U.S. Fish & Wildlife Service

California-Nevada Fish Health Center
FY 2009 Investigational Report:

Myxosporean Parasite (*Ceratomyxa shasta* and *Parvicapsula minibicornis*) Incidence and Severity in Klamath River Basin Juvenile Chinook Salmon, April-August 2009

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SUMMARY

Juvenile Klamath River Chinook (*Oncorhynchus tshawytscha*) experience high incidence of infection with the myxosporean parasites *Ceratomyxa shasta* and *Parvicapsula minibicornis* during the spring and summer outmigration period. Klamath River Chinook were assayed by quantitative real-time polymerase chain reaction (QPCR) or histology to determine parasite infection rates from April to August, 2009. The incidence of *C. shasta* in Chinook salmon above the Trinity River confluence was 43% by QPCR and 54% by histology, the highest level observed by histology during Klamath River parasite monitoring studies conducted from 2005 to 2009. The incidence of *P. minibicornis* in Chinook salmon above the Trinity River confluence for the same period was 82% by QPCR and 85% by histology, which is similar to the highest levels observed in 2007 (81%) and 2008 (89%). The QPCR assay results from natural and marked Iron Gate Hatchery (IGH) and Trinity River Hatchery (TRH) Chinook salmon suggest that Klamath River reaches above the Trinity River confluence were more infectious for *C. shasta* earlier in the sampling period this year, and particularly for natural fish. Additionally in 2009, the Trinity to Estuary reach had higher *C. shasta* incidence of infection, compared to previous study years. In coded-wire tagged (CWT) IGH Chinook salmon screened by QPCR, *C. shasta* was detected in 36% of fish examined. The highest *C. shasta* prevalence of infection occurred in the IGH-CWT Chinook residing 3 Weeks at Liberty (WAL) upon recapture post hatchery release. The incidence of *C. shasta* infection in marked TRH Chinook salmon sampled in the Klamath River was 13%, higher than the 10% average incidence historically observed in Chinook salmon sampled within the Trinity River, or in the Klamath River below the Trinity River confluence.

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INTRODUCTION

Juvenile Klamath River Chinook (*Oncorhynchus tshawytscha*) experience high incidence and severity of infection with the myxosporean parasites *Ceratomyxa shasta* and *Parvicapsula minibicornis*. Both parasites have a similar distribution and are found throughout the Klamath River system including the lower reaches of the Williamson and Sprague Rivers, Agency Lake, Klamath Lake, Copco Reservoir, and the entire Lower Klamath River from Iron Gate Dam to the estuary (Hendrickson et al. 1989; Stocking et al. 2006; Bartholomew et al. 2007; Stocking and Bartholomew 2007). Both parasites share the vertebrate (salmonid) and invertebrate (*Manayunkia speciosa*) hosts and have overlapping distributions throughout the Pacific Northwest (Ching and Munday 1984; Hoffmaster et al. 1988; Bartholomew et al. 1989; Hendrickson et al. 1989; Bartholomew et al. 1997; Kent et al. 1997; Jones et al. 2004; Bartholomew et al. 2006, Stocking et al. 2006). In previous studies, native Klamath River salmonids have demonstrated various degrees of *C. shasta* resistance (Foott et al. 1999, Foott et al. 2004; Foott et al. 2007, Stone et al. 2008). Regardless of this resistance, Foott et al. (2004) observed that 100% of Klamath River Chinook salmon became infected and over 80% died within 17d following a 3d exposure in the Klamath River. A prognosis study conducted in 2008 examined daily levels of parasite levels (*C. shasta* and *P minibicornis* DNA copy number) and cumulative mortality in Iron Gate coho and Trinity Hatchery Chinook juveniles, following 72 hour river exposure above Beaver Creek. In this study, *C. shasta* infections resulted in a 17.3 mean day to death (MDD) and 87.1% cumulative percent mortality (CPM) in Chinook and 20.6 MDD and 98.5% CPM in coho juveniles (True, unpublished data). The observed high incidence of infection in resistant indigenous fish indicates an extremely high parasite challenge (Foott et al. 2004). Dual infections with both parasites are common and may have a synergistic effect which increases the lethality of infection (Nichols and True 2007). However, the contribution of each myxozoan parasite towards clinical disease in infected Chinook is difficult to evaluate. In sentinel studies, and the monitoring program, fish succumb to clinical Ceratomyxosis before an independent assessment of tissue changes and parasite DNA levels attributable to *P. minibicornis* can be fully determined.

In 2009, two changes were made in how data is reported for the Klamath River Fish Health Monitoring program. First, Cycle Threshold (C_T) values obtained with the QPCR assay have been transformed to a more meaningful metric of parasite DNA copy number. Parasite DNA quantities are based on the standard curves for each parasite assay using known quantities of parasite DNA. This change in the reporting metric for QPCR provides a more meaningful quantification of parasite infectious load, and a directly comparable unit between groups of fish and for annual comparisons. Secondly, clinical disease incidence by histology has been expanded to include a pathology score for both kidney and intestine tissues. The pathology score does not affect the overall incidence of infection reported for histological assessments, but provides a numeric index of the disease state in sample groups.

Additional diagnostic examinations were also performed in 2009 with the primary purpose of documenting bacterial and external parasite infections in moribund juvenile salmon. In particular, we were interested in the occurrence of *Ichthyophthirius multifiliis* (Ich) and *Flavobacterium columnare* (columnaris) infections in juvenile salmon. These two pathogens are associated with disease in returning adult salmon (Belchik et. al 2004, McCovey and Strange 2008). Given the

elevated water temperatures of the lower river during July and August, both juveniles and adult salmon tend to congregate in thermal refugia (Bartholow 2005, Belchik et.al 2004, Foott et al. 2001). It is possible that juvenile Chinook could act as reservoirs of infection for the early returning adults.

The objectives of this study were: 1) examine the pathogen incidence in Iron Gate Hatchery (IGH) and Trinity River Hatchery (TRH) Chinook prior to and post release; 2) examine the parasite incidence in the juvenile Chinook salmon population within the river throughout the spring out-migration period; 3) compare parasite incidence in 2009 to previous years; and 4) examine the diagnostic incidence of other significant pathogens in moribund Chinook in select reaches.

METHODS

Sample Sites

Fish were collected in the Klamath River from below Iron Gate Dam (Klamath RM 190) to the Klamath River Estuary and on the Trinity River between Lewiston Dam (Trinity RM 111) and the Trinity River confluence with the Klamath River (Klamath RM 43.5). Klamath and Trinity Rivers were divided into sample reaches at major tributaries, with study cooperators collecting fish in each reach (Table 1). When possible, existing salmonid downstream migrant trapping sites were utilized for collection, but seining was required to achieve the desired sample size in some weeks. Collection sites were preferably located in the lower portion of each reach, but when abundance was low fish from anywhere within a reach were accepted.

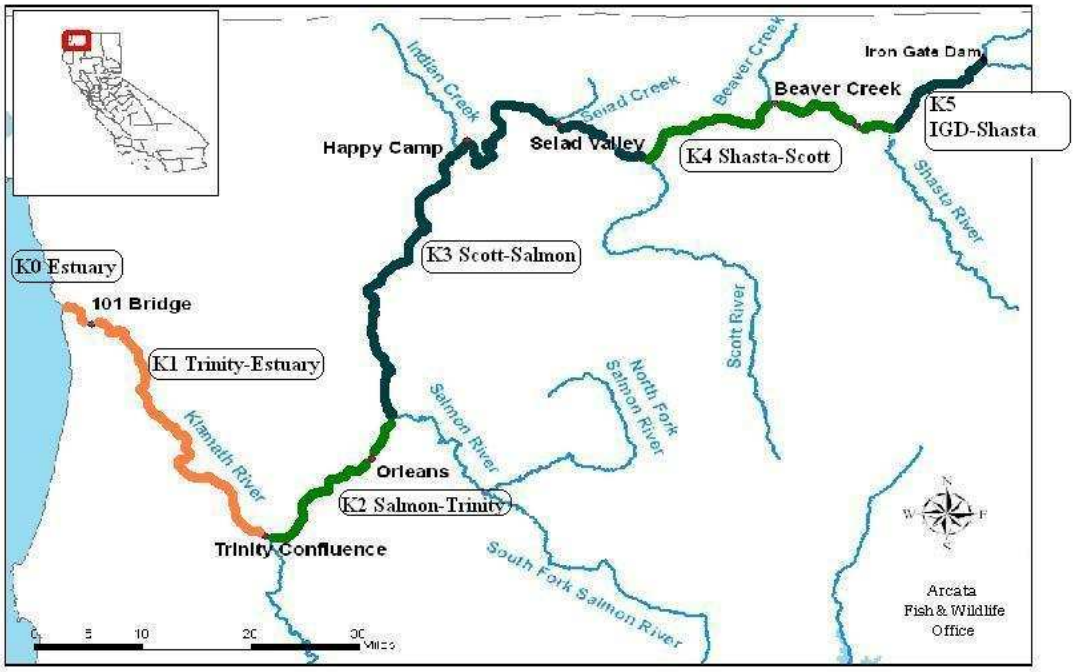

Figure 1. Klamath River watershed, major tributaries, and sample reaches: Iron gate dam to Shasta River (K5), Shasta River to Scott River (K4), Scott River to Salmon River (K3), Salmon River to Trinity River confluence (K2), Trinity River to Estuary (K1), Klamath River Estuary (K0). (map courtesy of Arcata FWO)

Table 1. Sample reach locations (reach code), river mile, and cooperating agencies performing fish collections on the Klamath and Trinity rivers.

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
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

Sample Groups

Pre-release IGH and TRH Chinook salmon - Prior to being released from the hatchery, 53 Chinook were sampled from the IGH population on 20 May, and 48 fish were sampled from TRH population on 12 May. All fish from each pre-release examination were assayed by QPCR for both parasites, and a subset of 10 fish from each hatchery were examined histologically for tissue abnormalities.

Mixed-origin Chinook – These juvenile Chinook salmon were collected in select reaches of the Klamath and Trinity Rivers. This sample consisted of 30 Chinook salmon for the QPCR assay and 10 Chinook salmon for the histology assay. In the Shasta to Scott (K4) reach and Salmon to Trinity (K2) reach of the Klamath River, mixed-origin Chinook were collected every other week.
In the upper and lower reaches of the Trinity River, mixed-origin Chinook were collected during the weeks of 12 May and 27 May. Prior to the release of hatchery fish, these fish were primarily naturally produced with the possibility of a few hatchery origin Chinook salmon used for trap efficiency calibration in the sample. After IGH releases, which occurred from 19 May – 9 June, mixed-origin Chinook collected in the Klamath River could have been of either hatchery or natural origin.

Marked IGH and TRH Chinook salmon – Twenty-five percent of the Chinook salmon released from IGH on the Klamath River and TRH on the Trinity River were marked with an adipose fin clip and implanted with a coded-wire-tag (CWT). In the Klamath River, any CWT Chinook encountered by the sample crews were collected for analysis by QPCR. No CWT Chinook were collected in the Trinity River; however, significant recapture effort occurred in the Klamath River below the Trinity River confluence. Heads from any marked IGH or TRH Chinook salmon recovered were assigned unique identification numbers to track lab assay results to individual fish. Tags were extracted and read by the USFWS Arcata Fish and Wildlife Office (AFWO). Chinook salmon release groups at IGH occurred on 19 May, 21 May, 26 May, 2 June, and 9 June. The CWT codes were unique for each release date, with the exception of the 9 June release group which included 2 tag codes for a single release date. Volitional releases occurred at TRH from 1 June through 15 June; 8 June was used as the date of release for all marked TRH Chinook salmon. The date each group of CWT Chinook salmon was released from the hatchery and date of recapture was used to calculate weeks at liberty (WAL) for individual fish.

Diagnostic Examinations of Moribund Chinook in the Trinity River to Estuary Reach

Evaluations of moribund Chinook in the Trinity River to estuary (K1) reach were conducted with the primary purpose of documenting bacterial and external parasite infections in moribund juvenile salmon. Of particular interest is the occurrence of *Ichthyophthirius multifiliis* (Ich) and *Flavobacterium columnare* (columnaris) infections in juvenile salmon. Moribund fish were selected from beach seine collections at the mouths of Pecwan, Tec Tah, Roach, Lower Crow, and Blue creeks on 18 June, 8 July, and 11 August. Selected external and internal clinical signs were recorded and laboratory samples (lesion imprints, spleen imprints, kidney inoculums onto Brain Heart Infusion agar and fixed tissues for histology) were collected from the examined group.

Sample Periods

In each reach, fish were accumulated over a calendar week until the desired sample size was achieved. Weekly prevalence of infection was calculated for a reach by dividing the number of fish in which a parasite was detected by the total fish assayed for a calendar week. Fish collection started the week of 21 April in the Shasta to Scott (K4) reach and 6 May in the Salmon to Trinity (K2) reach. Collection in each reach continued until the target Chinook salmon sample numbers per week (30 fish) could no longer be captured. Collection of CWT Chinook salmon began after hatchery release and collection crews accumulated as many CWT Chinook salmon as time allowed each week. Collection of CWT Chinook salmon in a given reach continued until fewer than 10 fish could be recovered in a single week's effort.

QPCR Assays

Fish collected for the quantitative real-time polymerase chain reaction (QPCR) assay were euthanized, placed in a plastic bag labeled with date and reach, and arranged between frozen gel pack sheets in an ice chest. Samples were frozen, and subsequently collected from cooperator's freezers by Fish Health Center staff every other week. In the laboratory, fish were thawed, fork length was measured, clinical disease signs notated, and necropsy performed to collect tissue samples. The intestine (both small and large) and entire kidney from each fish were removed and combined into an individually numbered 2 ml cluster tube. Tissue samples were then frozen at -20 ºC until DNA extraction was performed.

Combined intestine and kidney tissues were digested in 1ml NucPrep Digest Buffer containing 1.25 mg/ml proteinase K (Applied Biosystems, Foster City, CA) at 55ºC for 2 hours with constant shaking. A subsample of digested tissue homogenate was diluted 1:33 in molecular grade water and extracted in a 96 well vacuum filter plate system (Applied Biosystems Model 6100 Nucleic Acid Prep Station). Extracted DNA was stored at -20ºC until the QPCR assays were performed.

Samples were assayed in a 7300 Sequence Detection System (SDS) (Applied Biosystems), using probes and primers specific to each parasite. The combined tissues were tested for *C. shasta* 18S rDNA using TaqMan Fam-Tamra probe and primers (Hallett and Bartholomew 2006) and tested in a separate assay for *P. minibicornis* 18S rDNA utilizing TaqMan Minor-Grove-Binding (MGB) probe and primers (True et al. 2009). Reaction volumes of 30µL, containing 5µL DNA template, were used for both assays under the following amplification conditions: 50ºC for 2 min.; 95ºC for 10 min; 40 cycles of 95ºC for 15s and 60ºC for 1 min. Plasmid standards, extraction control and no template control (NTC) wells were included on each assay plate. Cycle threshold (C_T) values were calculated by the SDS software (v 1.3.1, Applied Biosystems) and converted to parasite Plasmid Molecular Equivalent (PME), a measure of specific parasite DNA copy number derived from the

standard curve of each specific assay (Figure 2) . Validation studies examining the dynamic range and endpoint of the assays indicated 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 (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. It should be noted that these thresholds are statistically conservative to preclude false positive test results and therefore slightly underestimated the true infection incidence of both parasites in this aquatic animal population. Appendix II provides a further technical description of how assay thresholds are determined.

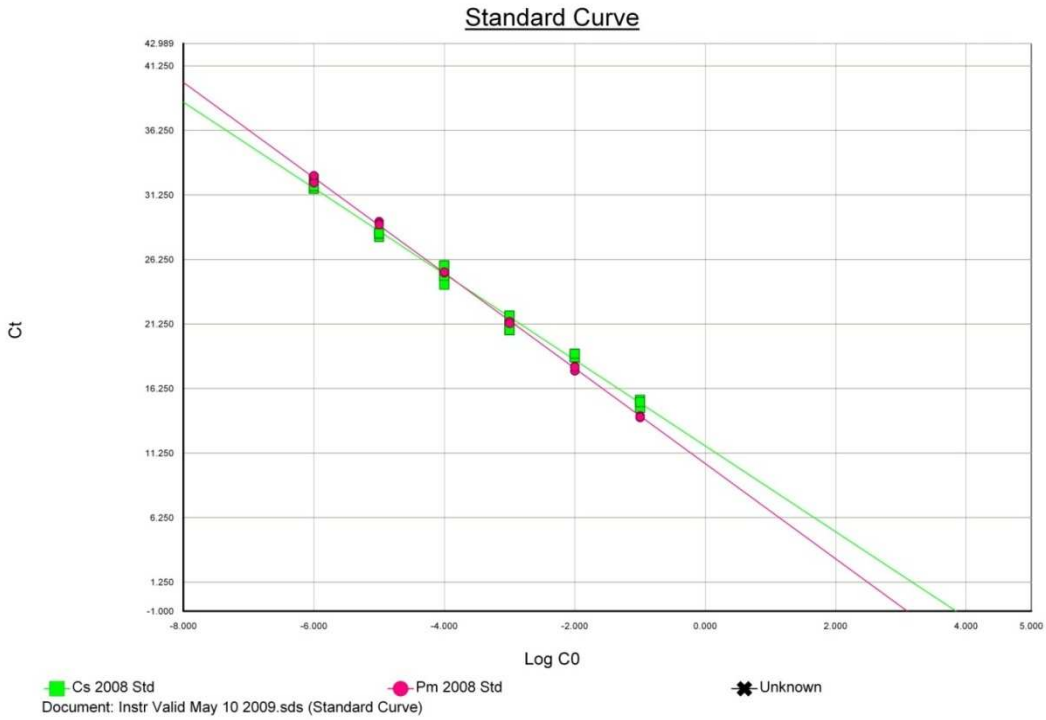

Figure 2. Standard curves for *C. shasta* and *P. minibicornis* QPCR assays using plasmid standards developed in 2008 with known concentrations of parasite DNA. The amplification efficiency of each assay was calculated using the formula $E=(10^{-1/slope}-1) \times 100$ (Applied Biosystems Guide to Quantitative Gene Expression). Amplification Efficiency is similar for both QPCR assays at 95.7% for *C. shasta* and 93.4% for *P. minibicornis* .

Histology Assays

Ten to 20 fish from the bi-weekly beach seine collections were randomly selected for histology. Rapidly after euthanization, the peritoneum was cut open and entire fish placed in Davidson's fixative and held for 24-48 hours. The fixative was replaced with 70% ethanol for storage until the gross examination and histological processing was performed. Each histological cassette contained kidney, intestine, liver, and 1 to 2 gill filaments. Specimens were processed for 5µm paraffin sections and stained with hematoxylin and eosin (Humason 1979). All tissues for each fish were placed on one slide and identified by a unique number code. Each slide was examined at 40X to 400X magnification.

Histological rankings of 'clinical disease' were expanded in 2009 to include a pathology score: a numeric index of disease severity for kidney and intestine. Pathology score is based on the degree of specific tissue abnormalities and parasite distribution (0 = normal, 1= focal, 2 = multi-focal, and 3 = diffuse distribution) listed in Table 2. A kidney pathology score was calculated by summing the score of each kidney lesion (interstitial hyperplasia, necrotic interstitium or tubule, interstitial granuloma, glomerulonephritis, and protein casts within the glomeruli or tubules). The mean kidney pathology score was reported for each collection group to demonstrate severity of disease. Similarly for the intestine, the sum of lesion scores (lamina propria hyperplasia, necrotic epithelium / sloughing, necrotic muscularis) was used to calculate a collection group's mean intestinal pathology score.

Table 2. Parasite abbreviations and tissue abnormalities listed in the histological result tables.

Kidney		
	P. minibicornis Troph.	*Parvicapsula minibicornis* trophozoite stage
	P. minibicornis Myxosp.	*Parvicapsula minibicornis* myxospore stage
	Metacercaria	Immature trematode stage
	C. shasta troph.	*Ceratomyxa shasta* trophozoite stage
	Chloromyxum sp.	Chloromyxum species trophozoite stage
	Pathology Score	**Mean kidney pathology score for sample group**
Intestine		
	C. shasta troph.	*Ceratomyxa shasta* trophozoite stage
	C. shasta myxosp.	*Ceratomyxa shasta* myxospore stage
	Helminth	Trematode, nematode, or cestode
	Pathology Score	**Mean intestine pathology score for sample group**
Gill	Glochidia	Larval mussel stage within lamellae
	Metacercaria	Immature trematode stage
	Invasive *C. shasta*	Single cell trophozoite-like stage
	Amoeba	Amoeba associated with lamellae
	Multif. Hyperplasia	Multifocal hyperplastic regions on lamellae
Other	Adipose steatitis	Inflammation of visceral fat tissue
	Adipose lipofuscin	Oxidized lipopigments within adipose cells
	Liver *C. shasta*	*Ceratomyxa shasta* trophozoite stage in liver

<u>2004-2009 Comparisons</u>

Histology data from this study was used to compare incidence of infection of fish in 2009 to previous juvenile Klamath River salmonid health monitoring studies (Nichols and Foott 2006; Nichols et al. 2007; Nichols and True 2007; Nichols et al. 2008). The histology data included in the analysis was limited to the months of May, June and July of each year and to mixed-origin Chinook sampled in the Klamath River above the Trinity River confluence. Limiting the data offered several advantages:

- Sampling start and end dates varied each year but included these months
- This date range brackets the typical peak of juvenile Klamath River Fall Chinook salmon outmigration (Leidy and Leidy 1984; Wallace and Collins 1997)

- Infection incidence during the "tails" of the migration (typically lower infection rates in early spring) were not given the same weight as the peak of migration
- The Trinity River population was excluded as it is largely uninfected with *C. shasta*
- Our target sample size was typically met during this period, reducing sample variation due to small sample size

While QPCR data was generated for 2005-2008, tissue collection and extraction protocols were modified in 2006, resulting in non-standardized tissue volumes for the QPCR assay that cannot be compared between all previous years. QPCR data from 2007-2009 is standardized and direct comparisons of annual parasite incidence of infection were made from 2007 forward.

Statistical Analysis

Prevalence of infection (defined as cases of disease present at a given time) and incidence of infection or disease (defined as the frequency of occurrence over a period of time, or for the population as whole), for *C. shasta and P. minibicornis* , are reported with 95% confidence intervals, denoted as ci, for each sample reach prevalence of infection data. Pearson's chi square analysis was used to compare *C. Shasta* incidence of infection between reaches, groups (i.e. CWT groups) and years when differences in apparent incidence data are noted (Prism® V5.02, GraphPad Software, La Jolla, CA). Associated P values are reported for comparisons of *C. Shasta* parasite incidence of infection for: K4, K2 and K1 sample reaches; annual comparison of the *C. shasta* incidence of infection in the Trinity River confluence to Estuary (K1) reach; and annual comparison of *C. shasta* incidence of infection in TRH CWT recovered in K1 and K0 reaches.

For IGH CWT Weeks at Large (WAL) analysis, comparisons of parasite mean DNA copy number were graphed for positive test results and the entire sample population in Figures 18 (*C .shasta*) and 20 (*P. minibicornis*). TRH CWT parasite DNA copy number was graphed in a similar manner in Figures 22 (*C. shasta*) and 23 (*P. minibicornis*). Mean parasite mean DNA copy number for all positive fish in the sample group are represented by a red dashed line whereas mean DNA copy number for the entire sample population (all fish tested, including negative and positive test results) are graphed in a black solid line to illustrate the parasite DNA loads for the infected fish in the sample set, compared to the sample group as a whole. Standard error whiskers and sample numbers (N) for each week are included in these figures.

RESULTS

Pre-release IGH and TRH Chinook Salmon

Light infections of *C. shasta* were detected by QPCR in 2% (1/53) of Chinook salmon sampled 20 May at IGH, prior to hatchery release. The single positive fish was lightly infected near the detection threshold of the QPCR assay (C_T 37.7 or 5.6 DNA copy number). Infections of *P. minibicornis* were detected by QPCR in 2% (1/53) of pre-release Chinook salmon sampled at IGH. The single positive fish had a moderate infection level (C_T 33.1 or 641 DNA copy number). Histology did not detect *C. shasta* or *P. minibicornis* in a subsample of 10 fish from Iron Gate Hatchery.

Trinity River hatchery Chinook had a higher prevalence of infection for both *C. shasta* and *P. minibicornis* by QPCR, compared to IGH. *C. shasta* was detected in 19% (9/48) with a mean C_T of 35.3 (SD=2.7). Parasite copy number ranged from a minimum of 7 to maximum of 937 with an average DNA copy number of 255. *P. minibicornis* was detected in 17% (8/48) of fish tested, with a mean C_T of 37.4. Parasite copy number ranged from a minimum of 21 to maximum of 846 with an average DNA copy number of 219. Histology did not detect *C. shasta* or *P. minibicornis* in a subsample of 10 fish from Trinity River Hatchery.

Iron Gate Dam to Shasta R. (K5)

In the IGD to Shasta (K5) reach, *C. shasta* was detected by QPCR in 9% (6/65, 95% confidence interval [ci]= 4-19%) of mixed-origin Chinook. Infection prevalence was low (10%) in late May, and *C. shasta* was not detected in fish sampled during the weeks of 1 June through 8 June (Figure3). The peak prevalence of *C. shasta* infection occurred 15 June with 21% (3/14, ci=5-51%) of fish testing positive by QPCR. Histology sampling was not performed in this reach, due to the relatively low incidence of *C. shasta* we have observed historically in this reach and the proximity to Iron Gate hatchery (primarily early infections).

In the IGD to Shasta reach, *P. minibicornis* was detected by QPCR in 58% (38/65, ci= 46-70%) of mixed-origin Chinook. Infection prevalence was over 55% in the first sample collected in late May, increased in early June and reached 100% by mid June (Figure 3).

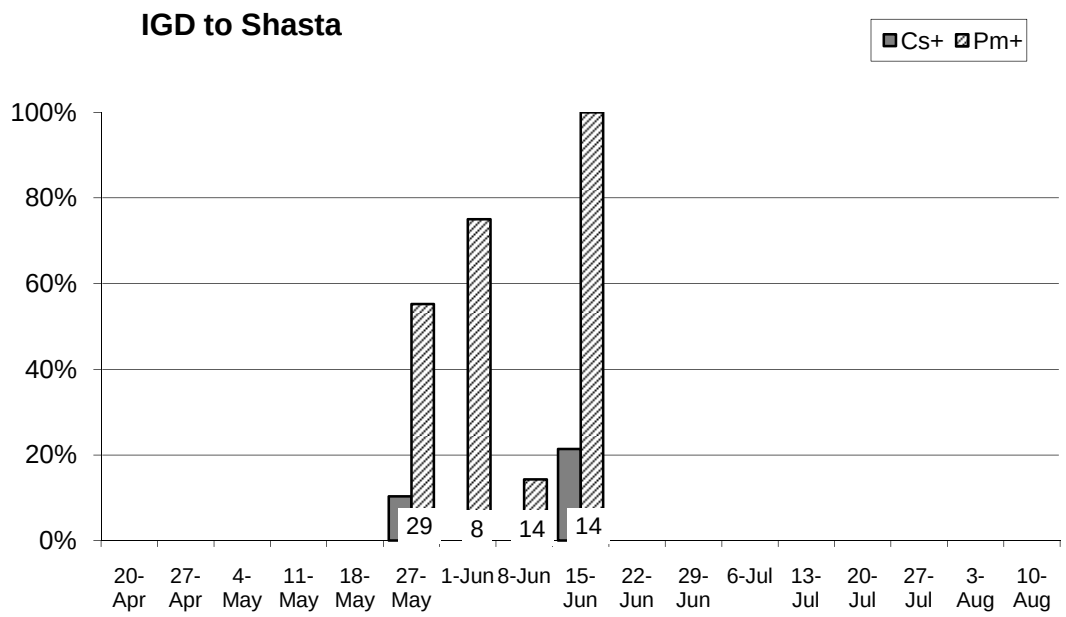

Figure 3. Weekly incidence of *Ceratomyxa shasta* and *Parvicapsula minibicornis* infection by QPCR in juvenile Klamath River Chinook salmon captured in Iron Gate Dam to Shasta River (K5) reach on the Klamath River. Sample numbers collected each week are displayed at the bottom of each column; Cs was not detected on 1 June and 8 June.

Shasta R. to Scott R. (K4)

In the Shasta to Scott reach (K4), *C. shasta* was detected by QPCR in 50% (131/261, ci=44-56%) of mixed-origin Chinook. Infection prevalence peaked at 97% in early May, decreased over a three week period, and peaked again (59-67%) from 15 June to 22 June. The second peak appeared to be descending when sampling in this reach ended in late June (Figure 4).

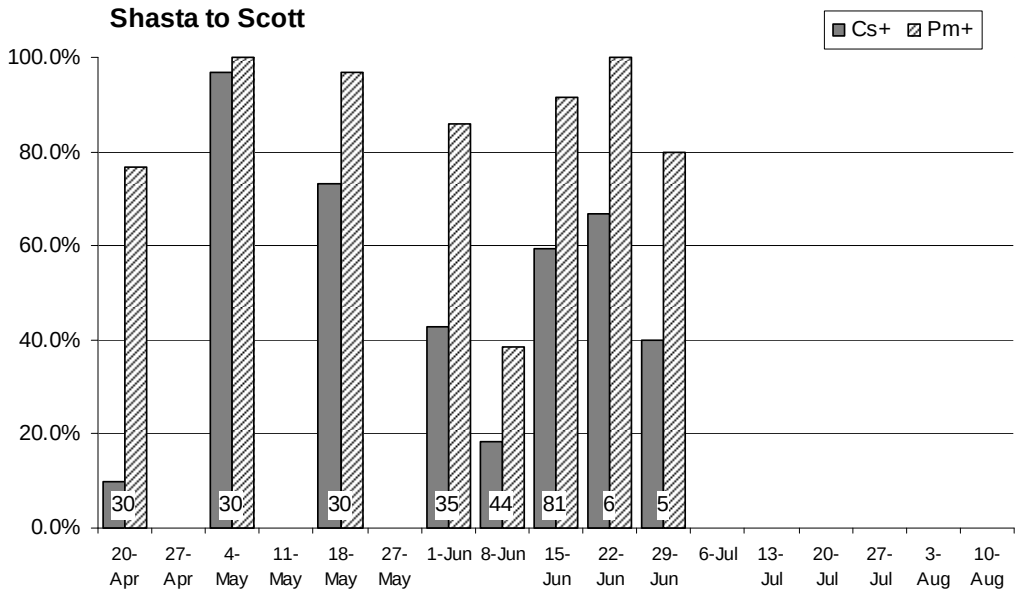

Figure 4. Weekly incidence of *Ceratomyxa shasta* and *Parvicapsula minibicornis* infection by QPCR in juvenile Klamath River Chinook salmon captured in the Shasta to Scott River (K4) reach on the Klamath River. Sample numbers collected each week are displayed at the bottom of each column.

In the Shasta to Scott reach (K4), *C. shasta* incidence of infection by QPCR was quite high in natural fish collected in early to mid May (97% and 73% respectively), and peaked again 15 June (59%), when hatchery fish were likely included in the sample sets (IGH releases occurred 19 May through 9 June). Parasite infectious load (log of the mean *C. shasta* DNA copy number) lagged incidence data by approximately 1 week: natural Chinook carried 3.1 logs of *C. shasta* DNA on 4 May when prevalence of infection was 97%. Parasite copy number increased to 3.8 logs two weeks later on 18 May, when prevalence decreased to 73% (Figure 5) . By 1 June, parasite load was decreasing (2.7 logs DNA) as prevalence decreased to 43%. This decrease in weekly prevalence is likely due to the inclusion of uninfected hatchery fish entering the reach, and 'diluting' the overall incidence of infection for the subsequent mixed origin Chinook population. Prevalence of infection, and parasite load increased again on 15 June, as prevalence of infection increased to 59%, indicating that *C. shasta* positive fish during this period also carried relatively heavy parasite loads.

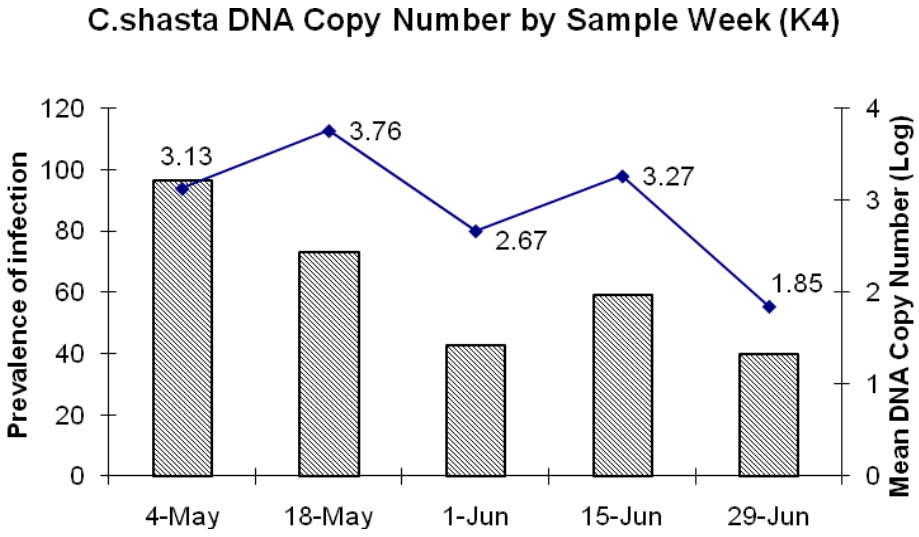

Figure 5. Bi-weekly *C. shasta* prevalence of infection (bars) and mean log parasite DNA copy number (lines) in intestinal tissue of Chinook collected in the Shasta to Scott (K4) reach, as tested by QPCR.

In the Shasta to Scott reach, *P. minibicornis* was detected by QPCR in 82% (213/261, ci=76-86%) of Chinook tested. Infection incidence reached 100% by the second sample in early May and remained high through the end of sampling in this reach in late June (Figure 4).

Histologically, *C. shasta* was detected in 54% (26/48, ci= 39-69%) of mixed origin Chinook. *Ceratomyxa shasta* was not observed in the intestine of natural salmon collected on 20 April, however, presumptive *C. shasta* trophozoites were seen in the gill lamellae of 2 fish in this sample set (Table 3). Two weeks later, prevalence of *C. shasta* infection was 89% and showed severe intestinal pathology (Figure 6). This pattern continued in the 18 May collection group and declined in the 1 June collection. This reach had the highest incidence of presumptive *C. shasta* trophozoites observed in the gill tissue at 61% (28/46, ci =45-75%).

By histology, *P. minibicornis* was detected in 89% (43/48, ci=77-96%) of mixed-origin Chinook. *Parvicapsula minibicornis* trophozoites were seen in 70 – 100% of the collection groups (Table 3) with highest kidney pathology score occurring on 18 May (Figure 7). Encysted metacercaria were seen in 61% (28/46, ci =45-75%) of the gill sections and 8% (4/48, ci =2-20%) of the kidney sections.

Table 3. Prevalence of parasite infection (number positive / total (%)) and tissue abnormalities observed in Chinook collected from the Shasta to Scott (K4) reach.

	Sample Date 4/20	5/4	5/18	6/1	6/15
Kidney					
Pm Troph.	7 / 10 (70)	9 /9 (100)	10 / 10 (100)	8 / 10 (80)	9 / 9 (100)
Pm Myxosp.	0 / 10 (0)	0 / 9 (0)	1 / 10 (10)	0 / 10 (0)	0 / 9 (0)
Metacercaria	0 / 10 (0)	0 / 9 (0)	0 / 10 (0)	1 / 10 (10)	3 / 9 (33)
C. shasta troph.	0 / 10 (0)	0 / 9 (0)	1 / 10 (10)	1 / 10 (10)	0 / 9 (0)
Chloromyxum sp.	0 / 10 (0)	0 / 9 (0)	0 / 10 (0)	0 / 10 (0)	0 / 9 (0)
Pathology Score	0.000	5.778	8.400	5.400	2.111
Intestinal tract					
C. shasta troph.	0 / 10 (0)	8 / 9 (89)	8 / 10 (80)	3 / 10 (30)	7 / 9 (78)
C. shasta myxosp.	0 / 10 (0)	0 / 9 (0)	0 / 10 (0)	0 / 10 (0)	0 / 9 (0)
Helminth	0 / 10 (0)	1 / 9 (11)	0 / 10 (0)	1 / 10 (10)	0 / 9 (0)
Pathology Score	0.000	3.440	3.400	0.600	1.333
Adipose steatitis	3 / 4 (75)	5 / 6 (83)	5 / 5 (100)	5 / 9 (56)	2 / 8 (25)
Adipose lipofuscin	0 / 4 (0)	0 / 6 (0)	0 / 5 (0)	1 / 9 (11)	1 / 8 (13)
Liver *C. shasta*	0 / 10 (0)	0 / 8 (0)	3 / 10 (30)	0 / 9 (0)	0 / 9 (0)
Gill					
Glochidia	0 / 10 (0)	0/ 9 (0)	0 / 8 (0)	2 / 10 (20)	1 / 9 (11)
Metacercaria	4 / 10 (40)	5 / 9 (56)	6 / 8 (75)	4 / 10 (40)	9 / 9 (100)
Invasive *C. shasta*	2 / 10 (20)	6 / 9 (67)	8 / 8 (100)	4 / 10 (40)	9 / 9 (100)
Amoeba	0 / 10 (0)	0 / 9 (0)	0 / 8 (0)	0 / 10 (0)	0 / 9 (0)
Multif. Hyperplasia	3 / 10 (30)	0 / 9 (0)	5 / 8 (63)	5 / 10 (50)	9 / 9 (100)

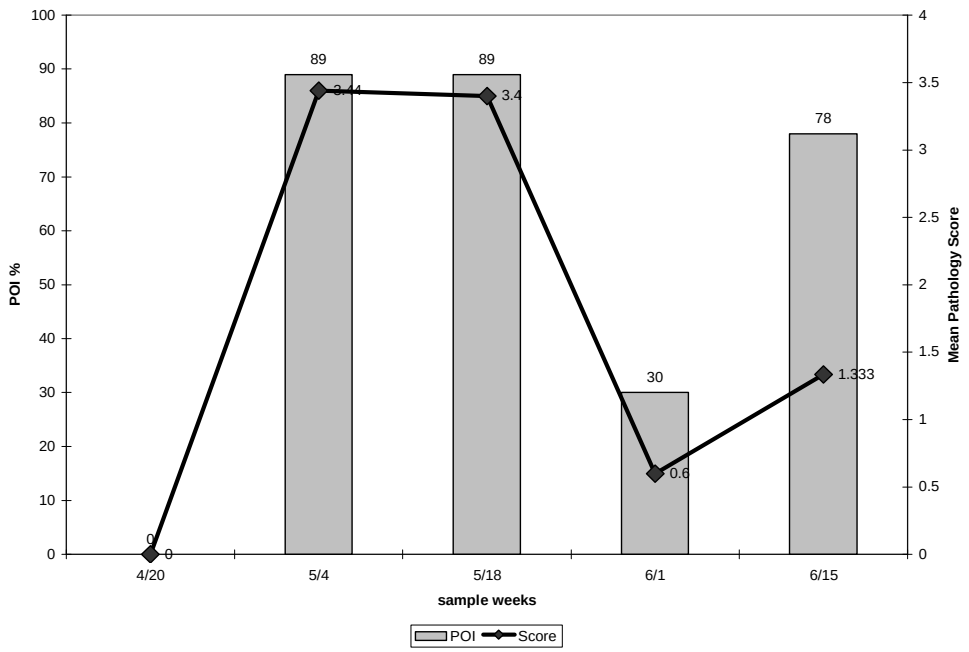

Figure 6. Prevalence of infection (bars) and mean intestinal pathology score (line) for *C. shasta* infections of Chinook collected in the Shasta to Scott (K4) reach.

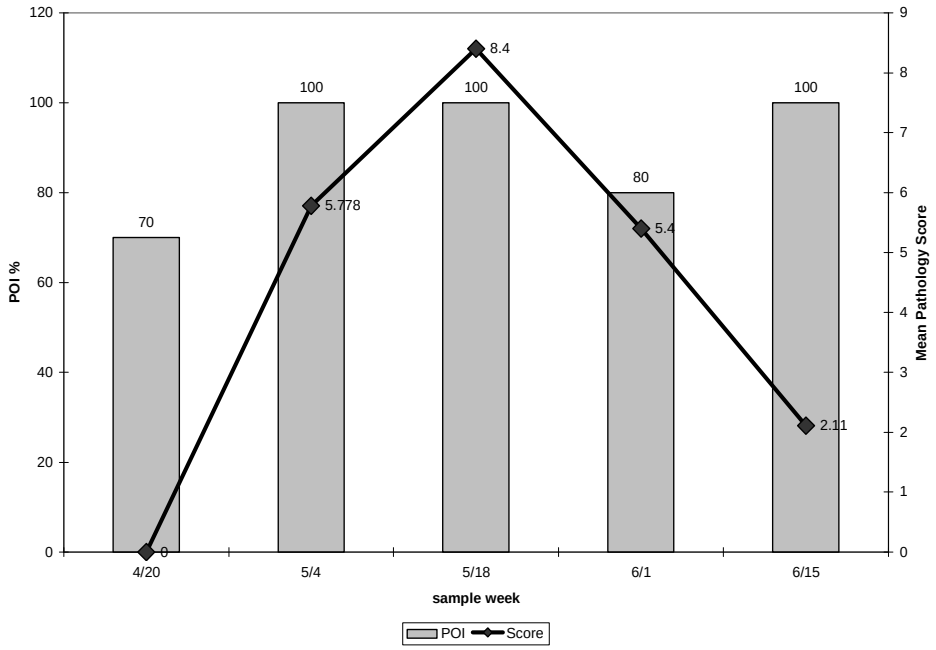

Figure 7. Prevalence of infection (bars) and mean kidney pathology score (line) for *P. minibicornis* infections of Chinook collected in the Shasta to Scott (K4) reach.

Salmon R. to Trinity R. reach (K2)

In the Salmon to Trinity (K2) reach, *C. shasta* was detected by QPCR in 44% (139/318, ci=38-49%) of mixed-origin Chinook. *Ceratomyxa shasta* was not detected on 4 May however prevalence of infection increased rapidly to 95% by 1 June, then decreased to less than 25% by 29 June (Figure 8). A second *C. shasta* prevalence of infection peak occurred a week later on 6 July (75%, however it should be noted that this was a small sample set consisting of 8 fish). *Ceratomyxa shasta* prevalence of infection was below 40% in July, and decreased to <20% in the last sample from this reach, collected 10 August.

In the Salmon to Trinity reach, *P. minibicornis* was detected by QPCR in 87% (277/318, ci=83-90%) of mixed-origin Chinook. Infection incidence reached 100% by early June and remained high through the end of sampling in this reach on 10 August (Figure 6).

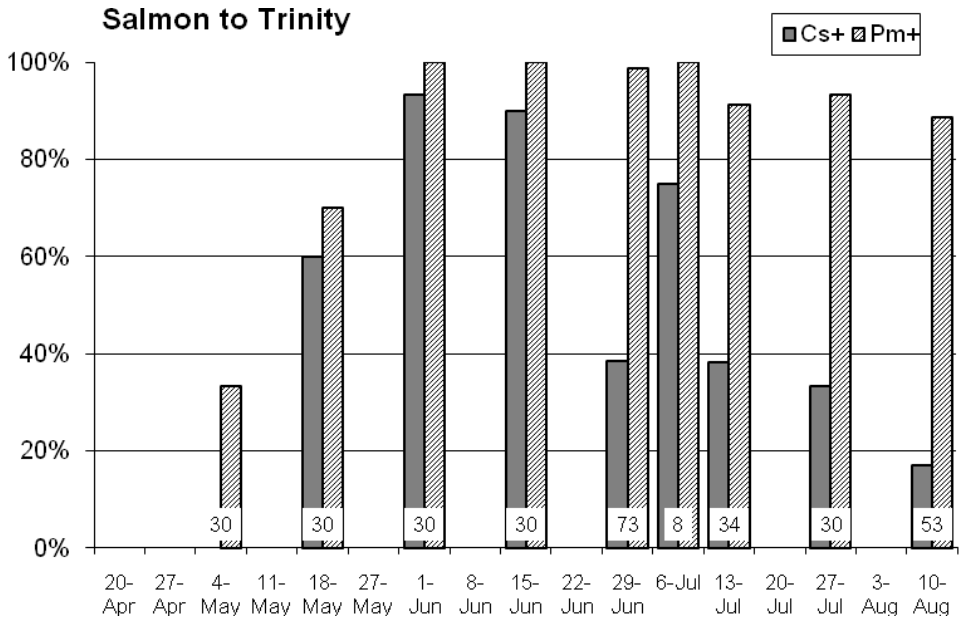

Figure 8. Weekly incidence of *Ceratomyxa shasta* and *Parvicapsula minibicornis* infection by QPCR in juvenile Klamath River Chinook salmon captured in the Salmon River to Trinity River confluence (K2) reach in the Klamath River. Sample numbers collected each week are displayed at the bottom of each column; Cs was not detected on 4 May.

In the Salmon to Trinity (K2) reach, *C. shasta* was not detected initially by QPCR in natural fish collected the week of 4 May, but prevalence of infection rapidly increased to 60% by 18 May (Figure 9). Hatchery fish were likely predominant in the sample set for 1 June through 29 June: *C. shasta* incidence of infection peaked at 95% on 1 June, then decreased slightly to 90% on 15 June when parasite infectious load was highest at >6 logs DNA copy number (Figure 9). A second *C. shasta* prevalence of infection peak occurred one month later on 13 July, and again parasite load peaked approximately 1 week later to 4.7 logs DNA copy number on 27 July.

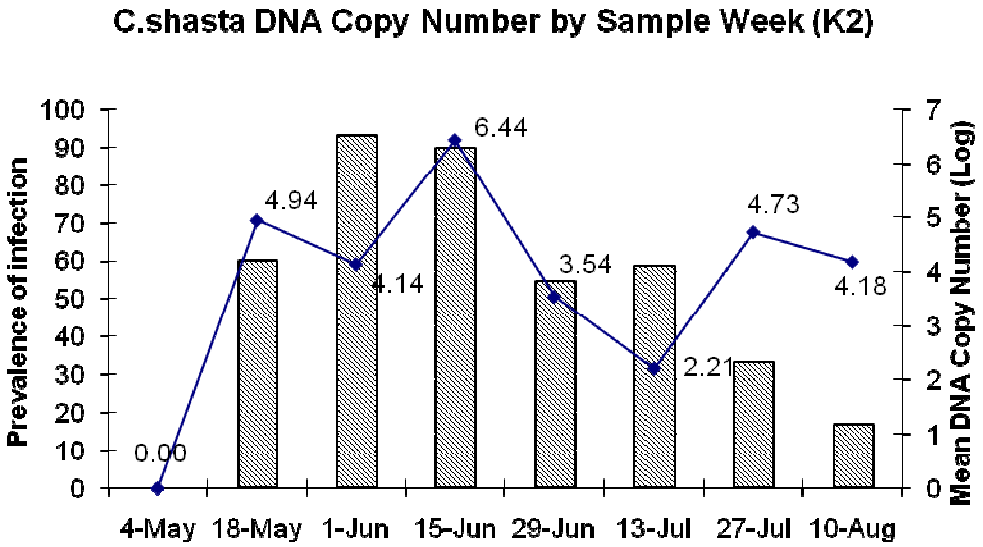

Figure 9. Bi-weekly *C. shasta* prevalence of infection (bars) and infectious load as determined by mean log parasite DNA copy number (lines) in intestinal tissue of Chinook collected in the Salmon to Trinity (K2) reach, as tested by QPCR.

Histologically, 10 to 20 salmon were collected initially on 18 May, and then every other week between the 15 June – 27 July (Table 4). The 13 and 27 July collection was inadvertently combined in the same storage container. Given hatchery release dates (IGH 19 May – 9 June, TRH 1 June – 15 June), the 18 May collection is natural origin salmon while later sample groups could be mixed hatchery and natural Chinook. The 18 May natural salmon had the highest prevalence of *C. shasta* infection and intestinal pathology score in this reach (Table 4). The incidence of *C. shasta* infection was 44% (24/55, ci=30-58%) in this reach with collection groups in June and July having both lower prevalence (20-27%) and intestinal pathology scores than the 18 May group (Figure 10).

Table 4. Prevalence of parasite infection (no. positive / total (%)) and tissue abnormalities observed within salmon collected from the Salmon to Trinity reach (K2). Tissues from the sample set collected 4 May were too degraded for evaluation, and the 13 July and 27 July collections were inadvertently mixed and are therefore reported as one combined sample set.

	5/18	6/15	6/29	7/13 &27
Kidney				
Pm Troph.	15 / 20 (75)	9 / 10 (90)	9 / 10 (90)	10 / 15 (67)
Pm Myxosp.	0 / 20 (0)	0 / 10 (0)	0 / 10 (0)	0 / 15 (0)
Metacercaria	1 / 20 (5)	2 / 10 (20)	2 / 10 (20)	4 / 15 (27)
C. shasta troph.	0 / 20 (0)	0 / 10 (0)	0 / 10 (0)	1 / 15 (7)
Chloromyxum sp.	1 / 20 (0)	0 / 10 (0)	0 / 10 (0)	1 / 15 (7)
Pathology Score	2.900	4.700	6.700	3.267
Intestinal tract				
C. shasta troph.	15 / 20 (75)	3 / 10 (30)	2 / 10 (20)	4 / 15 (27)
C. shasta myxosp.	0 / 20 (0)	0 / 10 (0)	0 / 10 (0)	0 / 15 (0)
Helminth	0 / 20 (0)	0 / 10 (0)	1 / 10 (10)	3 / 15 (20)
Pathology Score	4.200	0.100	0.300	0.800
Adipose steatitis	4 / 9 (44)	5 /7 (71)	5 / 6 (83)	7 / 10 (70)
Adipose lipofuscin	0 / 9 (0)	0 / 10 (0)	2 / 6 (33)	1 / 10 (10)
Liver *C. shasta*	0 / 20 (0)	0 / 10 (0)	0 / 9 (0)	2 / 15 (13)
Gill				
Glochidia	0 / 20 (0)	0 / 10 (0)	0 / 10 (0)	0 / 20 (0)
Metacercaria	12 / 20 (60)	8 / 10 (80)	10 / 10 (100)	18 / 20 (90)
Invasive *C. shasta*	1 / 20 (5)	1 / 10 (10)	1 / 10 (10)	0 / 20 (0)
Amoeba	0 / 20 (0)	0 / 10 (0)	0 / 10 (0)	1 / 20 (5)
Multif. Hyperplasia	2 / 20 (10)	3 / 10 (30)	9 / 10 (90)	13 / 20 (65)

Parvicapsula minibicornis prevalence of infection was 78% (43/55, ci=65-88%) by histology and ranged from 67 – 90% in the collection groups (Table 4). The peak kidney pathology score occurred on 29 June (Figure 11).

Encysted metacercaria within the gill was a common observation (96%, 48/50, ci=86-99%) with only 16% (9/55, ci=8-29%) incidence of infection in the kidney (Table 4). Presumptive *C. shasta* trophozoites were seen 6% (3/50, ci=1-17%) of gill sections examined. One fish in the combined 13 and 27 July group was observed with a single *Ichthyophthirius multifiliis* trophozoite in its gill. This was the only observation of this ciliate parasite in 310 histological samples examined over the 2009 project.

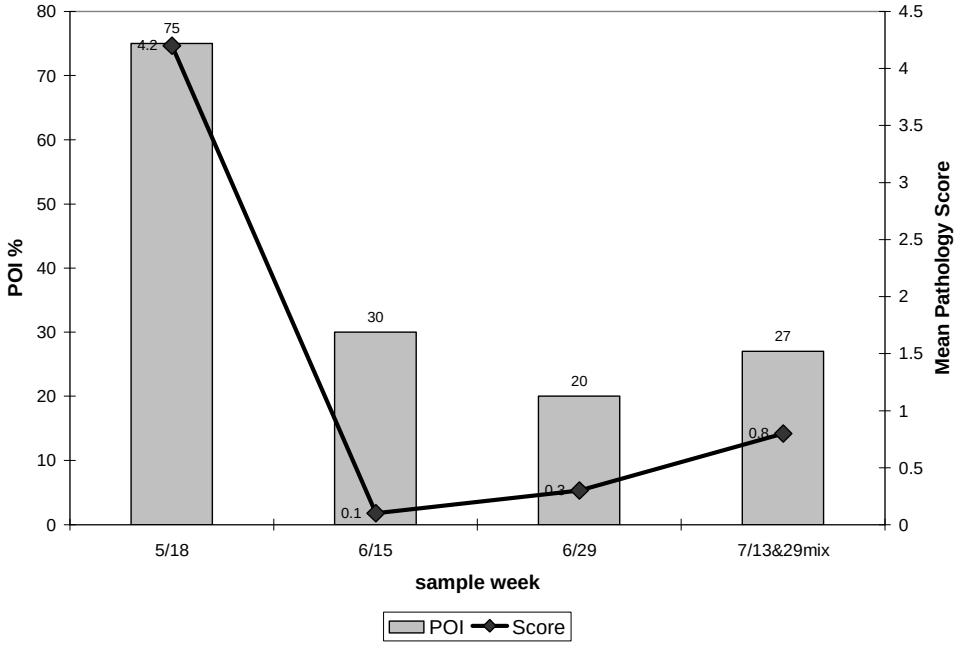

Figure 10. Prevalence of infection (bars) and mean intestinal pathology score (line) for *C. shasta* infections of Chinook collected in the Salmon to Trinity River (K2) reach on the Klamath River.

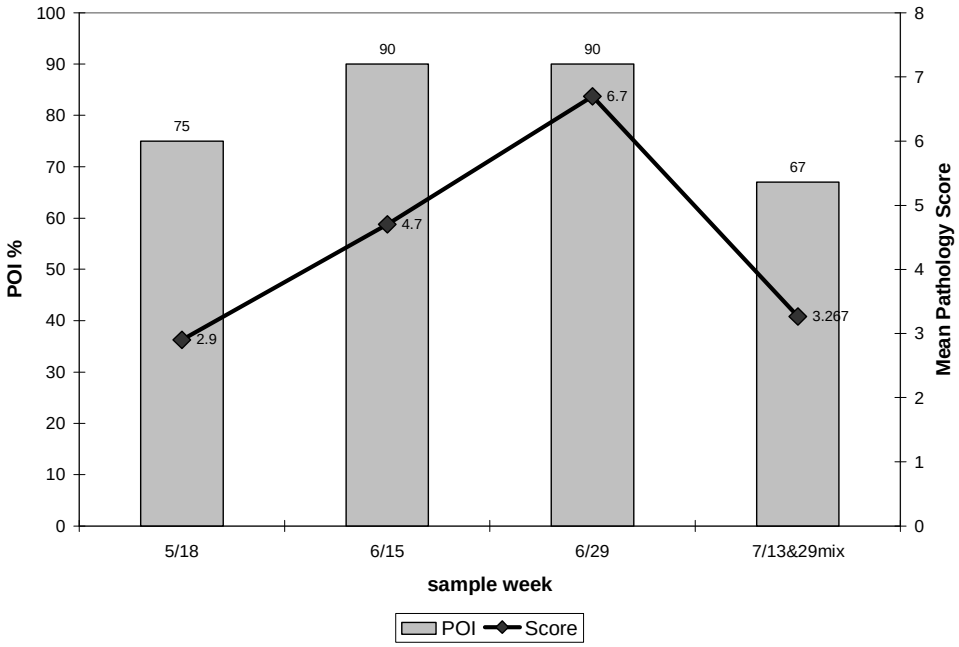

Figure 11. Prevalence of infection (bars) and mean kidney pathology score (line) for *P. minibicornis* infections of Chinook collected in the Salmon to Trinity River (K2) reach on the Klamath River.

Trinity R. to Estuary reach (K1)

In the Trinity to Estuary (K1) reach, *C. shasta* was detected by QPCR in 50% (155/313, ci=43-55%) of mixed origin Chinook, and by histology in 33% (31/95,ci=23-43%). Infection prevalence was high (77%) in the first samples collected 1 June, and remained between 40-76% throughout the June sampling period (Figure 12). *C. shasta* prevalence decreased by 20 July, and was not detected on 27 July and 3 August. *C. shasta* prevalence was increasing (28%) on the last sample date of 10 August (although this small sample set was comprised of 3 fish only).

In the Trinity to Estuary reach, *P. minibicornis* was detected by QPCR in 83% (259/313, ci=78-87%) and by histology in 78% (74/95, ci=68-86%) of mixed-origin Chinook. Infection prevalence by QPCR was high initially (97%) on 1 June, and reached 100% by 8 June. Prevalence remained over 80% throughout June, declined in late July to 32%, and early August to 20%, then increased in the final sample (N=3) collected on 10 Aug to 67% (Figure 12).

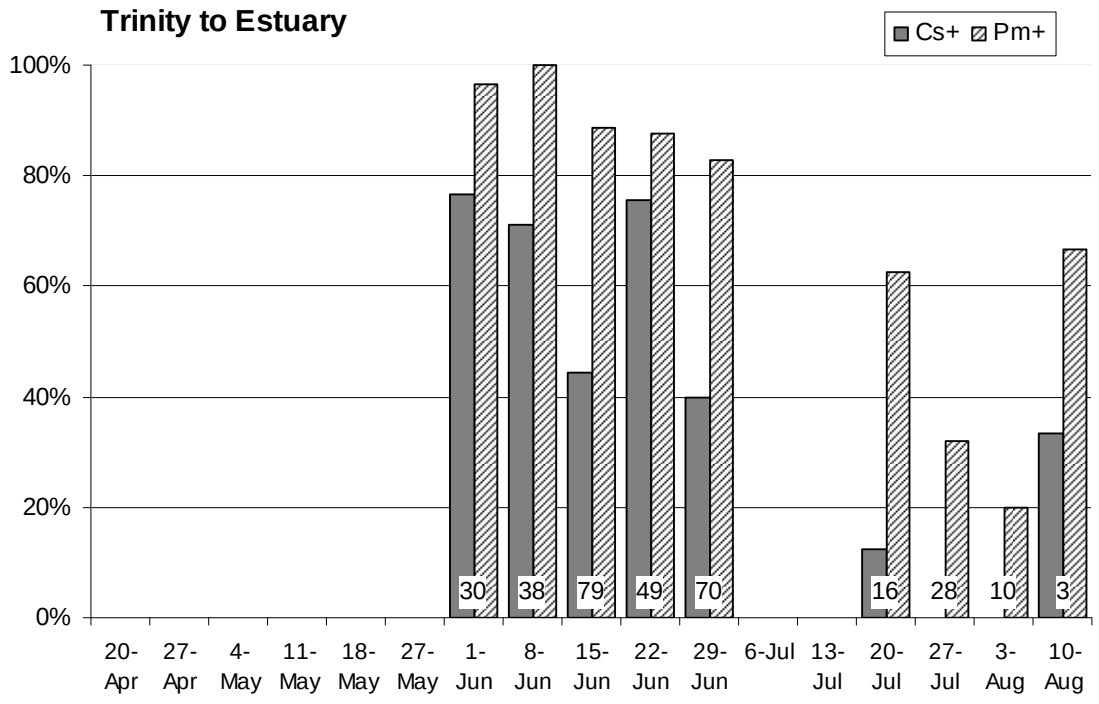

Figure 12. Weekly incidence of *Ceratomyxa shasta* and *Parvicapsula minibicornis* infection by QPCR in juvenile Klamath River Chinook salmon captured in K1 reach on the Klamath River (Trinity River confluence to Estuary). Sample numbers collected each week are displayed at the bottom of each column; Cs was not detected on 27 June and 3 Aug.

Histologically, 15 to 20 Chinook were collected in this reach every 2 weeks from 1 June – 27 July (Table 5). Given the hatchery release dates (IGH 19 May – 9 June, TRH 1 June – 15 June), and the migration time required for IGH Chinook to travel to this reach, the 1 June collection is likely natural origin salmon. Later sample groups could be mixed origin Chinook. It is noteworthy that the 1 June sample of natural salmon had both the highest prevalence of *C. shasta* infection and intestinal pathology score in this reach (Table 5 and Figure 13). A second peak in *C. shasta* prevalence and disease pathology score was observed in the 13 July collection group. Mean pathology score and prevalence of infection did not correlate in the 27 July sample. In general for this reach, Chinook salmon tended to be either in the early stage of infection (low pathology score and few trophozoites observed in the intestine) or late stage ceratomyxosis (e.g. 13-20% prevalence of systemic infection of liver or kidney).

Table 5. Prevalence of parasite infection (number positive / total (%)) and tissue abnormalities observed in Chinook collected from the Trinity R. to Estuary reach (K1). No gill tissue collected on 6/1 (n/d = not done).

	6/1	6/15	6/29	7/13	7/27
Kidney					
Pm Troph.	18 / 20 (90)	18 / 20 (90)	7 / 20 (35)	20 / 20 (100)	11 / 15 (73)
Pm Myxosp.	2 / 20 (10)	0 / 20 (0)	0 / 20 (0)	2 / 20 (10)	1 / 15 (7)
Metacercaria	2 / 20 (10)	3 / 20 (15)	9 / 20 (45)	16 / 20 (80)	9 / 15 (60)
C. shasta troph.	1 / 20 (5)	1 / 20 (5)	1 / 20 (5)	0 / 20 (0)	3 / 15 (20)
Chloromyxum sp.	2 / 20 (10)	2 / 20 (10)	4 / 20 (20)	0 / 20 (0)	0 / 15 (0)
Pathology Score	7.300	7.000	0.600	7.700	3.400
Intestinal tract					
C. shasta troph.	10 / 20 (50)	4 / 20 (20)	3 / 20 (15)	9 / 20 (45)	5 / 15 (33)
C. shasta myxosp.	1 / 20 (5)	1 / 20 (5)	0 / 20 (0)	2 / 20 (10)	0 / 15 (0)
Helminth	0 / 20 (0)	1 / 20 (5)	1 / 20 (5)	1 / 20 (5)	3 / 15 (20)
Pathology Score	3.400	0.700	1.950	2.050	0.533
Adipose steatitis	11 / 11 (100)	15 / 15 (100)	11 / 11 (100)	15 / 18 (83)	11 / 12 (92)
Adipose lipofuscin	3 / 11 (27)	3 / 15 (20)	0 / 11 (0)	4 / 18 (22)	4 / 12 (33)
Liver *C. shasta*	5 / 20 (25)	2 / 20 (10)	1 / 20 (5)	1 / 20 (5)	2 / 15 (13)
Gill					
Glochidia	n/d	4 / 20 (20)	3 / 20 (15)	1 / 19 (5)	0 / 20 (0)
Metacercaria		17 / 20 (85)	17 / 20 (85)	18 / 19 (95)	19 / 20 (95)
Invasive *C. shasta*		1 / 20 (5)	1 / 20 (5)	3 / 19 (16)	0 / 20 (0)
Amoeba		1 / 20 (5)	2 / 20 (10)	0 / 19 (0)	0 / 20 (0)
Multif. Hyperplasia		9 / 20 (45)	15 / 20 (75)	10 / 19 (53)	18 / 20 (90)

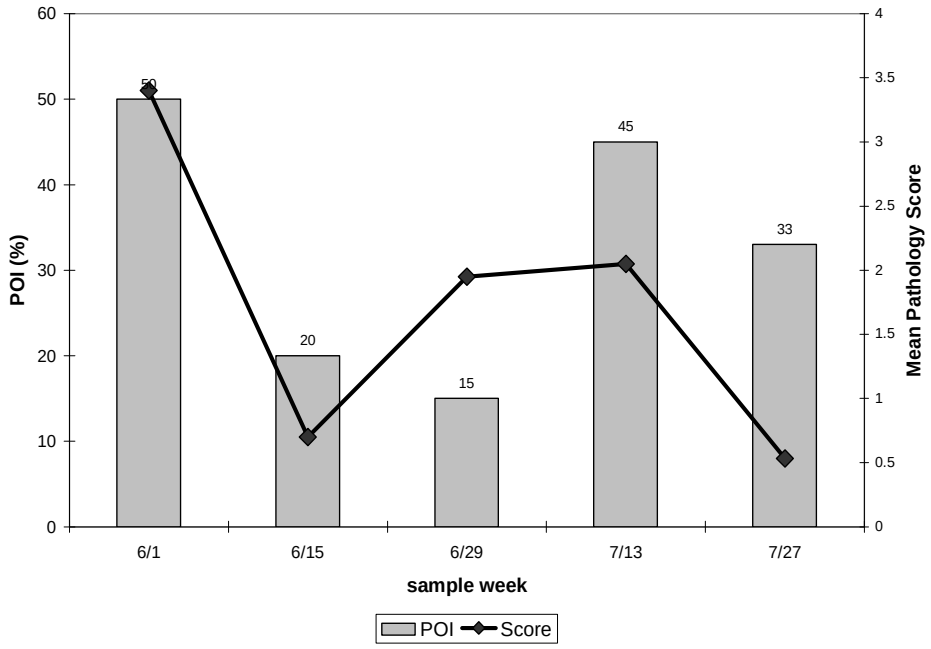

Figure 13. Prevalence of infection (bars) and mean intestinal pathology score (line) for *C. shasta* infections of Chinook collected in the Trinity to Estuary (K1) reach on the Klamath River. Sample numbers are displayed above each column bar.

Except for 29 June sample, *Parvicapsula minibicornis* infection was ≥ 73% in the sampled groups with high pathology scores (Table 5 and Figure 14). The majority of the kidney sections showed severe interstitial hyperplasia, tubule and glomerular pathology, and were grossly swollen. While similar for most collection dates, prevalence of infection pattern for *P. minibicornis* did not track *C. shasta* in the 15 June sample set (Figures 13 and 14). Encysted metacercaria in the gill was a common observation (≥ 85% prevalence per sample group).

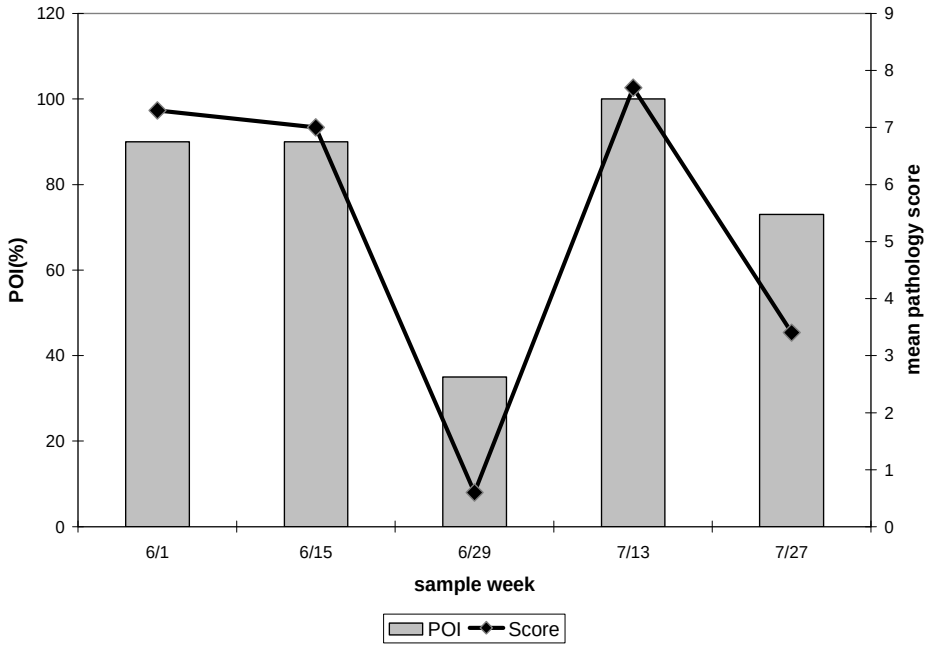

Figure 14. Prevalence of infection and mean kidney pathology score for *P. minibicornis* infections of Chinook collected in the Trinity to Estuary (K1) reach on the Klamath River. Sample numbers are the same for *C. shasta* and *P. minibicornis* histological assays, and are given in Figure 13.

Multi-focal lamellar hyperplasia without metacercarial cysts was also seen in 45 – 90% of the gill sections. It is likely metacercarial infection was associated with the hyperplastic lamellae but out of the field of the section.

No bacteria were seen in 5 hyperplastic gill sections (29 June) stained with McDonald's gram stain. Presumptive *C. shasta* trophozoites were seen 5 – 16% of the gill lamellae and vessels (Table 5).

Several other parasites were detected by histology in Chinook from the Trinity River and Trinity to estuary reach (K1). Another myxozoan trophozoite was observed in kidney glomeruli and tubules, and presumed to be *Chloromyxum* sp. based on differentiation from *P. minibicornis* by its elongated plasmodium and location within the Bowman's capsule and tubule lumen (rather than singular trophozoites within the vasculature of the mesangium) (Figure 15). Amoeba were also seen in a low number of gill sections in the Trinity River to Estuary (K1) reach but were not associated with epithelial hyperplasia (Figure 16). Metacercaria were common in Klamath River Chinook gill sections (61-96% prevalence per reach) but less so in Trinity River samples (20%). Many gills from Klamath River smolts had multi-focal regions of epithelial hyperplasia (41 – 66%. prevalence per reach) with no associated parasite.

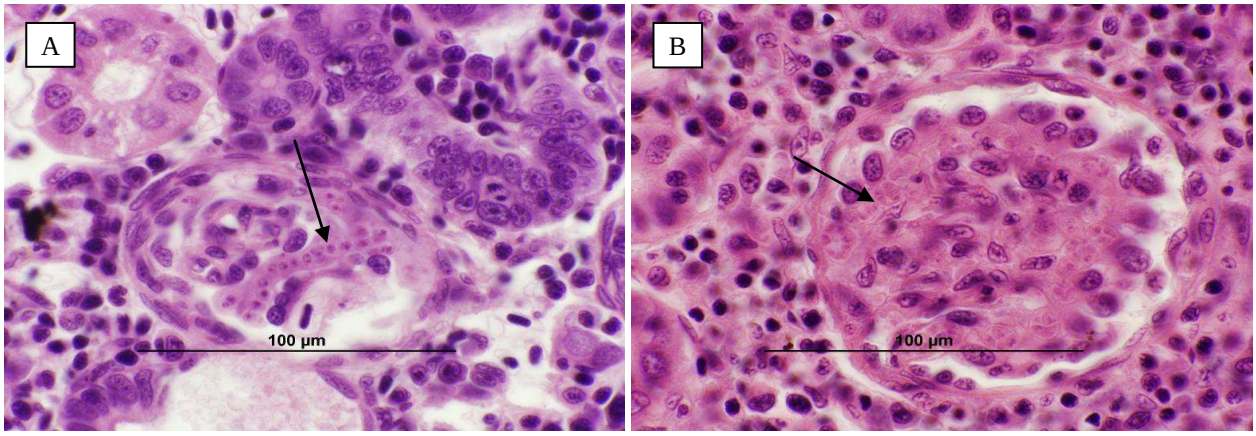

Figure 15. Presumptive *Chloromyxum sp.* (A) and *Parvicapsula minibicornis* (B) in kidney glomerulus of juvenile Chinook salmon. Micrograph (A) from lower Trinity River sample and (B) from Klamath River. H&E stain.

Figure 16. Presumptive amoeba associated with Chinook gill tissue (Trinity R. to estuary (K1) reach). H&E stain.

Klamath River Estuary

In the Klamath Estuary, *C. shasta* was detected by QPCR in 42% (87/207, ci=35-49%) of mixed-origin Chinook. Infection prevalence followed a normal bell shaped curve with the exception of the 1 June sample that consisted of a single positive fish. Infection prevalence peaked at 60% (18/30, ci=40-77%) in mid June and gradually decreased through the end of June and early July sample periods (Figure 17).

In the Klamath Estuary, *P. minibicornis* was detected by QPCR in 67% (138/207, ci=60-73%) of mixed-origin Chinook. Infection prevalence followed a normal bell shaped curve with the exception of the 1 June sample that consisted of a single positive fish. Infection prevalence peaked at 80% (24/30, ci= 61-92%) on 22 June and decreased to 20% (3/15, ci=4-48%) in the last sample collection from this reach on 6 July (Figure 17).

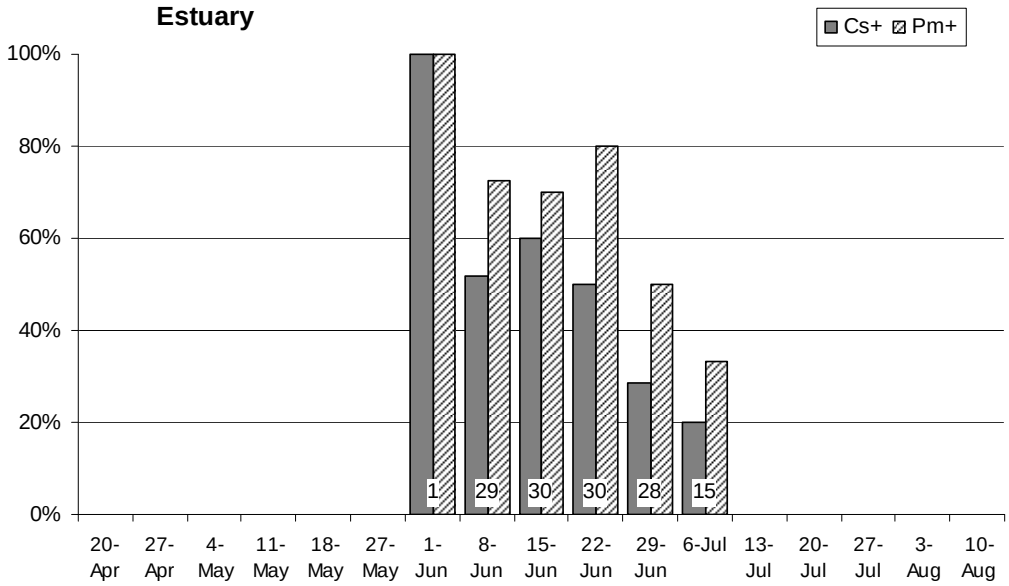

Figure 17. Weekly incidence of *Ceratomyxa shasta* and *Parvicapsula minibicornis* infection by QPCR in juvenile Klamath River Chinook salmon captured in the estuary (K0) reach of the Klamath River. Sample numbers collected each week are displayed at the bottom of each bar column. Note: Only 1 fish was collected and tested on 1 June.

Trinity River

Chinook salmon were collected from the upper Trinity River from the Pear Tree rotary screw trap (PTT) and from the lower Trinity River from the Willow Creek rotary screw trap (WCT) on 12 and 27 May. Kidney and intestine from each fish were tested by QPCR for parasite incidence of infection. *C. shasta* was detected in 12.1 % of these natural TR Chinook in the Trinity River, with no appreciable difference noted in the upper basin collection site (PTT = 12.1%) compared to the lower basin site (WCT – 11.6%). *P. minibicornis* was detected in 5.7% of Chinook examined from the Trinity River (PTT = 6.8% and WCT = 5.0%). Trinity River Hatchery coded-wire tagged Chinook were captured in the Trinity to Estuary (K1) and Estuary (K0) reaches, following TRH volitional releases, to evaluate parasite incidence of infection for TRH produced Chinook salmon (see CWT section for results).

Histological samples were collected at both Pear Tree and Willow Creek rotary screw traps on 12 and 27 May (Table 6). Neither *C. shasta* nor *P. minibicornis* parasites were observed on these sample dates. The myxozoan parasite *Chloromyxum* sp. was seen in glomeruli of 16 of 39 (41%) kidney sections and was differentiated from *P. minibicornis* by its elongated plasmodium and location within the Bowman's capsule and tubule lumen. No inflammation was associated with this parasite. Metacercaria were observed in 13% of the kidney sections and 20% of gill sections. Unlike previous years, glochidia were not detected in gill sections of Trinity River Chinook (Table 6).

Table 6. Prevalence of parasite [no. positive / total (%)] infection and tissue abnormalities observed in Chinook collected from Trinity R. rotary screw traps at Pear tree trap (rm 94) and Willow Creek (rm 14) on 12 and 27 May. Poor gill fixation prevented evaluation for hyperplasia in 12 May samples (n/d).

	Pear tree 5/12	Pear tree 5/27	Willow Crk 5/12	Willow Crk 5/27
Kidney				
Pm Troph.	0 / 10 (0)	0 / 9 (0)	0 / 10 (0)	0 / 10 (0)
Pm Myxosp.	0 / 10 (0)	0 / 9 (0)	0 / 10 (0)	0 / 10 (0)
Metacercaria	0 / 10 (0)	2 / 9 (22)	1 / 10 (10)	1 / 10 (10)
C. shasta troph.	0 / 10 (0)	0 / 9 (0)	0 / 10 (0)	0 / 10 (0)
Chloromyxum sp.	5 / 10 (50)	2 / 9 (22)	1 / 10 (10)	8 / 10 (80)
Pathology Score	0.000	0.000	0.02*	0.000
Intestinal tract				
C. shasta troph.	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 9 (0)
C. shasta myxosp.	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 9 (0)
Helminth	0 / 10 (0)	1 / 10 (10)	0 / 10 (0)	1 / 9 (11)
Pathology Score	0.000	0.000	0.000	0.000
Adipose steatitis	1 / 6 (17)	3 / 3 (100)	1 / 5 (20)	2 / 2 (100)
Adipose lipofuscin	2 / 6 (33)	0 / 3 (0)	2 / 5 (40)	0 / 2 (0)
Liver *C. shasta*	0 / 10 (0)	0 / 9 (0)	0 / 9 (90)	0 / 9 (0)
Gill				
Glochidia	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)
Metacercaria	0 / 10 (0)	8 / 10 (80)	0 / 10 (0)	0 / 10 (0)
Invasive *C. shasta*	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)
Amoeba	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)
Multif. Hyperplasia	n/d	0 / 10 (0)	n/d	0 / 10 (0)

* multifocal necrosis in kidney, no parasites in section

Diagnostic Evaluations of Moribund Chinook in the Trinity R. to Estuary Reach (K1)

Diagnostic examinations were conducted to assess the presence and prevalence of other major fish pathogens and parasites. White spots were seen in 13 – 29% of gills and due entirely to metacercaria (58 – 90% prevalence) and to a lesser extent encysted glochidial mussel larvae (10 – 50% prevalence) infection (Table 7). No *Ichthyophthirius* parasites were detected. *Flavobacterium columnare* bacteria (Columnaris disease) was observed in 13 – 29% of the sampled fish and was largely confirmed by gram-stain of the observed gill lesions. *Aeromonad-Pseudomonad* bacteria were isolated from 29 – 78% of the kidney cultures however it is unlikely that these infections were causing disease as gram-negative bacteria were not seen in associated spleen imprints. In addition, a common clinical sign of gram-negative bacterial septicemia (pinpoint or petechial hemorrhage on the skin and at fin base) was only seen in13-14% of the examined fish. *Ceratomyxa shasta* infection and ceratomyxosis were quite prevalent (50%) in the 8 July and 12 August collection.

On 12 August, five dead Steelhead adults were found in Blue Hole (~0.5 mi upstream of Blue Creek). All these fish had columnaris gill lesions and hemorrhagic intestines. Histological samples were assayed from a moribund Steelhead trout (160mm FL) captured at the mouth of Blue Creek and diagnosed with severe ceratomyxosis. Ceratomyxosis was also diagnosed histologically in 1 Chinook salmon on 12 Aug with bilateral exophthalmia and external fungal infection of the eye. Trophozoites were seen in the kidney but not in the intestinal tract of this fish. While *P. minibicornis* was detected in 40 – 80% of the histological sections of kidney, it was associated with morbidity in the 18 June sample; these fish had swollen kidneys and were anemic. It is likely that *Parvicapsula* was the primary pathogen associated with disease in the 18 June fish as *C. shasta* was only observed in one fish in the collection group (Table 7).

Table 7. Moribund Chinook diagnostic evaluation data: frequency of clinical signs and pathogen detection. Column abbreviations include: Hem/Cat = hemorrhagic or catarrhal exudates within intestine, Pet Hem = petechial hemorrhages at fin base or ventral skin, AP = *Aeromonad-Pseudomonad* bacteria, Staph = *Staphylococcus sp.*, Fc = *Flavobacterium columnare*, F-GNR = Filamentous gram negative rod (presumptive for *F. columnare*, imprt = gram stained spleen imprint, Intes Path Score (+) = mean pathology score for intestinal sections with *C. shasta*, KD Path Score (+) = mean pathology score for kidney sections with *P. minibicornis.*

	6/18	7/8	8/12
Creek mouth sites	Tec Tah Lower Crow	Pecwan, Blue Tec Tah	Tec Tah, Blue, Roach
Water temp.	20C	21C	21.8C
Sample No.	14	15	15
Forklength range	60 - 80 mm	75 – 95 mm	78 – 90 mm
<u>Clinical signs:</u>			
White spot-gill	4 / 14 (29)	4 / 15 (27)	2 / 15 (13)
Pale gill color	10 / 14 (71)	5 / 15 (33)	3 / 15 (20)
Swollen kidney	11 / 14 (79)	5 / 15 (33)	3 / 15 (20)
Ascites fluid	0 / 14	3 / 15 (20)	1 / 15 (7)
Hem/Cat Intestine	1 / 14 (7)	4 / 15 (27)	5 / 15 (33)
Pet Hem fin base	2 / 14 (14)	2 / 15 (13)	2 / 15 (13)
Notes:	1 bilateral exophthalmia	1 Lamprey wound	Dead steelhead
Fc lesion	4 / 14 (29)	2 / 15 (13)	3 / 15 (20)
Gram stain F-GNR	2 / 3	2 / 2	2 / 3
Cultured Bacteria	5 / 14 AP 3 / 14 Staph	2 / 7 AP	7 / 9 AP
GNR-spleen imprt	0 / 13	0 / 14	n/d
C. shasta-histology	1 / 10	6 / 16 (50)	5 / 10 (50)
Intes Path Score (+)	0.0	6.2	2.6
P. minibicornis-histology	8 / 10 (80)	9 / 12 (75)	4 / 10 (40)
KD Path Score (+)	**8.3**	3.1	4.8
Metacercaria-gill	7 / 10 (70)	7 / 12 (58)	9 / 10 (90)
Amoeba- gill	0 / 10	2 / 12 (17)	0 / 10
Glochidia - gill	5 / 10 (50)	4 / 12 (33)	1 / 10 (10)

Marked (CWT) Chinook Salmon

Iron Gate Hatchery

The constant fractional mark rate at Iron Gate Hatchery increased to 25% in 2009 (Buttars and Knechtle 2009) providing an opportunity to capture a larger proportion of IGH CWT Chinook in the monitoring study. The larger sample size for CWT analysis provides improved assessment of myxozoan infection level at weeks post hatchery release. *Ceratomyxa shasta* was detected in 52% (232/448, ci=47-57%) of all marked IGH Chinook screened by QPCR and 36% of IGH-CWT collected above the confluence of the Trinity River (Table 8).

Table 8. Historic incidence of *C. shasta* infection (% positive), as diagnosed 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, 1995-2009. Similar data for coded-wire tagged (CWT) fish 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	All Chinook (IG and TR Hatcheries, all reaches) Percent Positive by Assay		Trinity CWT- QPCR (Below TR confluence, K1/K0)	Iron Gate CWT-QPCR (Above TR confluence in reach K5, K4, and K2)
	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	1/67 (1%)	6/18 (33%)
2007	21	31	46/332 (14%)	15/22 (68%)
2008	37	49	8/257 (3%)	9/13 (69%)
2009	54	45	13/100 (13%)	82/228 (36%)
Average (SE)	**35% (4)**	**40 % (4)**	**10% (3)**	**54% (8)**

[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 tissue volumes were not standardized and cannot be directly compared to subsequent sample years.

C. shasta prevalence of infection in IGH CWT Chinook was higher this year in the initial weeks following hatchery release (0-2 WAL) compared to previous years. In 2008, *C. shasta* was not detected in IGH CWT Chinook recaptured at 1 WAL, rose to 8% in the 2 WAL group and peaked at 67% in the 3 WAL sample group. *C. shasta* prevalence of infection decreased at 4 WAL to 25% and decreased steadily through 6 WAL.

By contrast, in 2009 *C. shasta* prevalence of infection increased steadily from 19% in the 0 WAL (less than 7 days post release) group, to 41% at 1 WAL, peaked at 78% at 2 WAL, and generally followed a normal bell shape curve for 0-6 WAL sample groups (Figure 17). Very few CWT Chinook were recaptured that had resided for 7-10 WAL post hatchery release (1 to 3 fish per WAL sample set). *C. shasta* was not detected in these longer rearing groups, with the exception of 1

positive fish in the 8 WAL sample group (8 weeks post hatchery release for this CWT code corresponds to capture of this marked fish during late July).

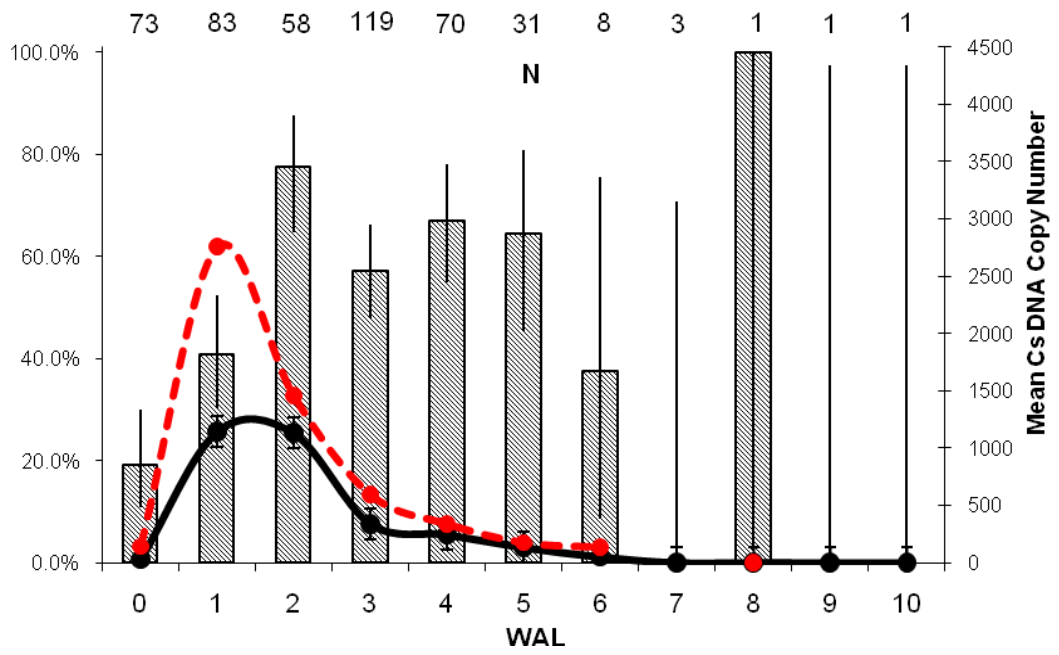

Figure 18. *Ceratomyxa shasta* prevalence of infection assayed by QPCR in Iron Gate Hatchery CWT Chinook recovered in the Klamath River by Weeks At Liberty (WAL); the number of weeks from hatchery release. The red dashed line indicates the *C. shasta* mean DNA Copy Number for positive results in the sample group (all positive fish tested in the WAL group); the black line is the mean *C. shasta* DNA Copy Number for the sample population (all fish tested, including negatives for a group). Whiskers are standard error for Cs POI and sample number (N) is located at the top of the chart above each bar.

For IGH CWT Weeks at Large (WAL) analysis, comparisons of parasite mean DNA copy number were graphed for positive test results and the entire sample population in Figures 18 (*C. shasta*) and 20 (*P. minibicornis*). Mean parasite mean DNA copy number for all positive fish in the sample group are represented by a red dashed line whereas mean DNA copy number for the entire sample population (all fish tested, including negative and positive test results) are graphed in a black solid line to illustrate the parasite DNA loads for the infected fish in the sample set, compared to the sample group as a whole.

For IGH CWTs, *C. shasta* parasite DNA levels for positive fish were high during initial exposure, as demonstrated in both the 1WAL group, and the slightly higher values observed in the 2-3 WAL groups when compared to the entire sample population. As longer exposure periods in the Klamath River main stem occurred in the 4-6 WAL groups, the mean parasite number for the sample group and the population are more closely aligned. The similar mean DNA copy number in the positive fish compared to the entire sample group, indicates a larger proportion of positive fish and similar parasite DNA levels in the sample population at that time, for these WAL exposure groups.

Incidence of *C. shasta* infection, by reach in which CWT Chinook were collected from, is shown in Figure 19. The highest incidence of *C. shasta* infections in IGH CWT occurred in the Trinity to Estuary (K1) and Estuary (K0) reaches, at 67% and 65% respectively. The proportions of total IGH CWT Chinook recovered in each reach were fairly well distributed among all reaches. Approximately 75% of IGH CWTs were equally distributed among three reaches: Shasta to Scott (K4) at 26.1%, the Trinity to Estuary (K1) at 24.1%, and the Estuary (K0) at 24.8% respectively. The remaining 25% of CWTs were recovered in roughly equal proportions from the IGD to Shasta reach (K5) at 12.7% and the Salmon to Trinity reach (K2) at 12.3%. The upper reach from IGD to the Shasta (K5) and the Salmon to Trinity (K2) reach had the lowest incidence of *C. shasta* infection, at 9% and 38% respectively.

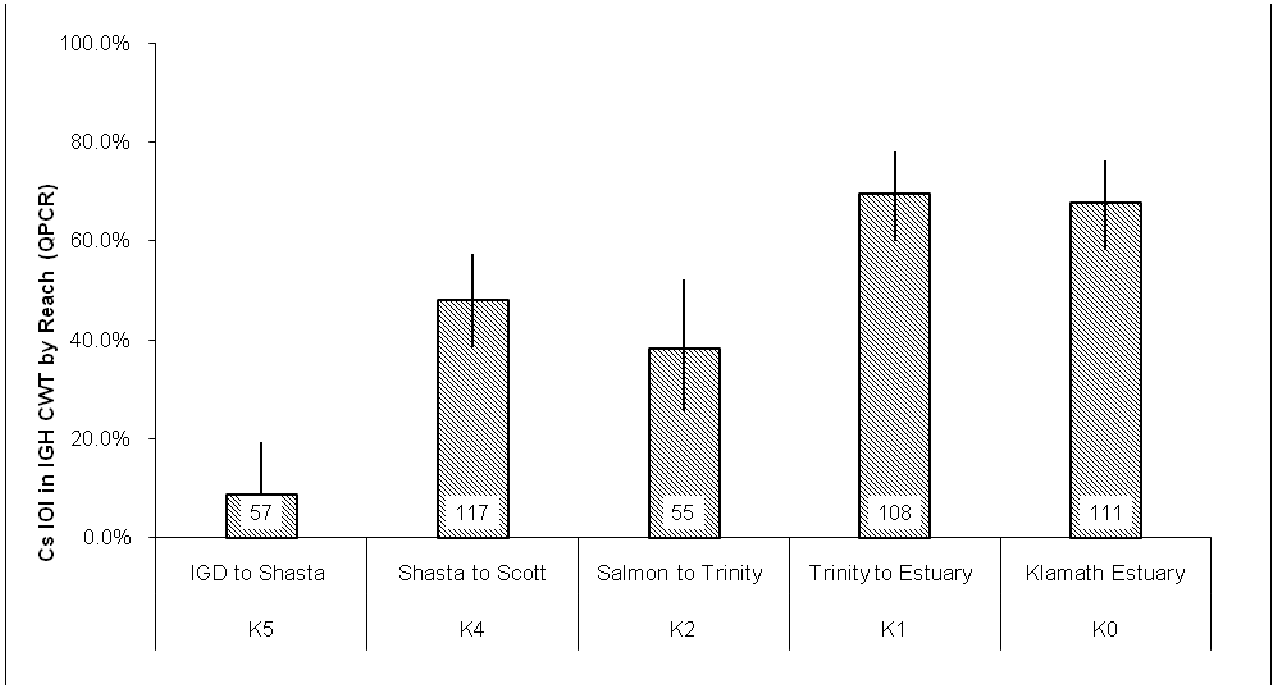

Figure 19. *Ceratomyxa shasta* incidence of infection by QPCR in Iron Gate Hatchery CWT by reach that marked Chinook were collected from. Whiskers indicate 95% confidence interval and sample number is at the base of each bar.

Parvicapsula minibicornis was detected in 86% (386/448, ci=83-89%) of all marked IGH Chinook salmon screened by QPCR. Incidence of infection was 41% (30/73, ci= 30-53%) in Chinook collected within 6 days of release (<7 days post release = 0 WAL) and rose rapidly to 83% by 1 WAL and 93% by 2 WAL (Figure 20). *P. minibicornis* was detected in 100% (70/70) of IGH CWT Chinook by 4 WAL. IGH CWT sample numbers for 6-10 WAL were very small, with 1 – 8 fish per collection group, however *P. minibicornis* prevalence remained at 100% in fish collected from 17 July to 10 August (WAL 7-10). Due to the nearly 100% incidence of *P. minibicornis* observed in juvenile Chinook in the Klamath River during June and July, the mean DNA copy number for *P. minibicornis* positive fish compared to the entire IGH-CWT sample population are nearly identical with the exception of initial exposure at < 1 WAL.

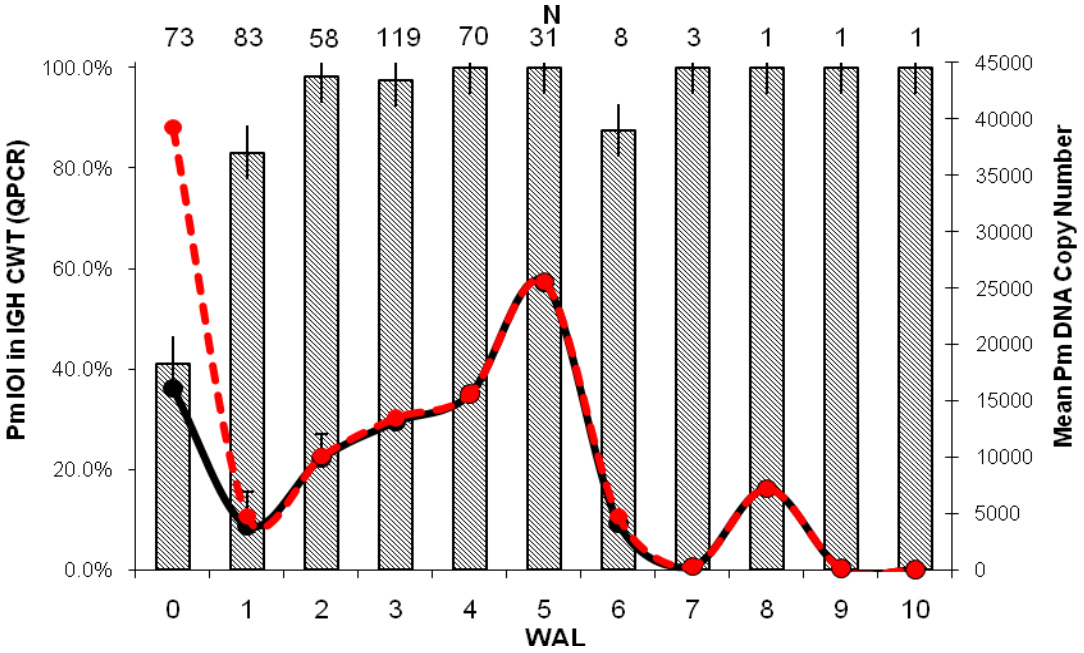

Figure 20. *Parvicapsula minibicornis* incidence of infection assayed by QPCR in Iron Gate Hatchery CWT Chinook recovered in the Klamath River by Weeks At Liberty (WAL); the number of weeks from hatchery release. The red dashed line indicates the *P. minibicornis* mean DNA Copy Number for positive samples in the group (all positive fish tested at each weekly interval); the black line is the mean *P. minibicornis* DNA Copy Number for the sample population (all fish tested, including negatives). Whiskers indicate standard error for Pm POI and sample number (N) is located at the top of the chart above each bar.

Incidence of *P. minibicornis* infection, by reach in which IGH CWT fish were recovered from, followed a similar trend as previous monitoring studies (Figure 21). Chinook are infected with *P. minibicornis* in the upper two reaches and incidence of infection in the Salmon to Trinity reach (K2) through the Estuary reach (K0) is near, or at 100%.

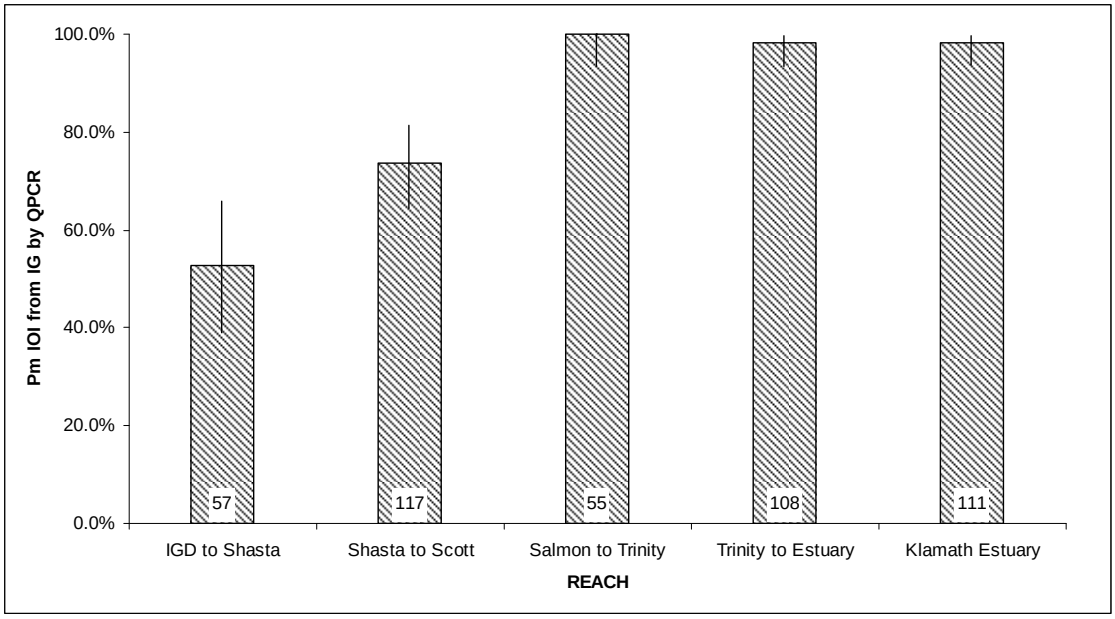

Figure 21. *Parvicapsula minibicornis* incidence of infection assayed by QPCR in Iron Gate Hatchery CWT Chinook recovered in the Klamath River by weeks since hatchery release (WAL). Whiskers indicate 95% confidence interval and sample number is at the base of each bar.

Trinity River Hatchery

Ceratomyxa shasta was detected in 13% (14/109, ci=7-20%) of the marked TRH Chinook screened by QPCR. Chi square analysis found this incidence was not statistically significant compared to *C. shasta* prevalence of infection levels (12.1%) observed in natural Chinook sampled in the upper and lower Trinity River basin in mid to late May. *Ceratomyxa shasta* was detected in 33% (3/9, ci=8-70%) of Chinook recaptured at 1 WAL and fell to 14% (2/14, ci=2-43%) in the 2 WAL group. A similar trend occurred in 5-6 WAL groups where *C. shasta* was detected in 31% and 14% of the groups respectively (Figure 22).

For TRH CWT Weeks at Large (WAL) analysis, comparisons of parasite mean DNA copy number for the positive test results and the entire sample population are illustrated in Figures 22-23. Mean parasite mean DNA copy number for all positive fish in the sample group are represented by the red dashed line whereas mean DNA copy number for the entire sample population (all fish tested, including negative and positive test results) are graphed as a black solid line. These graphs illustrate the parasite DNA loads for the infected fish in the sample set, compared to the sample group as a whole.

C. shasta DNA copy number tracked peak prevalence well for both fish testing positive and for the entire TRH CWT sample population (mean copy number for all fish tested in the sample set, including negatives, Figure 22). The highest *C. shasta* DNA copy number occurred on initial

exposure of fish post release (less than 7 days = 0 WAL). The highest infectious load for the population (all fish tested) occurred by 1 WAL. A similar increase in *C. shasta* DNA levels occurred at 5 WAL sample group (and 9 WAL, although this sample set included only 1 fish).

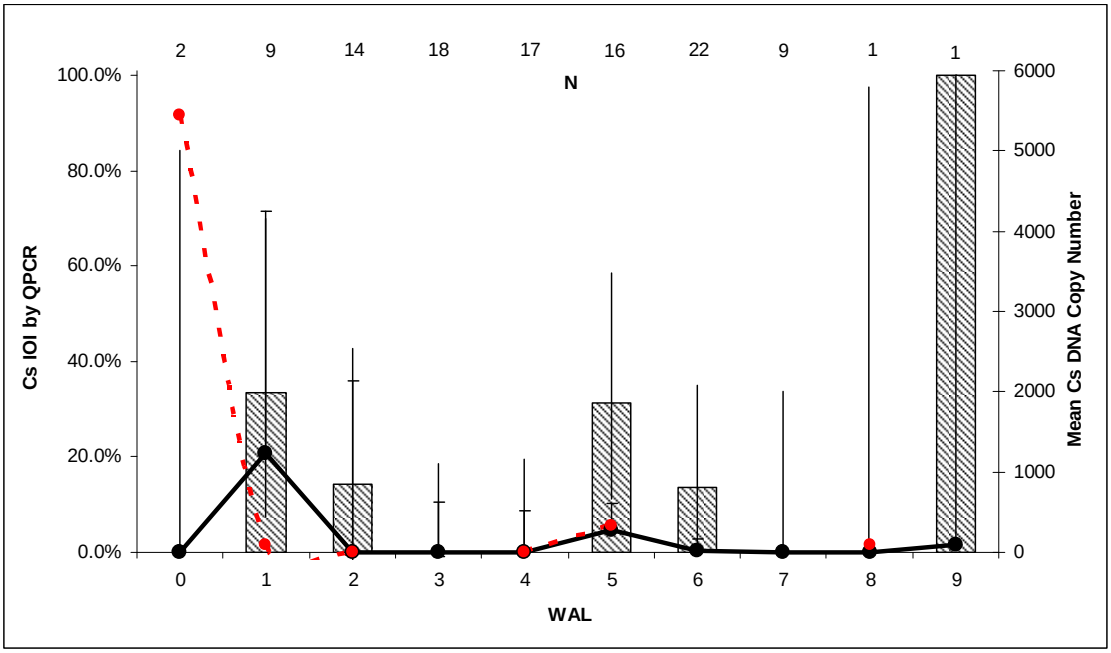

Figure 22. *Ceratomyxa shasta* incidence of infection and mean DNA copy number by QPCR in Trinity River Hatchery CWT Chinook salmon recovered in the Klamath River by Weeks At Liberty (WAL). Whiskers indicate 95% confidence interval and sample number is at the base of each bar. The red dashed line indicates the mean DNA Copy Number for positive samples in the WAL groups (all positive fish tested); the black line is the mean *C. shasta* DNA Copy Number for the sample population (all fish tested, including negatives). Whiskers indicate 95% confidence interval and sample number is located at the top of the chart above each bar.

Parvicapsula minibicornis was detected in 26% (28/109, ci= 18-35%) of the TRH CWT Chinook screened by QPCR. Prevalence was 22% (2/9, ci=3-60%) in fish representing the 1 WAL group (22 June) and rose to 55% (12/22, ci= 32-76%) by 6 WAL (20 July). DNA copy number was high (>15,000 copies) on initial exposure, and a second peak in parasite DNA copy number (>5000 copies) occurred in the 5-6 WAL sample groups, which corresponds to CWT capture dates between 13-20 July (Figure 23).

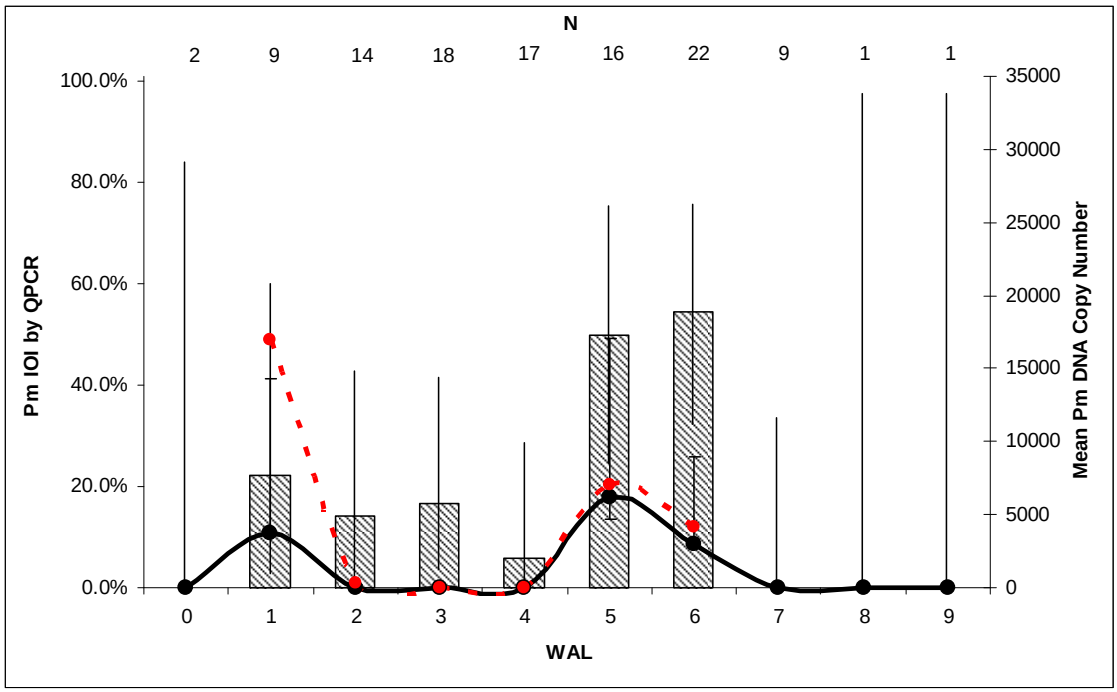

Figure 23. *Parvicapsula minibicornis* incidence of infection and mean DNA copy number by QPCR in Trinity River Hatchery CWT Chinook salmon recovered in the Klamath River by weeks since hatchery release (WAL). The red dashed line indicates the mean DNA Copy Number for positive samples in the WAL groups (all positive fish tested); the black line is the mean *P. minibicornis* DNA Copy Number for the sample population (all fish tested, including negatives). Whiskers indicate 95% confidence interval and sample number is located at the top of the chart above each bar.

Annual Comparison- Histology Above the Trinity River Confluence

Annual comparison of myxozoan incidence of infection for juvenile Chinook collected above the Trinity confluence, between May and July, are provided to assess trends in parasite prevalence by histology, and annual incidence as it relates to past monitoring studies.

Histology

The incidence of *C. shasta* by histology in mixed-origin Chinook captured during May, June and July in the Klamath River above the confluence of the Trinity River was 54% (50/93, ci= 43-64%), the highest observed compared to previous years (Table 9). While the trophozoite stage was prevalent, observations of myxospore stages of both *C. shasta* and *P. minibicornis* were uncommon (only 4-5% in the Trinity River to estuary reach). The prevalence and severity of myxospore infection determined by histology cannot be expanded to the entire Klamath River smolt population as the collections were done by beach seine and did not target moribund or dead fish.

Table 9. Historical incidence of infection for C. *shasta* and *P. minibicornis* in histological sections of intestine and kidney (respectively) from juvenile Chinook salmon collected between May 1 and July 30 in the Klamath River (Iron Gate dam to confluence of Trinity R.)

	2004	2005	2006	2007	2008	**Average 2004-2008**	**2009**
C. shasta	34%	35%	21%	21%	37%	**30%**	50 / 93 (**54%)**
P. minibicornis	77%	92%	58%	81%	89%	**79%**	79 / 93 (**85%)**
N	735	134	112	81	116		

The incidence of *C. shasta* infection, as determined by histology, was highest in the Shasta to Scott reach (K4) in 2009 at 54% while incidence was 44% for the Scott to Trinity (K2) and 33% for the Trinity to Estuary (K0) reaches.

The incidence of *P. minibicornis* by histology in mixed-origin Chinook captured during May, June and July in the Klamath River above the confluence of the Trinity River was 85% (79/93, ci= 76-91%). *Parvicapsula* infection was $\geq$ 73% in the 3 Klamath reach collection groups (K4, K3 and K2) in 2009, with a majority of the kidneys showing severe inflammatory response. Compared to previous studies, the overall incidence of *P. minibicornis* infection in mixed-origin Chinook was average, with levels comparable to 2007 and 2008, and lower than 2005 (Table 9).

DISCUSION

The incidence of both *C. shasta* and *P. minibicornis* infections in juvenile Chinook salmon has been monitored in fish health studies in the Klamath River since 2004 (Nichols and Foott 2006; Nichols et al. 2007; Nichols and True 2007; Nichols et al. 2008). The May-July 2009 incidence of *C. shasta* by histology (54%), the metric used for annual comparisons, is the highest level observed during the Klamath River fish health monitoring studies conducted from 2005 to 2009. The incidence of *P. minibicornis* by histology in Chinook salmon was similar to the highest levels observed in 2007 (81%) and 2008 (89%), but lower than the record level of observed in 2005 (92%).

Pre-release exams of Iron Gate and Trinity Hatchery Chinook indicate that hatchery fish were exposed to *C. shasta* and *P. minibicornis* at both facilities prior to release. *C. shasta* prevalence of infection was 2% at IGH by QPCR and undetected by the less sensitive histological screening method. *C. shasta* prevalence of infection was higher in TRH Chinook at 19% by QPCR, compared to IGH Chinook. *P. minibicornis* prevalence of infection was low (2%) for IGH Chinook and 17% for TRH Chinook, similar to the TRH *C. shasta* prevalence of infection of 19%. The parasite DNA levels for both myxozoans on pre-release exams were very low, indicating exposure of hatchery Chinook to these parasites, but at levels considered below those required to induce a diseased state.

Natural fish incurred early infections in 2009, and higher incidence of infection compared to mixed origin Chinook sampled after hatchery releases. In the Shasta to Scott (K4) reach, the peak weekly prevalence of *C. shasta* infections (97%) was observed in natural fish sampled in early May (hatchery releases began 19 May). We assume this high infection rate is a result of residing in highly infectious waters, and that naturally produced Chinook that rear in the main stem have a greater probability to incur disease mortality than hatchery Chinook and tributary fish which spend less time in the Klamath River. Natural fish sampled in the Salmon to Trinity (K2) reach had lower *C. shasta* prevalence of infection (60%) in mid May, compared to the Shasta to Scott (K4) reach. *C. shasta* was not detected in 30 natural Chinook sampled 4 May, however prevalence increased to 60% by 18 May, before hatchery Chinook entered the system. It is also noteworthy that the 1 June sample of natural Chinook in the Trinity to Estuary (K1) reach, sampled by histology, had the highest prevalence of *C. shasta* infection and intestinal pathology score of all sample groups collected in this downstream reach. In summary, natural fish experienced earlier infections and high *C. shasta* incidence of infection levels in both the upper Shasta to Scott (K4) reach and the lower Trinity to Estuary (K1) reach by both QPCR and histological assays in 2009.

Similar to naturally produced Chinook, we observed higher *C. shasta* incidence of infection in mixed origin Chinook sampled after IGH releases, in the Shasta to Scott (K4) and Salmon to Trinity (K2) reaches. *C. shasta* prevalence followed a bimodal distribution in the Shasta to Scott (K4) reach, related to presence of natural fish (prior to hatchery release) and mixed origin Chinook (post hatchery release). As hatchery Chinook entered the main stem and became infected in late May and early June, *C. shasta* prevalence of infection in mixed origin Chinook rose to >40% by 1 June and peaked at 65% within 3 weeks, on 22 June. It is noteworthy that the highest incidence of *C. shasta* trophozoites in gill tissue by histology, an indication of early infection pathology associated with parasite entry into host tissue, was observed in this reach. Additionally, parasite infectious load, measured as *C. shasta* DNA copy number present in fish tissue, is highly suggestive of an alternating cycle of high and low prevalence of infection based on when Chinook cohort groups are becoming infected.

In the Salmon to Trinity (K2) reach, *C. shasta* was detected by QPCR in 44% (139/318, ci=38-49%) of mixed-origin Chinook. *Ceratomyxa shasta* prevalence of infection rose rapidly to 95% in early June, two weeks after IGH releases. *Ceratomyxa shasta* prevalence decreased by 29 June and remained <40% throughout the remainder of the sample period (with the exception of 6 July where prevalence reached 75% in a small sample set of 8 fish).

In addition to higher *C. shasta* incidence of infection in natural and mixed origin Chinook in the upper reaches (K4 and K2), we observed higher infectivity levels in the Trinity to Estuary (K1) reach in 2009. In past monitoring studies, the upper reaches from the Shasta and Scott (K4) and Scott and Salmon (K2) have produced the highest overall *C. shasta* incidence data for mixed origin Chinook. Conversely, the Trinity to Estuary (K1) reach historically had lower infection levels than the upper Klamath River reaches, presumably due to the influx of uninfected Trinity River Chinook. In 2009, *C. shasta* incidence of infection by QPCR (50%) in this reach was not statistically different (P=0.213) from incidences observed in the two reaches above the Trinity confluence: Shasta to Scott = 50%, and the Salmon to Trinity = 44%. However, when *C. shasta* incidence of infection in the Trinity to Estuary (K1) reach was compared to prior years, the level observed in 2009 was significantly higher than the past three study years: 12.8% observed in 2008 (P=0.001), 16.2% in 2007 (P= 0.001) and 19.1% in 2006 (P= 0.001). It is also noteworthy that *C. shasta* incidence of infection by histology (33%) was the highest observed in this reach, for mixed origin Chinook since monitoring studies were initiated in 2004. Histological observations for the Trinity to Estuary (K1) reach confirmed a dichotomous infection pattern for *C. shasta;* Chinook were observed to be either in the early infection stages with few trophozoites and no tissue damage, or advanced states of systemic Ceratomyxosis. This infection pattern, along with the high *C. shasta* prevalence of infection and changes in bi-weekly DNA copy number indicate newly infected Chinook were entering this sample reach, while severely infected fish were incurring mortality.

In the Estuary (K0), the incidence of infection in mixed origin Chinook of *C. shasta* and *P. minibicornis* were 42% and 67% (respectively); levels comparable to previous study years (Nichols and Foott 2006; Nichols et al. 2007; Nichols and True 2007; Nichols et al. 2008).

In 2009, the constant fractional mark at IGH increased to 25% which facilitated collection of over 550 coded wire tagged Chinook (448 IGH CWT and 109 TRH CWT). Coded-wire tagged data provides important information about disease progression for hatchery fish as it relates temporal exposure (WAL relationship to infection levels) and spatial exposure (infection incidence associated with various reaches). IGH Chinook CWTs were collected from the Shasta to Scott (K4), Scott to Salmon (K3), Salmon to Trinity (K2), Trinity to Estuary (K1) and Estuary (K0) reaches, whereas TRH CWTs were collected from K1 and K0 reaches only. The recovery rate of IGH CWT Chinook analyzed for this study was 0.036% (448/1,257,801 CWT Chinook) of the total 5.0 M Fall-run Chinook released in 2009.

While IGH CWT had low prevalence of infection (2%) for both parasites on pre-release exams, Chinook became infected with *C. shasta* immediately following release and prevalence increased to 40% in IGH CWT Chinook residing in the main stem less than 1 WAL (up to 6 days post release). Prevalence of *C. shasta* in this group rose rapidly to over 80% in the 2 WAL group. *P. minibicornis* reached nearly 100% prevalence by 3 WAL and incidence remained high throughout the remainder of the monitoring period.

The incidence of *C. shasta* infection in TRH CWT in 2009 was 13%, comparable to levels observed in 2007 (14%), but higher than incidence typically observed (2006 = 1%, and 2008 = 3%). The *C. shasta* incidence of infection for this group of fish, including 2009 and all previous study years, averages 10% (Table 8). Trinity River hatchery CWT Chinook had higher *C. shasta* incidence of infection than IGH CWT on pre-release exams conducted in 2009. The higher incidence of *C. shasta* infection in marked TRH Chinook in 2009 was statistically significant compared to the 3% level observed in 2008 (P= 0.0003) and the 1% observed in 2006 (P=0.0047), but was not significant when compared to the 14% observed in 2007 (P=0.8501).

For the Weeks at Large assessment, the highest prevalence of *C. shasta* infection in TRH CWT Chinook was 33% in the 1 WAL group. A second peak occurred in the 5-6 WAL groups where *C. shasta* was detected in 31% and 14% of the Chinook sampled, respectively.
In contrast to 2009 data, *C. shasta* was not detected in 2008 TRH CWT Chinook until 5 WAL. TRH Chinook appear to have experienced an earlier exposure to *C. shasta* and higher incidence of infection in 2009 in the Trinity River, as well as in the Klamath River below the Trinity River confluence.

In summary, we observed increased *C. shasta* incidence of infection in the Klamath River Fish Health Monitoring Program for natural fish, Iron Gate Hatchery Chinook and Trinity Hatchery Chinook in 2009. Trinity Hatchery Chinook had higher levels of both *C. shasta* and *P. minibicornis* on pre-release examination compared to IGH Chinook, indicating exposure to these myxozoan parasites prior to hatchery release in early June. Trinity Hatchery CWT Chinook *C. shasta* incidence of infection levels were higher in the Trinity to Estuary (K1) reach than in 2 of 3 previous study years. Of special concern in 2009, is the apparent extension of the infectious zone into the reaches below the Trinity River confluence, where *C. shasta* incidence of infection in mixed origin Chinook was not statistically different from levels observed in the Shasta to Scott (K4) and Salmon to Trinity (K2) reaches.

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AUTHOR ROLES
The contributions of each author have been summarized below.

- Kimberly True – project coordination, data management and statistics, QPCR methods and analysis, DNA extraction and molecular assay QA, assembly and editing of final report.
- Scott Foott – histology, diagnostic assessments, and histological report sections.
- Anne Bolick - Necropsy, DNA extraction, QPCR assay, assistance with report figures and charts.
- Scott Benson – Necropsy, DNA extraction and QPCR assays.
- Ryan Fogerty – Field collection, histology processing and necropsy.

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APPENDIX I - Reviewers' comments

Listed below are the paraphrased comments by reviewers of a draft of this report and the authors' replies.

Reviewer #1

1) Thank you for the opportunity to review the 09 report. I read it and did not find anything to comment on. I found it very thorough and very informative.

Reviewer#2

Title

Summary

Introduction

Methods

1) Page 7 - Question regarding the term "grossed" - necropsy? dissection? I am not familiar with this term.

"Grossed" or "grossing" are abbreviated forms of gross examination, which is the process by which pathology specimens are inspected with the naked eye to obtain diagnostic information, while being processed for further microscopic examination. The word "grossed" was replaced with "gross examination" in the text.

Results

2) Page 26 - Use the chloromyxum description below, when Chloromyxum is first described.
'Chloromyxum sp. was differentiated from P. minibicornis by its elongated plasmodium and location within the Bowman's capsule and tubule lumen (rather than singular trophozoites within the vasculature of the mesangium)'.

Moved the description to appear when Chloromyxum sp. is first described on page 23.

3) Page 24-25, Table 6 & 7 – Keep date format consistent with other tables.

Table 6 & 7 formatting changed to month/day formatting (i.e., 5/12), consistent with other histology tables.

4) Page 27 – any specific lesions (in reference to *Flavobacterium columnare* findings).

Lesions were observed in gill tissue – edited "lesions" to "gill lesions".

5) Page 38 – What are these P values from, there is no mention of statistics in the methods.

The lead author neglected to include the statistical methods in the document sent to reviewers. Statistical methods are described in the Methods section, and appropriate text where needed. Reviewers 2 and 3 were asked to review the statistical methods in a second review, prior to completion of the final draft.

6) Minor edits for sentence structure, spacing, and spelling were corrected per Reviewer's comments.

Discussion

7) One overall comment is regarding the use of prevalence vs. incidence, I know you defined the two in the Methods, but they seem to be used interchangeably in the discussion.

Prevalence and incidence of infection definitions were provided in the Methods section as follows: prevalence of infection (defined as cases of disease present at a given time) and incidence of infection or disease (defined as the frequency of occurrence over a period of time, or for the population as whole). For discussions regarding a weekly infection level or trends over a partial period comprising the entire sampling period (i.e., the month of May) prevalence is used. In the discussion section, incidence is used to describe the overall frequency for a distinct group of fish or sub-population (i.e., CWT Chinook, or fish collected within one reach), or for the population as a whole for annual comparisons. Discussion section was reviewed for proper usage, and edited as appropriate to the specific data and content being addressed.

Reviewer#3

Title
Summary

1) Page 2 - There should be a space between "*C.*" and "*shasta*" and *P. minibicornis*.

Corrected spacing for all references to *C. shasta* and *P. minibicornis*.

2) Page 2 - What does "CWT" stand for?

Specified abbreviation for coded-wire tagged (CWT) in first occurrence.

Introduction

3) Page 3 - "coho is not capitalized, but "Chinook" is capitalized.

Checked and corrected all capitalization for references to Chinook and coho.

Methods

4) Page 4- Any way to label the map with the reach codes? Also the print on this map is small and difficult to read…can the print be made larger, or maybe just enlarge the whole map?

The map was enlarged and reach labels added.

Results
Discussion

5) Page 37 - It seems interesting that infection rate is lower in K2 than in K4, since K2 is downstream of K4. I realize this isn't new information, but since this is the discussion section, are you interested in hypothesizing as to the cause? Is it simply that habitat for the worms is less abundant/available/suitable, or do you suspect a different mechanism?

Stocking (2006) found the reaches below IGD more infectious for Chinook than lower reaches such as K2, primarily due to polychaete habitat and abundance in the upper section of the Klamath River. I think the larger question may be s whether proximity to polychaete habitat/abundance is a stronger factor in assessing disease risk, than exposure period in the main stem (for example IGH Chinook sampled in the lower reaches are presumed to have experienced longer exposure periods than TRH entering lower in the river). The report discusses the cyclical nature of prevalence data for mixed origin Chinook, as newly infected fish enter the main stem, migrate through reaches, and incur mortality. While this is not a mechanism per se, several studies have verified expected mortality levels for multiple temperature regimes, including those that constitute a "typical river year" for outmigrant Chinook (i.e., 15-18°C in June). The Chinook we sample in K2 are a mixed origin population, including natural production from tributaries, and the influx of TRH Chinook entering below the TR confluence. Therefore we cannot discern spatial exposure (initial exposure reach or number of reaches) or temporal exposure (how long they have been in the main stem). The fact that K2 is below K4 does not necessarily mean we'd expect to see higher infectivity down river, especially over a distance of ~80 river miles (the upper limit of K2 (RM66) and the lower limit of K4 (RM144)). We rely on marked Chinook, with known residency

time in the main stem and reach recapture location, to provide data about spatial and temporal exposure period as it relates to observed disease status.

6) Page 37- In reference to the following statement, "In summary, natural fish experienced earlier infections and high *C. shasta* incidence of infection in both the upper Shasta to Scott (K4) reach and the lower Trinity to Estuary (K1) reach by both QPCR and histological assays in 2009." – What do you think might be the cause?

Environmental conditions, such as drought conditions over the past three years and the subsequent reduction in annual flows, may be associated with the increase disease incidence we observed in natural fish in 2009, as well as the increased *C. shasta* incidence observed in the lower Trinity to Estuary (K1) reach. The specific environmental factors involved, and how they relate to disease susceptibility, are beyond the scope of this report.

7) Page 38 - In reference to the following statement, "*Ceratomyxa shasta* incidence of infection by histology (33%) was the highest observed in mixed origin Chinook for this reach, since monitoring studies were initiated in 2004." - Care to hypothesize on a cause?

The objectives of this study are to examine parasite incidence in natural and hatchery Chinook salmon during juvenile migration, and compare disease status to previous study years to provide disease status trends. As stated above, environmental factors associated with drought and reduced flows may play a role in increased disease incidence, however analysis of these factors are beyond the scope of this report.

8) Page 39 - In reference to the following statement, " The higher incidence of *C. shasta* infection in marked TR Chinook in 2009 was statistically significant compared to the 3% level observed in 2008 (P=0.0003) and the 1 % level in 2006 (P=0.0047), but not significant when compared to the 14% in 2007 (P=1.0). - The % differences and corresponding P values don't make sense to me…perhaps your sample sizes are dramatically different?

TR-CWT sample size does vary by year, depending on availability of marked Chinook collected in the Trinity confluence to Estuary (K1) and Estuary (K0) reaches. Sample size has ranged from 76 CWT Chinook in 2006, the smallest sample set, to the largest sample size of 332 in 2007. A pair wise chi square analysis of the 2009 *C. shasta* incidence of infection, compared to previous year's incidence data, found a statistically significance when compared to both 2008 and 2006 incidence data. However, *Ceratomyxa shasta* incidence in 2007 of 14% was not significant compared to 2009.

Sample Year	***C. shasta* Prevalence (Percent Positive)**	**Statistically Significant (Pair wise chi square)**	**P Value**
2009	14/109 (13%)		
2008	8/257 (3%)	Yes (2009-2008)	P= 0.0003
2007	46/332 (14%)	No (2009-2007)	P= 0.8501
2006	1/76 (1%)	Yes (2009-2006)	P= 0.0047

Technical support documentation for the statistical software GraphPad Prism 5.0 states the P value generated by Fisher's Exact Student's Test can be more precise for small sample sizes, however P value differences are trivial for large sample sizes (chi square is appropriate in those cases). A second statistical analysis was run using Fisher Exact Student Test to verify the accuracy of P values generated by the initial pair wise chi square analysis. Fisher's Exact Student Test gave the following P values for pair wise analysis of *C. shasta* incidence of infection in TRH CWT Chinook: P = 0.001 (2009-2008); P= 1.0 (2009-2007); P= 0.0048 (2009-2006). While the P values generated by each method are slightly different, there was no change in the statistical significance reported for pair wise annual comparisons between study years.

APPENDIX II

Assay Threshold for *Parvicapsula minibicornis* Quantitative Polymerase Chain Reaction (QPCR) assay

The two QPCR assays that are employed for myxozoan testing in the Klamath River Fish Health Monitoring program are fully described in the following publications:

Hallett SL and JL Bartholomew. 2006. Application of real-time PCR assay to detect and quantify the myxozoan parasite *Ceratomyxa shasta* in water samples. Diseases of Aquatic Organisms 71:109-118.

True K., M.K. Purcell and J.S. Foott. 2009. Development and validation of a quantitative PCR to detect *Parvicapsula minibicornis* and comparison to histologically ranked juvenile Chinook salmon (*Oncorhynchus tshawytscha*) from the Klamath River, USA. Journal of Fish Disease 32:183-192.

There is an important difference between apparent prevalence, as determined by any diagnostic test, and true prevalence of disease in an aquatic animal population.
True prevalence can never really be known for a population, unless all animals in the population are tested, and the testing method is 100% accurate. Because tests are never 100% accurate, it is important to fully validate diagnostic tests, and use the knowledge about how a specific test performs to interpret the test results appropriately for the study objectives.

For the *Parvicapsula minibicornis* (*Pm*) assay, dynamic range and reliable endpoint define assay sensitivity. To assess these parameters, QPCR assays were performed using serial dilutions of Pm plasmid DNA, with known copy number, and DNA extracted from naturally infected kidney tissue (confirmed clinical infection by histology). The reliable endpoint was determined by examining the standard deviation of the CT values of 4 replicate wells. Standard deviations above 0.30 were used to identify DNA concentrations in which replicates no longer conformed to assay precision as recommended by Applied Biosystems, Inc., Guide to Performing Relative Quantification of Gene Expression (www.appliedbiosystems.com).

It should be noted that when the assay threshold conforms to the statistically valid standard deviation of ≤0.30, a small proportion of test samples that contain very low copy numbers of parasite DNA may be excluded from the positive test group and prevalence data set (false negative or Type II error). Conversely, if larger standard deviation values are chosen to establish the assay positive threshold, a small proportion of false-positive samples would be included in the prevalence data set (Type I error). For the Klamath monitoring program, we have followed the instrument manufacturer's recommendation regarding assay threshold to preclude the inclusion of false-positive test results. We believe the small proportion of fish, with extremely low parasite DNA levels, are not biologically significant in terms of disease risk, or in reporting the overall prevalence of infection for this parasite. The *Pm* QPCR assay positive threshold precludes false-positive test results from the apparent prevalence data and therefore is conservative in estimating the true prevalence of disease in this aquatic animal population.