spore L^{-1} proves lethal for susceptible non-native rainbow trout routinely used for comparisons in sentinel studies (Bartholomew 2009, Bartholomew 2010). In June 2008, water sampling at Beaver Creek detected an average spore concentration of 147 spores L^{-1} over the 72 hour exposure period. Spore concentration increased daily from 86.1 to 198.7 spore L^{-1} by the third exposure day. Spore concentration for this study was well above the 10 spore L^{-1} threshold (Figure 4).

Composite water sampling was done over an 8-hour period and compared to individual water samples: both methods detected spore concentrations in the range of 61- 82 spores L^{-1} ,which was also similar to the overall 24 hour period sample of 86 spores L^{-1}. These composite and individual water samples were collected during the first 8 hours of day 1 exposure.

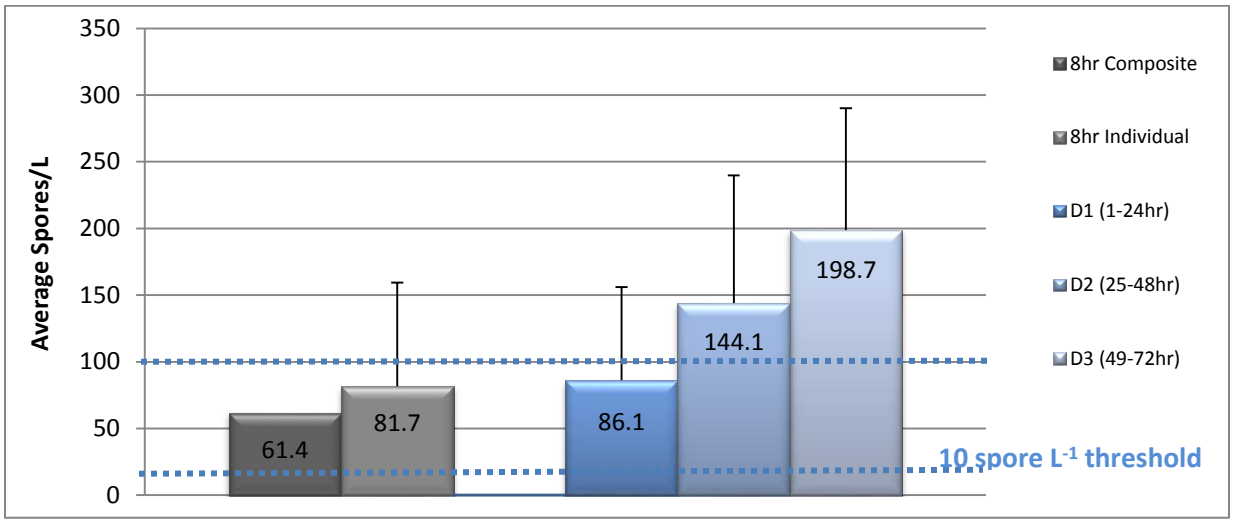

Figure 4. Waterborne exposure dose for *Ceratomyxa shasta* (average spore concentration L^{-1}) during each 24-hour period of the 72-hour exposure period. Eight hour composite sample (2L collected each hour and pooled) and 8-hour 2L individual samples (2L collected each hour and tested individually) were compared (shown in gray). Standard deviation shown with positive bars (the 8-hr composite was a single sample). Dashed blue line represents the 1 spore and 10 spore L^{-1} thresholds which are associated with mortality in susceptible Rainbow trout and resistant in- basin fish species, respectively.

Post Exposure Mortality and Mean Days to Death

Mean Days to Death (MDD) for TRH Chinook was 17.3 in the MC group. Mortality rose sharply between 13-14 dpe, peaked at 15 dpe, and decreased until 19 dpe. The mortality rate after 20 dpe was more chronic in nature with fewer than 5 fish mortalities/day, compared to the peak mortality periods of 20-40 fish per day.

Mortality for the DS group was also graphed to compare MDD in experimental groups that were sampled each day, to the MC group which was allowed to die naturally over the course of the study. It appears that the DS mortality frequency was slightly altered, in overall magnitude, by removal of five random fish each day.

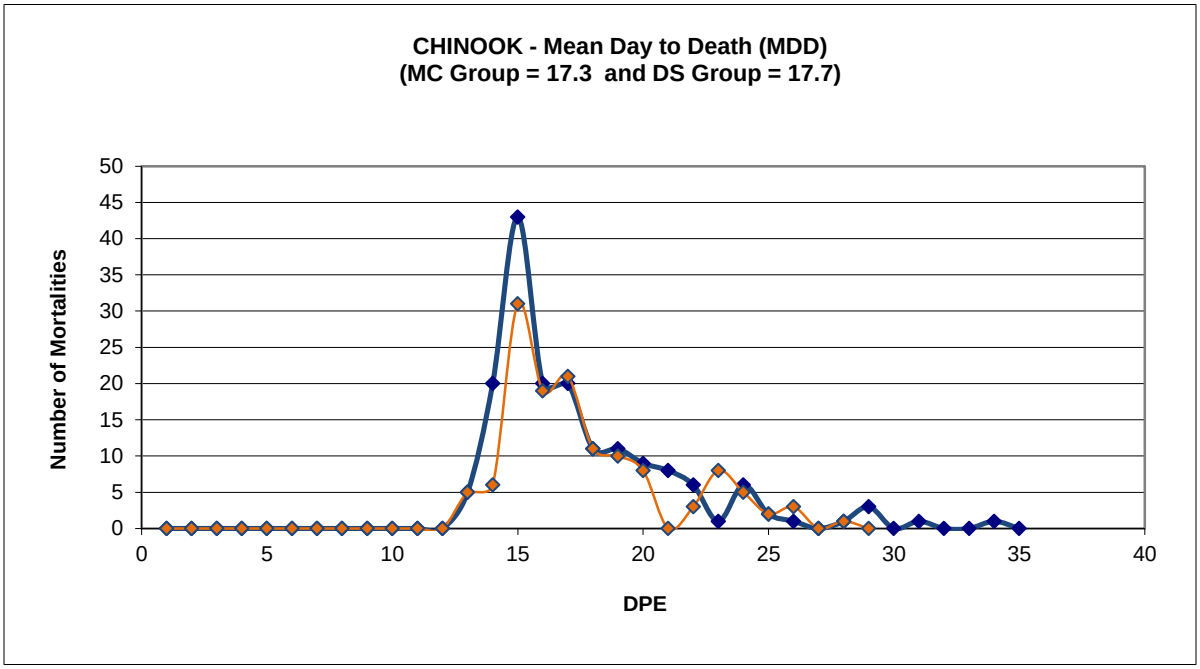

Figure 5. Mean Days to Death (MDD) for TRH Chinook following 72-exposure in the Klamath River, above Beaver Creek. The Mortality Control (MC) group is represented in blue, and Daily Sample (DS) group in orange.

Mean Days to Death (MDD) for IGH coho was 20.6 days in the MC group. The mortality frequency was more protracted for coho than Chinook, in both the delayed onset of 3 days and in the distribution of the mortality data. Mortality did not occur until 15 dpe, peaked at 20 dpe, and decreased more gradually from 21-30 dpe with 10-20 fish mortalities per day. The Daily Sample group was graphed as well and shows a slightly earlier MDD of 19.4 days and an altered mortality curve, both in peak and distribution, compared to the MC group.

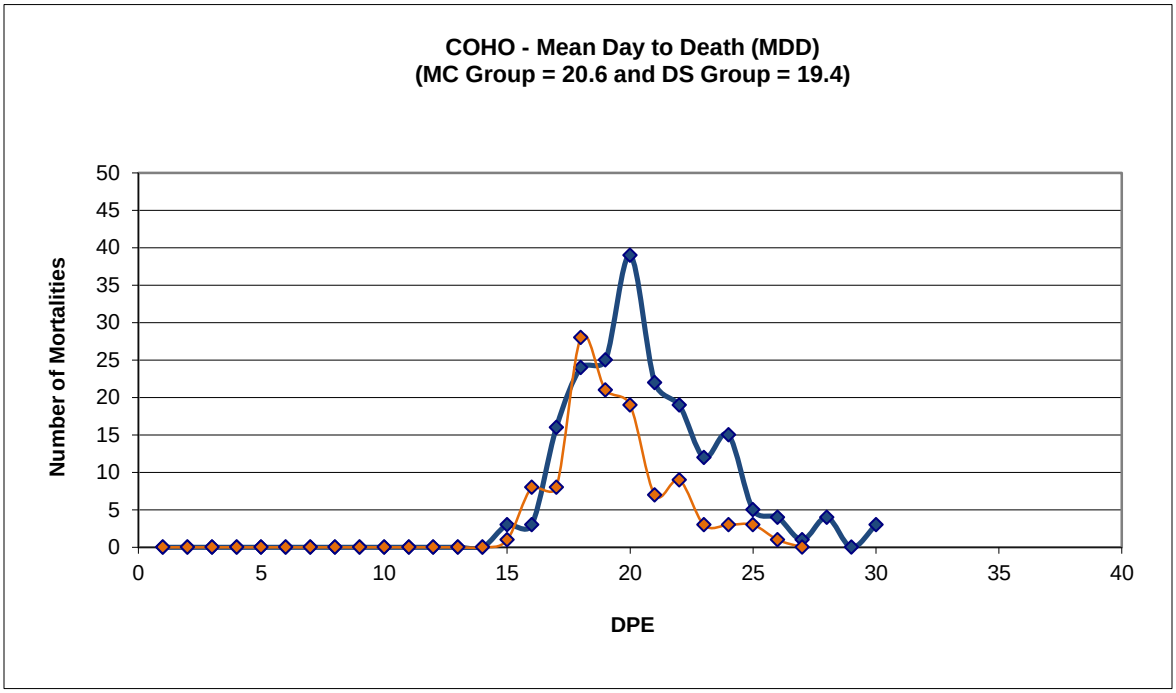

Figure 6. Mean Days to Death (MDD) for IG coho following 72-exposure in the Klamath River, above Beaver Creek. The Mortality Control (MC) group is represented in blue, and Daily Sample (DS) group in orange.

Cumulative Percent Mortality and Parasite Infection Levels by QPCR

Cumulative percent mortality for the TR Chinook Daily Sample group was 87.1% over the 30-day post exposure period (Figure 7). Quantitative parasite levels, expressed as log DNA quantity, were approximately 2 logs when *C. shasta* was first detected at 4 dpe. Parasite levels increased in a stair step manner from 5-17 dpe and peaked from 16-25 dpe when parasite DNA in intestinal tissues reached approximately 8-9 logs. Mean day to death corresponded with this peak event at 17.3 dpe. *C. shasta* DNA levels dropped somewhat the last 2 days (29-30 dpe), most likely as the most heavily infected fish died and were removed from the population.

The CPM mortality curve tracked *C. shasta* parasite levels well, whereas the *P. minibicornis* levels did not correspond to the increased mortality observed. Pm levels started quite high at ~5 logs but remained in the 5-7 log range throughout the 30 days with the exception of 23 dpe, when the highest peak occurred at 7.9 logs.

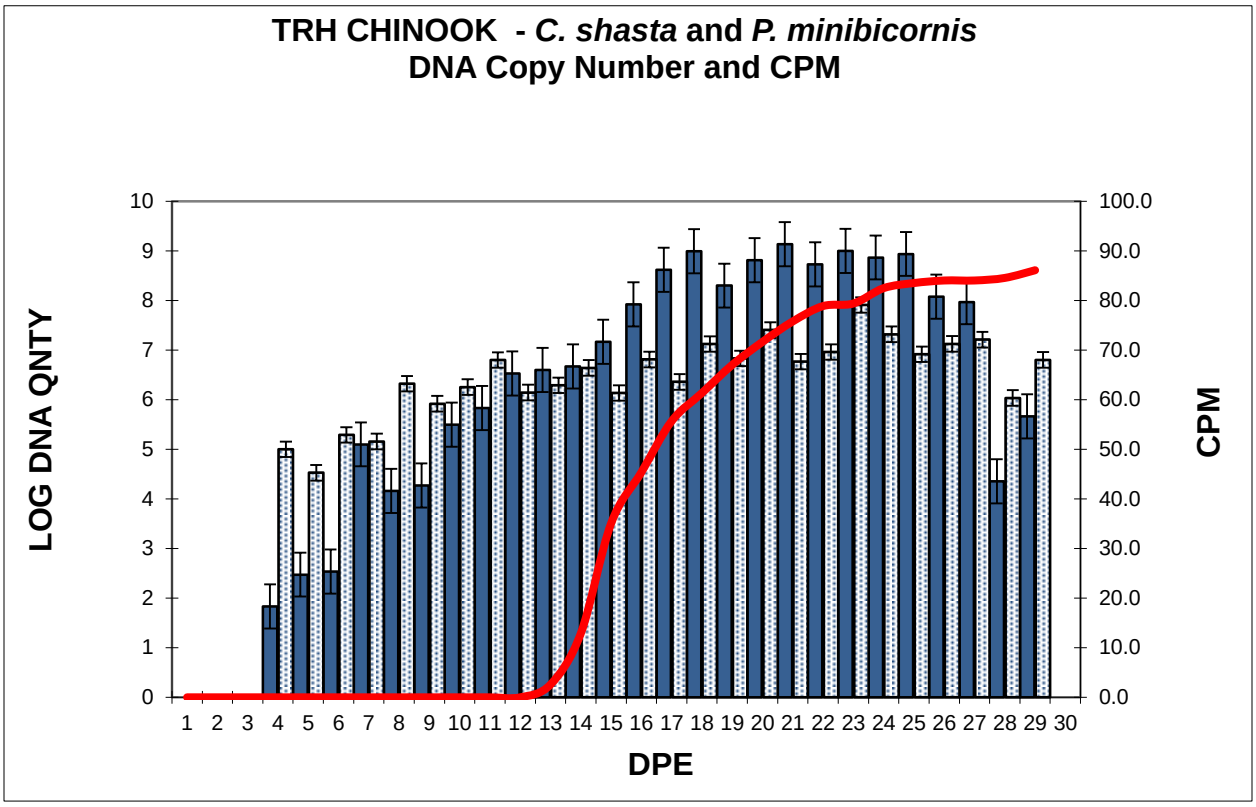

Figure 7. Cumulative Percent Mortality (CPM) (red line) and Log DNA Quantity in TRH Chinook at days post exposure (dpe). Dark blue bars = *C. shasta* in intestine and patterned bars = *P. minibicornis* in kidney tissue.

Cumulative percent mortality for IG coho Daily Sample group was 98.5% over the 27-day post exposure period. *C. shasta* DNA levels were 2 logs when first detected at 4 dpe, similar to levels observed in Chinook at onset of infection. Parasite levels increased rapidly at 6 dpe to 5 logs, then dropped one log before ascending from 10-15 dpe. The peak mortality occurred from 16-24 dpe when *C. shasta* levels in the intestine were over 8 logs and consistently remained between 8-9 logs. The MDD occurred at 20.6 days. Parasite levels dropped off at 27 dpe, most likely due to removal of the heavily infected mortalities from the experimental group.

Parvicapsula minibicornis DNA levels in coho juveniles were >3 logs during infection onset at 4-6 dpe, then rose in a stair-step manner and remained in the range of 5-7 logs throughout the 27 day study period. Pm DNA levels in coho did not track the cumulative percent mortality well, compared to *C. shasta*, and therefore do not suggest significant parasite proliferation in the kidney during peak mortality at 18-22 dpe. This concurs with the Pm POI by QPCR and pathology scores observed by histology in the KRFHMP studies: high *P. minibicornis*: prevalence of infection in out-migrating smolts, but no clear clinical level of infection that corresponds to mortality.

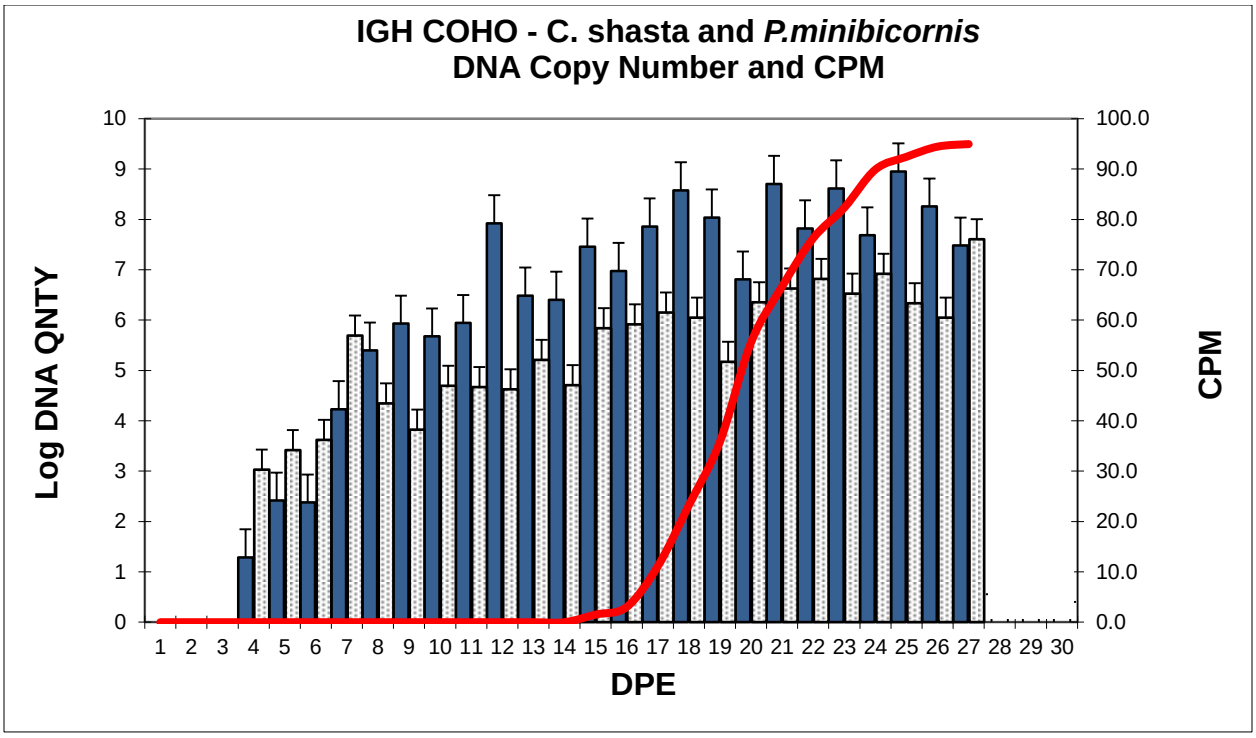

Figure 8. Cumulative Percent Mortality (CPM) (red line) and Log DNA Quantity in IGH coho at days post exposure (dpe). Dark blue bars = *C. shasta* in intestine and patterned bars = *P. minibicornis* in kidney tissue.

C. shasta C_T Values and DNA copy number at Mean Day to Death

C. shasta C_T values and DNA copy number was evaluate for DS Chinook and coho as part of daily QPCR testing (Table 5). Overall, the mean C_T values were relatively similar at the MDD for all 4 groups; values ranged from 11.1 to 21.9, representing approximately a 10.8 CT range for the mean day of death. This range of ~11 C_T values is roughly equivalent to 1000-fold difference in parasite DNA based on the relationship of 3.32 C_T values = 10-fold change in DNA concentration (theoretical for assays that are 100% efficient). Mean Cs copy number was above 100 million (M) in Daily Sample groups at MDD: Chinook were higest at 420M compared to 100M in coho. Mortality Control group mean Cs copy number was much lower: 220,000 in Chinook and 1.3 M in coho. The lower Chinook MC result arose from varied C_T values (infection levels) for the 5 fish tested at MDD. Of the five fish tested, 2 Chinook had C_T values at 18-19, closer to the overall mean for all groups, while the remaining 3 fish had C_T values ranging from 23-25. Additionally, the MDD for the CHK MC group did not track Cs parasite DNA as closely as the other 3 groups. In the CHK MC group (MDD 17.3), the highest parasite levels were observed the 2 days following the MDD: at 18 dpe mean copy number was 4.45M and at 19 dpe peaked at 18M copies. These DNA copy number values suggest the Chinook MC peak in copy number actually occurred 2 days following the MDD for the CHK MC group, compared to the other three.

In addition to CT value and DNA copy number in the Daily Sample groups, a comparison of archived frozen fish from the MC groups were analyzed at MDD to determine if mean C_T values and copy number ranges were similar at MDD for all groups (Table 5). A subset of 5 frozen Chinook carcasses (5 of 20 mortalites that occurred on 17 dpe) and 5 coho carcasses (5 of 39 mortalities on 20 dpe) were tested for *C. shasta* and compared to parasite levels in the cohort DS group at MDD (Table 5).

Table 5 – Summary of *C. shasta* infection levels in all exposed groups at Mean Day to Death (MDD). Mean C_T (SD), median C_T, and mean *C. shasta* copy number and ranges.

	DAILY SAMPLE GROUPS		**MORTALITY CONTROL GROUPS**	
	CHK	**COHO**	**CHK**	**COHO**
Fish sampled (N)	**5**	**5**	**5**	**5**
MDD	**17.7**	**19.4**	**17.3**	**20.6**
CPM	**96.4**	**95.7**	**87.1**	**98.5**
Mean C_T at MDD (SD)	**11.1 (2.1)**	**17.0 (6.4)**	**21.9 (2.7)**	**18.7 (2.0)**
Median C_T at MDD	11.4	17.1	22.7	18.3
Mean *C. shasta* Copy Number	4.2E8	1.0E8	2.2E5	1.3E6
Copy Number Range (MIN – MAX)	2.0E7– 5.0E8	9.0E3 – 2.8E8	5.2E3 – 7.2E5	1.3E5 – 3.0E6

QPCR Testing of Study Survivors

We were interested in the parasite level in fish that did not succumb to ceratomyxosis by the end of the study period (Table 6). The last coho and Chinook from DS groups were sampled on 27 dpe (n=2) and 29 dpe (N=8), respectively. These remaining fish, termed survivors were the last fish sampled and therefore were included with the QPCR and DNA copy number data each species group.

For the MC groups, the study was terminated on 31 dpe for coho (N=4 remaining fish) and 35 dpe for Chinook (N=22). All remaining fish from the MC group were tested by QPCR and included in the CPM data for each species group.

Interestingly, none of the surviving Chinook from either the DS or MC had detectable levels of *C. shasta* DNA present at 29-35 dpe respectively. Coho from the DS group were also negative for Cs while 2/4 (50%) of the MC coho were positive at 31 dpe (mean C_T value of 22.9).

However, all remaining fish tested 100% infected with *P. minibicornis* with moderate to high levels of parasite DNA present in the kidney. However the infection levels for the groups had not change significantly from early post exposure days to the termination of the study. This finding of similar Pm DNA levels in fish throughout the study suggests that *P. minibicornis* is not driving the overall clinical presentation of disease observed in the fish, or the cumulative percent mortality observed for the Chinook and coho MC groups.

Table 6 – Summary of infection levels in "surviving" fish (29-35 dpe) for all groups: date and dpe each group was terminated, number of survivors, prevalence of infection, mean CT value and DNA copy range for each myxozoan parasite detected.

	DAILY SAMPLE GROUPS		MORTALITY CONTROL GROUPS		NON-EXPOSED CONTROL GROUPS	
	CHK	**COHO**	**CHK**	**COHO**	**CHK**	**COHO**
Group Termination Date	8 July	6 July	14 July	10 July	14 July	14 July
DPE	29	27	35	31	35	35
No. Fish Survivors	**8**	**1**	**22**	**4**	**48**[a]	**25**
Cs Positive (POI)	**0/8**	**0/1**	**0/22**	**2/4 (50%)**	**0/48**	**0/25**
Mean C_T Value	NA	NA	NA	**22.9**	NA	NA
C_T Range	NA	NA	NA	**21.7 – 24.1**	NA	NA
DNA Copy Number Range	NA	NA	NA	**1.8E5 -1.2E6**	NA	NA
Pm Positive (POI)	**8/8 (100%)**	**1/1 (100%)**	**22/22 (100%)**	**4/4 (100%)**	**0/48**	**0/25**
Mean C_T Value	**23.1**	**20.2**	**23.6**	**24.2**	NA	NA
C_T Range	**27.9 - 20.6**	NA	**25.2 - 19.7**	**21.6-25.2**	NA	NA
DNA Copy Number Range	2.2E4 – 2.1E6	2.7E6	1.2E5– 3.7E6	2.3E4- 9.6E5		

[a] **Two mortalities occurred in the CHK-NEC Group (2/50 = 4% CPM), which were assayed by QPCR at the time of death and tested negative for both myxozoan parasites.**

QPCR Testing of Non-Exposed Control Groups

No mortality occurred in the coho NEC, and 2 mortalities occurred in the Chinook group. NEC groups were tested at the conclusion of the study and found negative for both parasites. Note that the pre-screen QPCR had detected Pm in 60% of Chinook and 57% of coho, and the majority of fish had low to moderate copy numbers with the exception of one Chinook with high parasite levels (Table 4). By the end of the study, Pm could not be detected in the NEC groups. This finding that fish apparently can resolve low to moderate levels of Pm by 29-35 days post exposure is also indicative of a clinical picture where Pm is not the primary driver of

disease in Klamath River stocks. This is not to say that Pm infection and mortality is not occurring. We occasionally examine juvenile Chinook in the KRFHMP that are positive for Pm and negative for *C. shasta* by histology, and present with clinical disease due to *P. minibicornis* (severe glomerulonephritis) or systemic infections. But these are singular *P. minibicornis* infections are rare and the majority of juvenile Chinook succumb to ceratomyxosis well in advance of *P. minibicornis* advancing to a clinical disease state.

Survival Proportions for the Mortality Control Groups

The estimated Kaplan-Meier survivor function for Chinook MC group is displayed in Figure 9. The survivor function (Collett 2003) displays the estimated probability that an individual fish survives (y-axis) for a time greater than or equal to days post exposure (x-axis). There are several characteristics of this survival function worth noting. The first, is the rather prolonged delay in observed *C. shasta* mortality post exposure. The second, is the sharp decrease in the survivor function soon after the initial mortalities were observed. And third, the rather flat shape of the survivor function at the later end of the study with 22 Chinook salmon surviving the duration of the 35 day post-exposure study period.

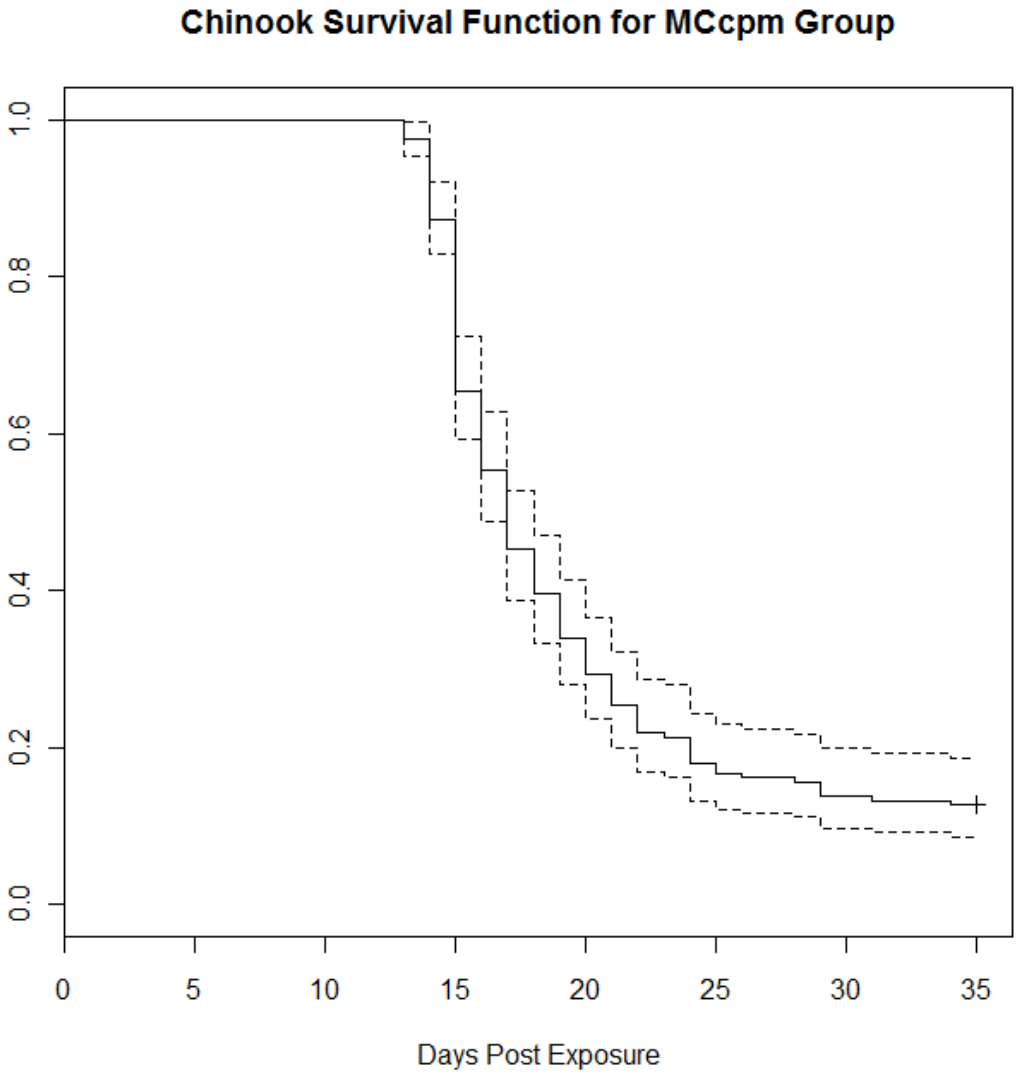

Figure 9. Kaplan-Meier survival function for Chinook Mortality Control (MC) group. Dashed lines represent 95% confidence intervals of the probability of survival to or beyond the associated number of days post exposure. The + at the end of the estimated survival curve indicates that 22 Chinook were still alive at the completion of the study.

The estimated Kaplan-Meier survivor function for coho juvenile salmon is displayed in Figure 10. The coho survival function reveals a longer time to initial mortality compared to the Chinook survivor function. The later time to initial mortalities is contrasted by a steeper decline in survival probabilities throughout a longer period of the study, and only 4 coho remaining alive at the completion of the study. The estimated number of days post exposure until 50% mortality can be expected is 20 dpe (95% CI: 19-21 dpe), and the estimated number of days post exposure until 75% mortality can be expected is 22 dpe (95% CI: 21-23 dpe)

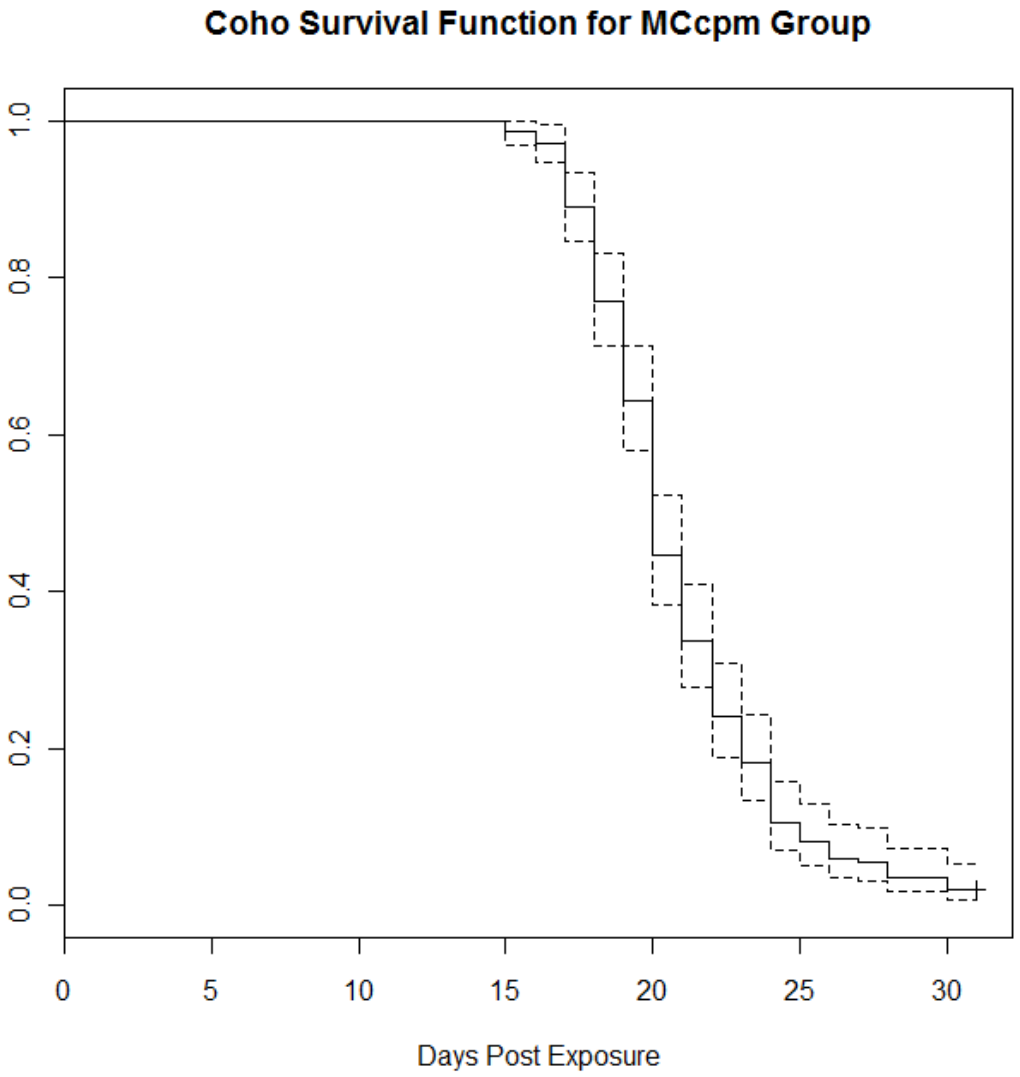

Figure 10. Kaplan-Meier survival function for the coho Mortality Control (MC) group. Dashed lines represent 95% confidence intervals of the probability of survival to or beyond the associated number of days post exposure. The + at the end of the estimated survival curve indicates that 4 coho were still alive at the completion of the study.

Histology - TRH Chinook

Eighty four specimens, collected between 4 and 29 dpe, were evaluated for infection and disease. The first *C. shasta* trophozoite was observed within the intestinal epithelium at 7 dpe (Table 7).

In the present study, intestine was not collected for histological assessments between 5 and 6 dpe and it is likely that early infections were missed by histology. Similar to Bjork and Bartholomew (2010), we have observed trophozoites within blood vessels of gill in salmon collected from the Klamath River. The parasite is not seen within the intestine of these presumptive early stage infections. An inflammatory response was initially observed in the lamina propria followed at 11 dpe by epithelial necrosis and sloughing (Bartholomew et al. 1989). A systemic *C. shasta* infection was indicated by the presence of trophozoites in 27% of the kidney sections beginning at 14 dpe. Severe ceratomyxosis ($\geq$ 50% of section with erosion, hemorrhage, or necrosis) was associated with an increase in trophozoite numbers (CS2 rating) and occurred in 39/84 (46%) of the Chinook salmon examined (Table 7 and Figure 11). Disease and parasite scores were similar between large and small intestine sections. Myxospores were first seen in the necrotic muscularis layer from specimens collected on 16 dpe with an overall incidence of 19% (16 of 84 specimens). Sporogenesis continued throughout the remainder of the study although the overall myxospore numbers were few, and less in Chinook than observed for coho (Table 7). Thirteen salmon (15%) sampled after 11 dpe were deemed “recovered or uninfected” as no *C. shasta* life stages or intestinal pathology was observed in their sections.

Myxospore production in dead fish (N=2) was assessed by hemacytometer counts in fish at time zero (euthanization of moribund fish) and at 72 hours of holding tissues at 18°C. At time zero, a few sporogonic stages but no myxospores were seen in the smears. The 72 hr decomposed samples contained 1.1 and 1.8 million myxospores, indicating rapid development within intestinal tissue of dead fish held at 18°C.

Table 7- Summary of key histological findings for *Ceratomyxa shasta* and *Parvicapsula minibicornis* in DS Chinook and coho groups, examined from 4-29 days post exposure (dpe). Ceratomyxosis (disease) was characterized as when >50% of the intestine section contained eroded epithelium, hemorrhage, or necrosis (>50% EHN).

Ceratomyxa shasta	**TRH Chinook**	**IGH Coho**
1st trophozoites observed	7 dpe	7 dpe
1st myxospores observed	16 dpe	15 dpe
Incidence of infection (all stages)	**64/84 (76%)**	**62/71 (87%)**
Incidence of myxospores	**16/84 (19.0%)**	**25/74 (33.8%)**
Disease incidence (>50% EHN)	**39/84 (46%)**	**51/71 (72%)**
Parvicapsula minibicornis		
1st trophozoites observed	8 dpe	7 dpe
1st myxospores observed	23 dpe	21 dpe
Incidence of infection (all stages)	**77/87 (89%)**	**63/74 (85%)**
Incidence of myxospores	**1/87 (1%)**	**6/74 (8%)**

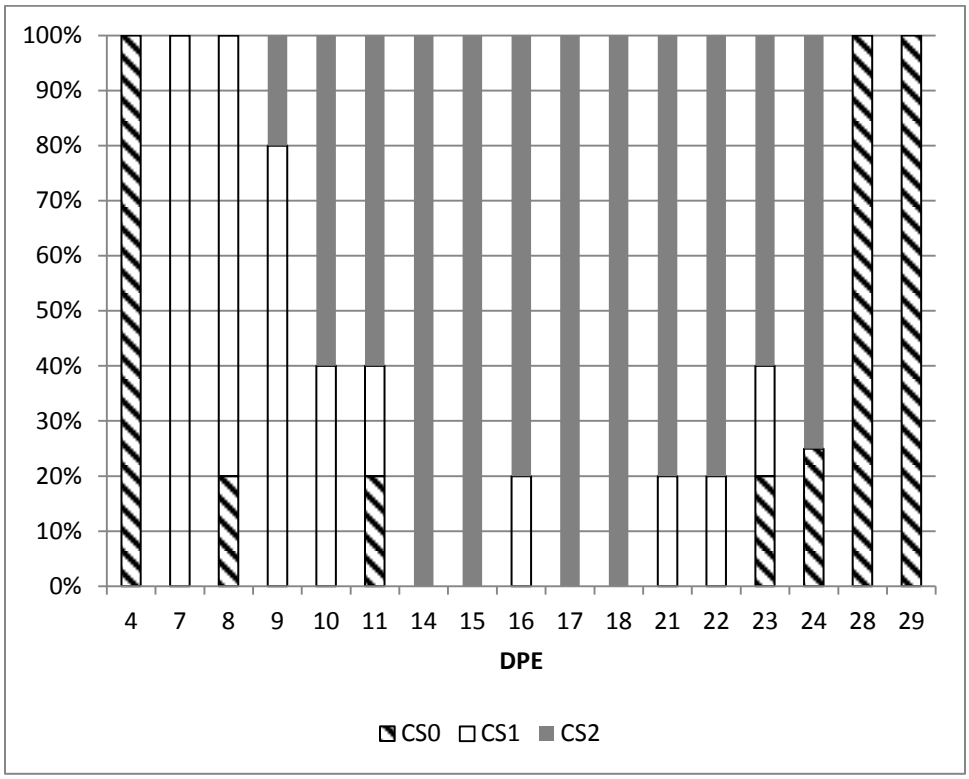

Figure 11- Frequency of C*. shasta* parasite quantity (CS0 =no parasite detected, CS1 = 1-10 trophozoites, CS2 >10 trophozoites) observed in histological specimens of Chinook salmon sampled over 29 days post exposure.

Parvicapsula minibicornis trophozoites were first observed in glomeruli at 8 dpe, increased in number with high trophozoite scores (Pm#3) beginning at 15 dpe, and moved into the tubules by 14 dpe (Table 7). Myxospores, within the kidney tubules, were seen in only 1 fish (23 dpe). Both *P. minibicornis* trophozoite number and kidney disease rating increased together beginning at 8 dpe through 23 dpe (Figure 12). Interstitial hyperplasia increased during the same period as shown by the steady decline in tubule/ μm^2 (Figure 12). Tubule density between 4 and 23 dpe was significantly different (T-test, t= 5.82, 9 d.f., P< 0.01) and occurred as early as 8 dpe (P< 0.029). Similarly, Bowman capsule space (surface area) increased between 8 and 18 dpe, declined sharply at 21 dpe (no measurements at 19 or 20 dpe) and then remained at the 15 – 18 dpe size through 23 dpe (Figure 13). The surface area of the Bowman capsule at 4 dpe was significantly smaller than at 23 dpe (T-test, t=-9.56, 9 d.f., P<0.001). Initially the dilated capsule was not filled with proliferating mesangium but presumptively due to fluid pressure. By 14 dpe, glomerular space was filled by inflammatory cells and later by periglomerular fibrosis. Lumsden et al. (2008) describe a presumptive type III hypersensitivity of membranous glomerulonephritis in Chinook salmon that was associated with chronic inflammatory disease. The authors describe immunoglobulin being observed in the thicken basement membranes of the glomerulus. It is likely that the systemic inflammatory response, to both *C. shasta* and *P. minibicornis,* could invoke similar type III hypersensitivity.

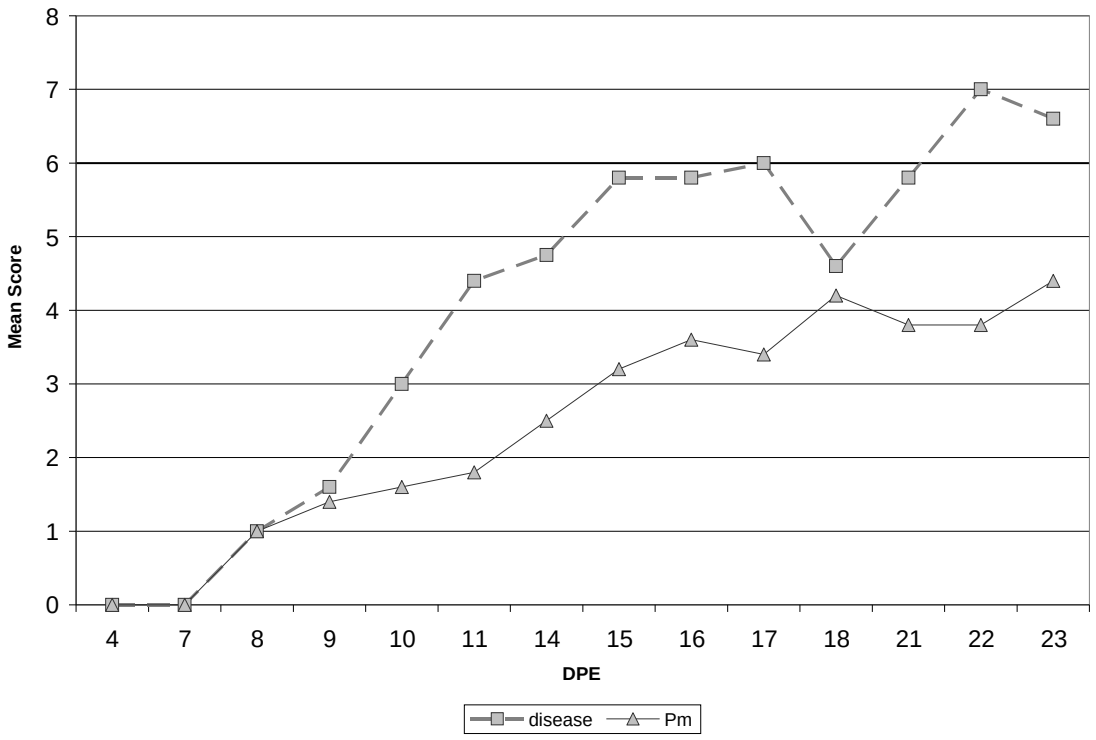

Figure 12. Mean kidney disease and P. *minibicornis* trophozoite (Pm) score of Chinook salmon kidney sections for a given dpe.

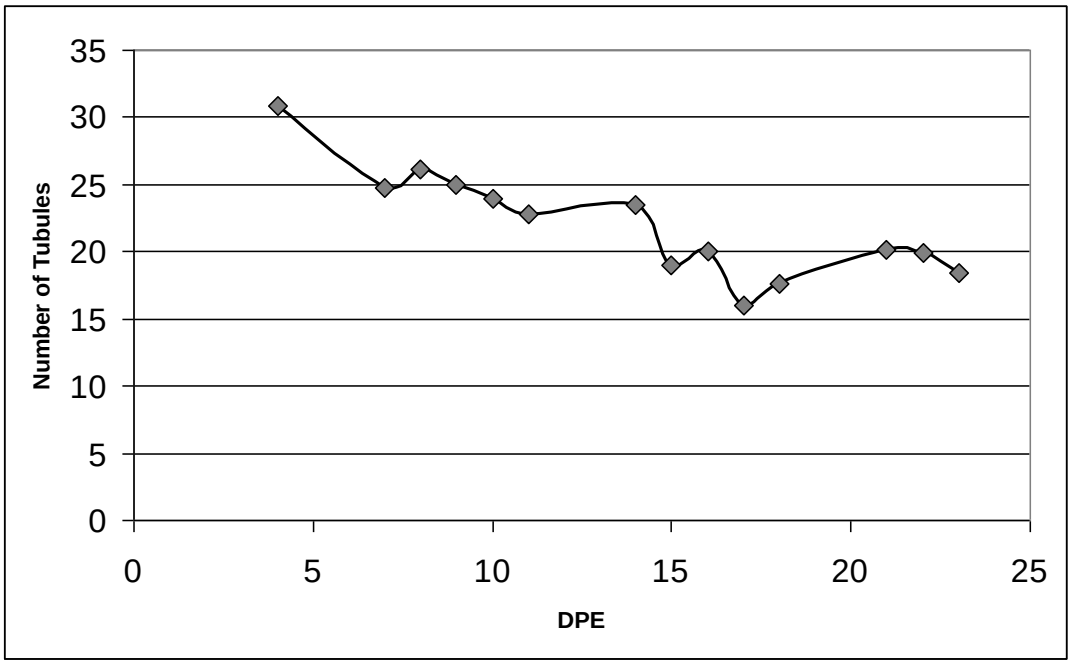

Figure 13. Mean number of tubules / μm^2 in Chinook kidney sections at 4 – 23 dpe.

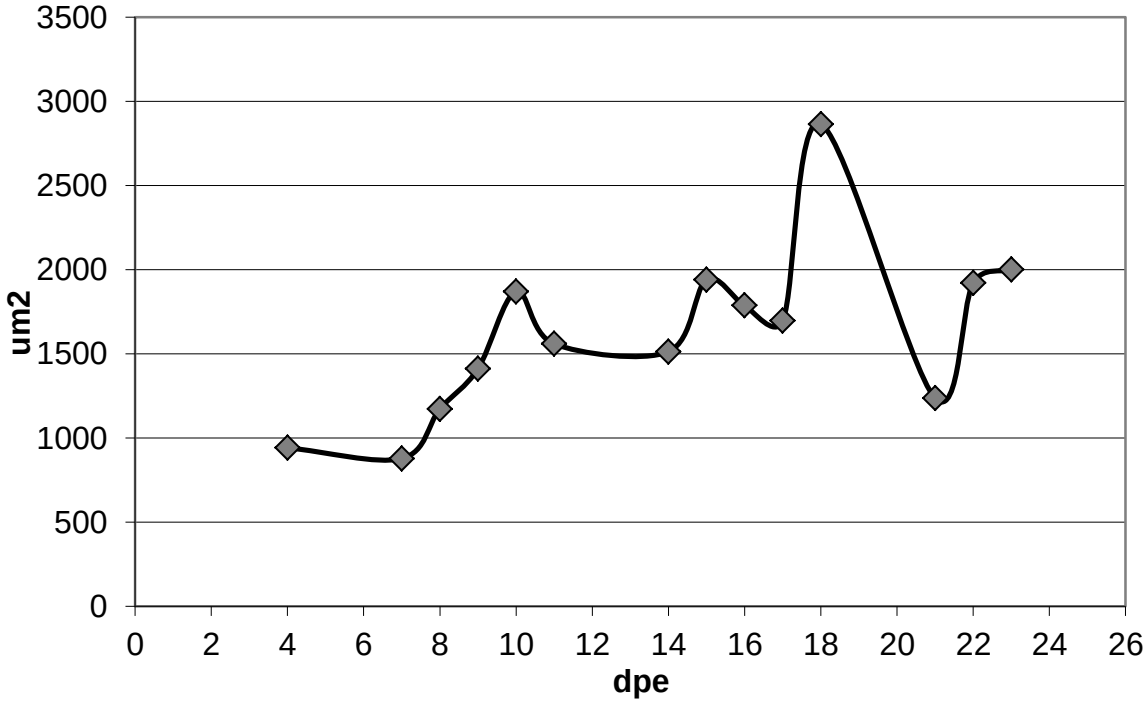

Figure 14. Mean surface area (μm^2) of Bowman's capsule in Chinook kidneys sampled from 4 to 23 dpe.

Histology - IGH Coho

Seventy-one specimens, collected between 4 and 24 dpe, were evaluated for infection and disease. Similar to Chinook, the first observation of *C. shasta* trophozoites within the intestine was at 7dpe with severe ceratomyxosis (>50% necrosis) occurring by 10 dpe. The incidence of severe ceratomyxosis was 68% (48 of 71). Myxospores were observed after 15 dpe and corresponded to when the muscularis layers became necrotic (Table7, Fig 14). Twice as many lower intestine sections contained myxospores as small intestine sections. Only 2 fish, sampled after 7 dpe, were deemed "recovered / uninfected".

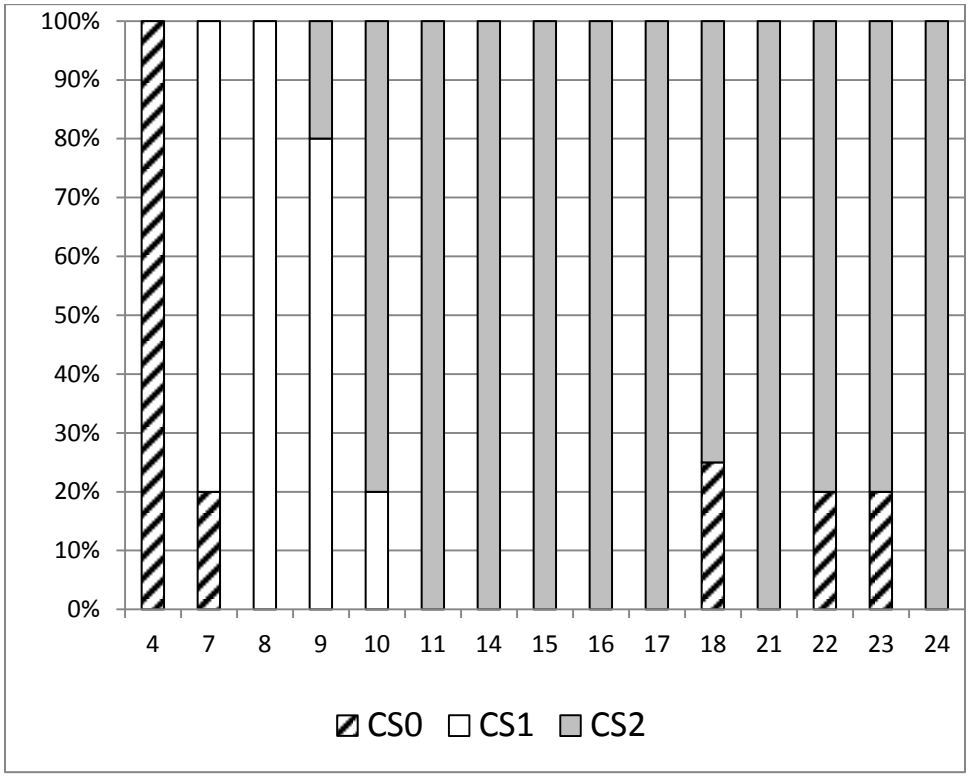

Figure 15. Frequency of *C. shasta* parasite quantity (CS0 = none, CS1 = 1- 10 trophozoites, CS2= > 10 trophozoites) observed in histological specimens of coho salmon sample over 24 days post-exposure.

P. minibicornis trophozoites were first seen in the glomeruli of 7 dpe coho and later in their tubule lumens at 14 dpe (Table 7). Myxospores were seen in 4 fish collected between 21 and 23 dpe. Interstitial hyperplasia and dilated Bowman's capsule was first seen in 8 dpe fish and was a common response to the infection throughout the remaining 15 days of the study. Unlike Chinook, coho did not develop severe glomerulonephritis as only 3 of 69 fish showed moderate lesions.

DISCUSSION

This study characterized disease dynamics of two myxozoan parasites in Klamath Basin juvenile Chinook and coho, following 72 hr in-river exposure under conditions (dose, temperature) that at the time of this study were not uncommon during the juvenile salmon outmigration period of June-July. Subsequent annual monitoring from 2009-2012 has demonstrated that 2008 was actually one of the highest *C. shasta* prevalence of infection years (Table 2, 2012 data not shown).

Fish were exposed at Beaver Creek, a site below Iron Gate Dam and within the infectious zone of the Klamath River. Over 800 juvenile Chinook and coho tissues were assessed by QPCR and to our knowledge this is the first study to utilize this molecular tool to describe in fine resolution the daily quantity of parasite load in target fish tissues over 29-35 days post exposure. We utilized complimentary histological assessments to determine the extent of tissue damage, and progression to a clinical "disease state": a point at which recovery is not expected. Histologically, we also describe the onset of *C. shasta* myxospore production in juvenile salmonids during clinical disease, as well as in decomposed fish tissue. Lastly, we utilized the expertise of Dr. Nicholas Som, statistician for USFWS – Arcata Fish and Wildlife Office, to develop survival curves and probabilities for survival for Chinook and coho salmon, under the exposure conditions and temperatures in this study. On-going work in parametric modeling of disease dynamics in the Klamath Basin is proceeding and data from this study and the Klamath Fish Health Monitoring Program will be utilized with the goal of fully elucidating the biological and environmental conditions that lead to myxozoan disease in Klamath Basin salmonid populations.

Ceratomyxa shasta exposure dose in fish was determined by water sampling utilizing the methods of Hallett and Bartholomew (2006). In early June 2008, water sampling at Beaver Creek detected an average spore concentration of 147 spores L^{-1} over the 72 hour exposure period. Spore concentration increased daily from 86.1 to 198.7 spores L^{-1} by the third exposure day. These finding concur with similar water sampling conducted by Oregon State University at this site in mid- June, where spore concentrations were found to be over 100 spores L^{-1} (Bartholomew 2009). Spore concentration for this study was well above the 10 spore L^{-1} threshold associated with high mortality observed in sentinel studies.

Disease dynamics were further characterized by cumulative percent mortality, mean day to death, prevalence of infection by each parasite, and finally by C_T value and DNA copy number detected in target tissues of each fish species. Both CPM (87.1%) and MDD (17.3) were lower for TR Chinook compared to IG coho (CPM of 98.5% and MDD of 20.3) For Trinity Hatchery Chinook, mortality rose sharply between 13-14 dpe, peaked at 15 dpe, and decreased until 19 dpe. The mortality rate after 20 dpe was more chronic in nature with fewer than 5 fish mortalities/day, compared to the peak mortality periods of 20-40 fish per day. Quantitative parasite levels, expressed as log DNA quantity, were approximately 2 logs when *C. shasta* was first detected at 4 dpe. Parasite levels increased in a stair step manner from 5-17 dpe and peaked from 16-25 dpe when parasite DNA in intestinal tissues reached approximately 8-9 logs. Mean day to death corresponded with this peak parasite load at

17 dpe. The CPM mortality curve tracked *C. shasta* parasite levels well, supporting field observations that this parasite primarily drives the clinical disease picture as well as the mortality observed in these Klamath basin juvenile salmon.

For Iron Gate Hatchery coho, mortality frequency was more protracted: both the delayed onset of 3.3 days and in the distribution of the mortality data. Mortality did not occur until 15 dpe, peaked at 20 dpe, and decreased more gradually from 21-30 dpe with 10-20 fish mortalities per day. The observed 3-day delay in onset of mortality in coho, and associated clinical disease signs, was observed in aspecies susceptibility study previously conducted by our laboratory (Stone et al. 2008) . In this study, *Ceratomyxa shasta* DNA levels were 2 logs when first detected at 4 dpe, similar to levels observed in Chinook at onset of infection. Parasite levels increased rapidly at 6 dpe to 5 logs, then continued ascending from 10-15 dpe. The peak mortality occurred from 16-24 dpe when *C. shasta* levels in the intestine consistently ranged between 8-9 logs. This peak corresponded with the 20.6 MDD for juvenile coho.

For *Parvicapsula minibicornis*, the CPM mortality curve did not track the parasite DNA copy number well for TRH Chinook or IGH coho salmon. In Chinook, Pm levels started high at ~ 5 logs at 4 dpe, but levels remained in the 5-7 log range throughout the 30 days post exposure, with the exception of 23 dpe, when the highest peak occurred at 7-9 logs. *Parvicapsula minibicornis* DNA levels in coho juveniles were >3 logs during infection onset at 4-6 dpe, then rose in a stair-step manner and remained in the range of 5-7 logs throughout the 27 day study period. Pm DNA levels in coho did not track the cumulative percent mortality well, compared to *C. shasta*, and therefore do not suggest significant parasite proliferation in the kidney during the peak mortality observed at 18-22 dpe. This concurs with the Pm POI by QPCR and pathology scores observed by histology in the KRFHMP studies: high *P. minibicornis*: prevalence of infection in out-migrating smolts, but no clear clinical level of infection that corresponds to mortality. Rarely, we observe single infections with *P. minibicornis* causing severe glomerulonephritis and mortality (True et al. 2011), however this is generally the exception for this parasite.

In summary, *C. shasta* parasite DNA copy number in intestinal tissue is similar for Chinook and coho at onset of ceratomyxosis (2-3 logs, 2-4 dpe), and during peak mortality: Chinook have a mean of 8.1 logs of parasite DNA present in intestinal tissues, compared to coho at 7.7 logs (15-19 dpe). The highest levels of *C. shasta* DNA occur from 18-25 dpe with mean DNA copy number for Chinook at 8.7 logs (SD 0.49) and 8.0 logs (SD 0.54) for coho. The most notable difference between species occurs in coho, where DNA levels in the intestine fluctuate by >1 log over a 1-3 day period. Chinook levels consistently increase in pattern. This could reflect a physiological difference in how coho react to, or briefly contain parasite proliferation in the intestine. There appears to be a pause in proliferation for short periods of time, then increasing numbers followed by another pause. This could explain the 3-day delay in mortality we observe for coho. However once a parasite number/threshold is reached, significant mortality (98.5%) is observed in coho juveniles, despite the brief delay in onset of disease.

Histologically, we observed the first *C. shasta* trophozoites within the intestinal epithelium at 7 dpe. A systemic *C. shasta* infection was indicated in 27% of the kidney sections beginning at 14 dpe. Severe ceratomyxosis was associated with an increase in trophozoite numbers and

occurred in 90/155 (58%) of the study fish. Additionally, we assessed the development of myxospores in juvenile fish by histology and in decomposed tissue from clinical/diseased fish. By histology, we observed the onset of myxospore production at 16 dpe in both species, and enumerated the prevalence of myxospore production by species. We found 19% of juvenile Chinook and 34% of juvenile coho produced myxospores under the conditions of this study: 72 hour challenge of >100 spores/L, at 18°C exposure and holding temperatures. Sporogenesis continued throughout the remainder of the study although the overall myxospore numbers were few, and less in Chinook than observed for coho.

The estimated Kaplan-Meier survivor function for Chinook MC group (Figure 9) is characterized by a prolonged delay in initial mortality, a sharp decrease in survival once mortality occurs, and a flat shape at the end of the study attributable to Chinook survivors (N=22). The estimated number of days post exposure until 50% mortality can be expected is 17 dpe (95% CI: 16-18 dpe). The estimated number of days post exposure until 75% mortality can be expected is 22 dpe (95% CI: 21-23 dpe).

The estimated Kaplan-Meier survivor function for coho MC group (Figure 10) reveals a longer time to initial mortality compared to the Chinook survivor function. The later time to initial mortalities is contrasted by a steeper decline in survival probabilities throughout a longer period of the study, and only 4 coho remaining alive at the completion of the study. The estimated number of days post exposure until 50% mortality can be expected is 20 dpe (95% CI: 19-21 dpe), and the estimated number of days post exposure until 75% mortality can be expected is 22 dpe (95% CI: 21-23 dpe). Coho appear to resist infection challenge for a short period of time, compared to Chinook juveniles exposed to the same parasite dose. Once the disease susceptibility “threshold” is overcome, by either direct proliferation of *C. shasta* trophozoites, or their inflammatory and necrotic effects on intestinal tissue, the clinical picture and mortality rates for coho change rapidly. This is indicated by the sharp drop in survival proportions at 19-21dpe that coincide with the MDD of 21 dpe.

Interestingly, none of the surviving Chinook from either the DS or MC had detectable levels of *C. shasta* DNA present at 29-35 dpe respectively. It is difficult to deduce if these survivors simply were not infected during the 72 hour exposure period, were able to resolve *C. shasta* infections during the ~30 days post exposure, or were simply innately more resistant to infection and development of ceratomyxosis than their cohorts. The 22 surviving Chinook juveniles could indicate the population may contain a subset of fish more resistant to the effects of the *C. shasta* parasite. This conclusion is supported histologically as well, as 13 Chinook (15% of fish examined after 11dpe) were deemed “recovered or uninfected” as no *C. shasta* life stages or intestinal pathology was observed in tissue sections.

Coho survivors (N=2) from the DS group were also negative for *C. shasta* by QPCR, while 2/4 (50%) of the MC coho were positive at 31 dpe (mean C_T value of 22.9). It would have been interesting to test serum from the survivors of both species, for the presence of *C. shasta* antibodies, to determine if titers suggested no exposure or resolution of infection after exposure. Unfortunately, we are not aware of a source for *C. shasta* antisera that could have been employed for this purpose.

All surviving fish tested 100% positive for *P. minibicornis*, with moderate to high levels of parasite DNA present in the kidney. However DNA log copy number did not change significantly from early post exposure to the termination of the study. Histologically, parasite numbers and tissue response was relatively stable. *P. minibicornis* POI did not appear to be driving the overall clinical presentation of disease observed in the juvenile fish. That is not to say that *P. minibicornis* cannot lead to clinical disease, as we have observed juvenile Chinook in the KRFHMP that are only infected with *P. minibicornis* and develop full blown glomerulonephritis leading to mortality (True et al. 2011). The role of synergistic infections may warrant additional study, however it is difficult logistically to expose fish to only *P. minibicornis*, due to the high prevalence of dual infections in juvenile fish in this river system.

The most important finding of this study, in addition to quantifying parasite levels and survival probabilities, is the histological observation of myxospore production in 19% of Chinook and 34% of juvenile coho examined. This rate of myxospore production may have been even higher than observed, if histology samples had been collected on all days post exposure, particularly during the later stages of infection >16 dpe (histology was not collected on weekends corresponding to 5-6, 12-13, 19-20 and 25-26 dpe). In decomposed fish tissue (N=2) 1.1 and 1.8 million myxospores were observed, indicating rapid development within dead fish intestinal tissue held at 18°C.

For many years, adult contribution has been thought to be the single source of myxospore production and input into the Klamath River basin (Stocking and Bartholomew 2007, Hedrickson et al. 1989). This study demonstrates that juvenile Chinook and coho can contribute myxospores, and their input may be significant under environmental conditions typical for the Klamath River in some years: high exposure dose, high river temperatures, juvenile survival to ~ 16 dpe, and mortality during outmigration. Considering the sheer size of the ~5M Fall Chinook salmon program at IGH, where all fish are released in late May to early June, myxospore contribution could be quite significant. For example, if the myxospore production rate of 20% (observed in this study) occurred for a release group of 4-5 million juvenile fish, and we conservatively assumed each fish only contributed 1 myxospore, the resulting input range would be 800,000- 1,500,000 myxospores. If each fish contributed 10 myxospores, the numbers become quite large (8-15M). And if numbers of myxospores detected in the 2 decomposed juvenile Chinook were common, the myxospore input would be extremely high (billions). Additionally, the myxospore deposition by juvenile mortality would no longer be confined to the river reach below Iron Gate Dam. Distribution would be based on when mortality occurred and would be distributed further downstream: likely in the reaches above the Trinity River confluence (based on typical juvenile Chinook salmon migration rates of 2-4 weeks from release to the estuary). Of course, polychaete populations would need to be present in lower reaches, and their abundance would have to be a significant factor in how successful juvenile contribution was in completing the parasites life cycle. Predation on infected, lethargic juvenile fish may also occur prior to mortality due to ceratomyxosis. If predation levels are high in the upper reaches, this would likely limit juvenile myxospore contribution as well as the area of direct deposition of juvenile carcasses.

In this study, juvenile fish experienced typical May and early June river temperatures in both the controlled wet lab environment and during the 72-hour exposure period. Post exposure, fish were maintained at, or near 18°C and still incurred high cumulative mortality rates for both Chinook (87.1%) and coho (98.5%). Higher in-river temperatures, as well as stressors associated with emigration and predator avoidance, would likely result in even higher rates of mortality in 2008, than observed in this temperature controlled study. OSU sentinel studies conducted June 17-21, 2008 demonstrated high *C. shasta* mortality rates for IGH Chinook (92.1%) and coho (84.2%) held for 72 hours at the same Beaver Creek exposure site. In 2008, OSU sentinel fish were placed in 4 temperature profiles post exposure (13,15,18 and 21°C) to evaluate MDD at various holding temperatures. Mean Days to Death decreased for each increasing temperature profile and IGH Chinook and coho suffered high losses at all temperatures. At 18°C specifically, MDD for IG Chinook was 19.3 and 25.2 for IG coho. The study concluded that irrespective of fish species exposed, the higher the holding temperature post exposure, the greater the percent loss of fish with *C. shasta* infection (Bartholomew 2009).

Conversely favorable environmental factors, outside the dose and temperature conditions that occurred in this study, would be expected to improve survival: early or rapid juvenile migration (reduced exposure time), reduced temperatures at release (slower polychaete development, reduced or delayed release of infectious actinospores, and delayed parasite proliferation in juvenile fish). These conditions were present in 2010 and 2011 fish health monitoring studies (Table 2) and decreased *C. shasta* prevalence of infection and clinical disease was observed for out-migrating Chinook salmon. Sporogenesis in other myxozoan species, such as *Myxobolus cerebralis*, has been shown to be highly temperature dependent (El-Matbouli et al. 1998, Baldwin et al. 2000). Based on the initial observation of myxospores by histology in this study, myxospore production is more likely in clinically diseased juvenile fish that survive 16 dpe, and develop pre-sporogonic stages of *C. shasta* at river temperatures above 18°C. In summary, the production of myxospores will be depended on the state of maturation (sporogenesis) of the parasite within the juvenile fish host.

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