U.S. Fish & Wildlife Service

California-Nevada Fish Health Center
FY 2010 Investigational Report:

Myxosporean Parasite (*Ceratomyxa shasta* and *Parvicapsula minibicornis*) Annual Prevalence of Infection in Klamath River Basin Juvenile Chinook Salmon, April-August 2010

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SUMMARY

Juvenile Klamath River Chinook salmon (*Oncorhynchus tshawytscha*) experience high prevalence of infection with the myxosporean parasites *Ceratomyxa shasta* and *Parvicapsula minibicornis* during the spring and summer outmigration period. Klamath River Chinook salmon were assayed by quantitative real-time polymerase chain reaction (QPCR) or histology to determine parasite infection rates from April to August, 2010. The annual metric of prevalence of *C. shasta* in Chinook salmon above the Trinity River confluence during the peak migration period (May-July) was 17% by QPCR and 15% by histology. The prevalence of *P. minibicornis* in Chinook salmon above the Trinity River confluence for the same period was 66% by QPCR and 58% by histology, compared to 82% by QPCR and 85% by histology in 2009. The prevalence of *C. shasta* below the Trinity River was 29% by QPCR and 17% by histology for sampled collected June-August in the lower basin.
P. minibicornis prevalence of infection below the Trinity River confluence for the same period was 88% by QPCR and 54% by histology.

The QPCR assay results from all groups of Chinook salmon sampled (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 less infectious for *C. shasta* in 2010 during the peak juvenile Chinook salmon migration period (May-July) than in any other sample year to date. In coded-wire tagged (CWT) IGH Chinook salmon screened by QPCR, *C. shasta* was detected in 28% of fish examined. The highest *C. shasta* prevalence of infection (56%) occurred in the IGH-CWT Chinook salmon residing 7 Weeks at Liberty (WAL) post hatchery release. *Ceratomyxa shasta* was detected in one marked TRH Chinook salmon sampled in the Klamath River. This low prevalence of infection in marked TRH Chinook salmon in 2010 contrasts with the 13% prevalence of infection observed in 2009, and the average historical mean of 7% for Chinook salmon sampled in the Klamath River below the Trinity River confluence. In summary, both the annual metric of *C. shasta* prevalence of infection by histology, and prevalence in Iron Gate and Trinity River coded-wire tagged Chinook salmon indicate that infectivity was very low relatively in 2010 compared to previous years in which monitoring studies were conducted. Cooler Spring and early summer river temperatures appear to have played a more significant role in disease dynamics in 2010.

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Cover Image – Blue Creek confluence in the lower Klamath River, photograph by Kimberly True.

INTRODUCTION

Juvenile Klamath River Chinook salmon (*Oncorhynchus tshawytscha*) experience high prevalence and severity of infection with the myxosporean parasites *Ceratomyxa shasta* and *Parvicapsula minibicornis*. The annual metric for *C. shasta* prevalence of infection by QPCR in study years 2006-2010 has ranged from 19-45% (Table 4) and *P. minibicornis* prevalence has ranged from 66-91% (data not shown in Table 4). 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. 2007a, 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 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 salmon and 20.6 MDD and 98.5% CPM in coho juveniles (True et al. 2011). The observed high prevalence of infection in relatively resistant indigenous fish indicates an extremely high parasite challenge (Foott et al. 2004) in most years. Dual infections with both parasites are common and may have a synergistic effect which increases the lethality of infection (Nichols and True 2007); however *C. shasta* drives the mortality curve observed in sentinel exposures (True et al. 2011). The contribution of each myxozoan parasite towards clinical disease in infected Chinook salmon is difficult to evaluate independently. In monitoring studies, nearly 90% of Chinook salmon are dual infected and typically succumb to clinical ceratomyxosis before *P. minibicornis* tissue changes and parasite DNA levels can be fully assessed.

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 prevalence by histology has been expanded to include a pathology score for both kidney and intestine tissues. The pathology score does not affect the overall prevalence of infection reported for histological assessments, but provides a numeric index of the disease state in sample groups. Additionally, with the increased constant fractional marking that was implemented at Iron Gate Hatchery in 2009, a larger number of hatchery fish can be identified. Sampling effort in 2009 and this year focused on capturing fish of known origin (natural Chinook salmon collected before hatchery releases and hatchery CWT Chinook salmon).

Diagnostic examinations were also performed in 2010 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 salmon could act as reservoirs of infection for the early returning adults.

The objectives of this study were: 1) examine the pathogen prevalence in Iron Gate Hatchery (IGH) and Trinity River Hatchery (TRH) Chinook salmon prior to and post release; 2) examine the parasite prevalence in the juvenile Chinook salmon population within the river throughout the spring out-migration period; 3) compare parasite prevalence in 2010 to previous years; and 4) examine the diagnostic prevalence of other significant pathogens in moribund Chinook salmon in select reaches.

METHODS

Sample Sites

Fish were collected in the Klamath River from below Iron Gate Dam (Klamath River Mile [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). Klamath and Trinity Rivers were divided into sample reaches at major tributaries, with study cooperators collecting fish in each reach (Figure 1, 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.

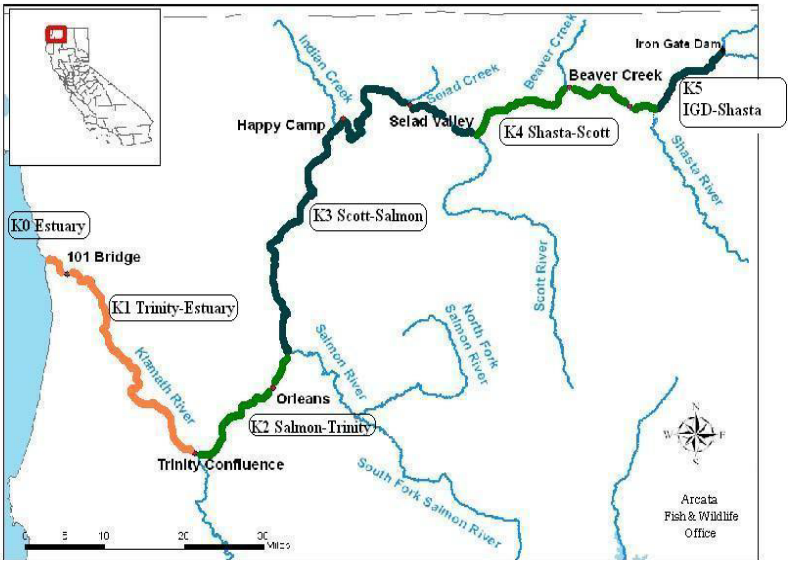

Figure 1. Klamath River watershed, major tributaries, and sample reaches: Iron Gate dam to Shasta River (K5), Shasta River to Scott River (K4), Scott River to Salmon River (K3), Salmon River to Trinity River confluence (K2), Trinity River to Estuary (K1), Klamath River Estuary (K0).

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
Scott to Salmon (K3)	Klamath 144-66	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 examinations of Chinook salmon were performed at Iron Gate Hatchery (IGH) and Trinity River Hatchery (TRH). Sixty fish were sampled from the IGH population on 24 May, and 70 fish were sampled from TRH on 13 May. All pre-release fish were assayed by QPCR for both parasites, and a subset of 10 fish from each hatchery were examined histologically for tissue abnormalities.

Natural production juvenile Chinook salmon were collected from upper Klamath reaches prior to first IGH release (1 June) and in the Trinity River prior to the first TRH releases (1 June). A total of 260 natural fish were sampled in the Klamath reaches from 5 April through 31 May (Shasta to Scott (K4), Scott to Salmon (K3) and Salmon to Trinity (K2)). An additional 118 fish were sampled in the upper Trinity River at the Pear Tree Trap (PTT) and in the Trinity to Estuary (K1) reach, for a total of 378 natural fish sampled in 2010. All natural fish were tested for both parasites by QPCR and histology to determine prevalence of infection, and any other tissue abnormalities in the natural Chinook salmon population. Natural fish are a component of the mixed-origin Chinook salmon group for reporting parasite prevalence of infection for each reach. Prevalence of parasite infections in natural fish is also discussed as a sub-set of the larger mixed-origin group, to provide relative comparisons between these two groups of Chinook salmon.

Mixed-origin Chinook salmon were collected in select reaches of the Klamath and Trinity Rivers every other week. This bi-weekly sample consisted of 30 Chinook salmon for the QPCR assay and 10 Chinook salmon for the histology assay. Once hatchery Chinook salmon were released, coded-wire tagged Chinook salmon were targeted for bi-weekly sampling, but when adequate numbers were not available, unmarked Chinook salmon (of unknown origin) were used to supplement the 30 fish sample set. Prior to hatchery releases, mixed–origin Chinook salmon would have been of natural origin, with the exception of a few hatchery Chinook salmon that may have been used for trap efficiencies studies. After IGH releases, mixed-origin Chinook salmon collected in the Klamath River could have been of natural origin or hatchery origin (CWT or unmarked).

Iron Gate and Trinity hatchery Chinook salmon were marked with an adipose fin clip and implanted with a coded-wire-tag (CWT) at a constant fractional mark rate of 25% for both facilities. In the Klamath River and Trinity River, a sample of 30 CWT Chinook salmon per week was collected by sample crews, if available, for analysis by QPCR. Limited numbers of CWT Chinook salmon were collected in the Trinity River at Pear Tree and Willow Creek rotary screw traps. Significant recapture effort for CWT Chinook salmon occurred in the Klamath River below the Trinity River confluence and in the Estuary.

Heads from any marked IGH or TRH Chinook salmon recovered were assigned unique identification numbers to track lab assay results to extracted tags, which were read by the USFWS Arcata Fish and Wildlife Office (AFWO). Chinook salmon release groups at IGH occurred on 1 June, 4 June, 6 June, 14 June, and 15 June. The CWT codes were unique for each release date, with the exception of the 15 June release group which included 2 tag codes for a single release date. Volitional releases occurred at TRH from 1 June through 8 June; 4 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), to assess temporal infections levels in individual fish.

Sample Periods

In each reach, fish were accumulated over a calendar week until the desired sample size was achieved. Bi-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 6 April in the Shasta to Scott (K4) reach and 3 May in the Scott to Salmon (K3), Salmon to Trinity (K2), and Trinity to estuary (K1) reaches. Fish collection started the week of 21 June in the estuary (K0). 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 was targeted after hatchery release, and collection crews were requested to accumulate 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 entire intestine and 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 Real Time PCR Sequence Detection Systems (SDS) 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) on the 7300 Sequence Detection System (Applied Biosystems, Foster City, CA). Separately, the combined tissues were tested for *P. minibicornis* 18S rDNA utilizing TaqMan Minor-Grove-Binding (MGB) probe and primers (True et al. 2009) on the StepOne Plus Sequence Detection System (Applied Biosystems Foster City, CA). 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 (7300 SDS v 1.3.1, StepOne SDS v. 2.0 Applied Biosystems) and converted to parasite plasmid molecular equivalents (referred to as DNA copy number), a measure of specific parasite DNA copy number derived from the standard curve of each specific assay (Figure 2). The fluorescence assay threshold, used to designate a positive test result, was slightly lower in the StepOne instrument compared to the 7300 SDS. Assay validation between two instruments was performed in 2009 using fish samples and plasmid controls with known parasite copy number. C_T values for each standard concentration as well as the positive threshold were determined for each instrument: 0.200 for the 7300 SDS and 0.100 for the StepOne SDS. Validation with samples and plasmid controls of a known parasite copy number was performed on both machines to determine the C_T value on each machine for those known copy numbers. Validation studies examining the dynamic range and endpoint of the assays indicated a C_T of 38.5 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 prevalence of both parasites in this aquatic animal population. Appendix II provides a further technical description of how assay sensitivity is used to determine thresholds for positive test results.

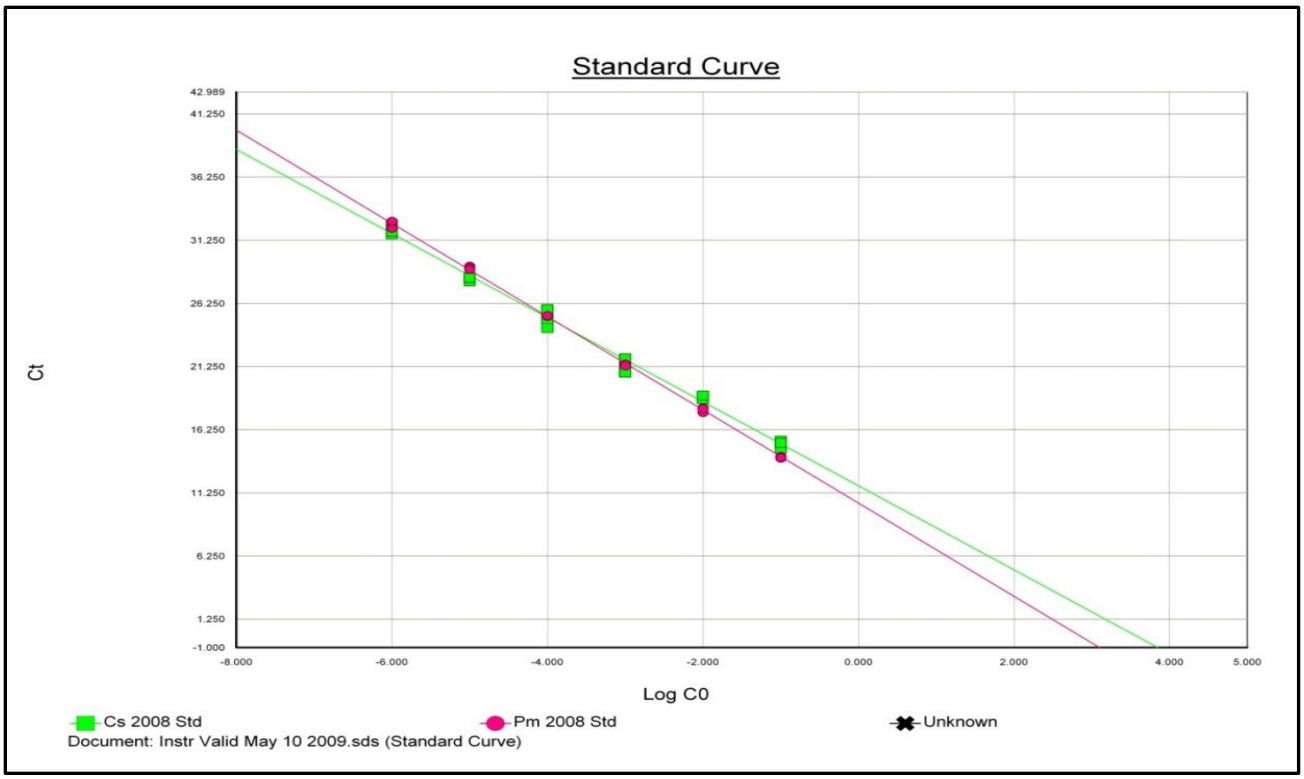

Figure 2. Standard curves for *C. shasta* and *P. minibicornis* QPCR assays using plasmid standards developed in 2008 with known concentrations of parasite DNA. "Unknowns" were not included in the assay. The amplification efficiency of each assay was calculated using the formula E= ($10^{-1/slope}$ -1) x 100 (Applied Biosystems Guide to Quantitative Gene Expression). Slope and amplification efficiency were similar for both QPCR assays: *C. shasta* slope = -3.12 and 95.7% efficient, *P. minibicornis* slope = -3.76 and 93.4% efficient.

Histological Assays

Ten fish from the bi-weekly 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' included 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	
Ich	*Ichthyophthirius multifiliis*
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

<u>2004-2010 Comparisons</u>

Histology data from this study was used to compare prevalence of infection of fish in 2010 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, True et al. 2010). 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 salmon 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 prevalence 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 has been generated each year the monitoring program has been conducted (2005-2010), tissue collection and extraction protocols were not standardized in 2005. This resulted in non-standardized tissue volumes for the QPCR assay that cannot be directly compared between

years. QPCR data from 2006-2010 is standardized and direct comparisons of annual parasite prevalence of infection can be made from 2006 forward.

Environmental conditions in 2010 will be discussed in context of disease prevalence; primarily mean daily river temperature and flow discharge below Iron Gate Dam.

Statistical Analysis and Terms Used

Prevalence of infection and annual prevalence (defined below) for *C. shasta and P. minibicornis* are reported with 95% confidence intervals, denoted as ci, for each sample reach prevalence of infection data. Prevalence of infection is used to describe bi-weekly ratios of infected Chinook salmon (numerator) in the sample population (number of animals examined). Annual prevalence is used to describe the overall prevalence of infection for the sampled population for the period of one calendar year. Definitions of the two terms used are as follows (Durfee 1978, USFWS Fish Health Policy FW713):

> Prevalence of infection (also referred to point prevalence): Number of cases of a disease which are detected in a population *at a designated point in time*. This is a census type of measurement, usually expressed as a ratio where the numerator is the number of cases detected at a point in time and the denominator is the population from which the cases were drawn.
>
> Annual prevalence (also referred to as period prevalence): Measures the total number of cases known to occur during a given period. Period prevalence is often mislabeled as incidence data because the factor time enters into it. However, it should be noted that incidence describes only *new cases in a specified population*, and *requires knowledge of when the animals became infected* to determine the rate of infection (incidence attack rate).

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 20 (*C .shasta*) and 21 (*P. minibicornis*). TRH CWT parasite DNA copy number was graphed in a similar manner in Figures 22 for *P. minibicornis,* but was not graphed for *C. shasta* as only one fish was found positive in this group. 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 number (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% (1/60) of Chinook salmon sampled 24 May at IGH, prior to hatchery release. The single positive fish was lightly infected near the detection threshold of the QPCR assay (C_T 37.2 or 22 DNA copy number). Infections of *P. minibicornis* were detected by QPCR in 2% (1/60) of pre-release Chinook salmon sampled at IGH. The single positive fish had a low infection level (C_T 37.4 or 50 copy number).

Neither *C. shasta* nor *P. minibicornis* were detected by QPCR in TRH Chinook salmon pre-release exams. In 2009, TRH Chinook salmon had a higher prevalence of infection for both parasites compared to IGH pre-release Chinook salmon, 19% and 17% respectively. No parasite or tissue abnormalities were seen in 10 fish examined histologically at either hatchery.

Natural Production Chinook Salmon

Natural Chinook salmon represent early infection status for *C. shasta* and *P. minibicornis*, as river temperatures are generally 8-10ºC cooler in the months of April and May compared to the peak hatchery salmon migration period of June-July. Natural production juvenile Chinook salmon were collected from upper Klamath reaches prior to first IGH release (1 June) and in the Trinity River prior to the first TRH releases (1 June). A total of 260 natural fish were sampled in the Klamath reaches from 5 April through 31 May (Shasta to Scott (K4), Scott to Salmon (K3) and Salmon to Trinity (K2)). An additional 118 fish were sampled in the upper Trinity River at the Pear Tree Trap (PTT) and in the Trinity to Estuary (K1) reach, for a total of 378 natural fish sampled in 2010.

Because natural fish are sampled before river conditions become more adverse for Chinook juveniles (primarily elevated river temperatures and associated actinospore production), infection levels are expected to be lower for this group. After hatchery fish are released, we are no longer able to differentiate natural Chinook salmon (produced in the main stem or tributaries) from unmarked hatchery Chinook salmon.

Prevalence of *C. shasta* and *P. minibicornis* infections were relatively low in natural fish sampled in the upper Klamath reaches from 6 April to 2 June. *C. shasta* was detected in 11% (31/280, ci= 3-9%) of Chinook salmon sampled above the Trinity River confluence (K4, K3, and K2) by QPCR, and in 6% (4/70, ci = 2-14%) of Chinook salmon sampled below the confluence, in the Trinity to Estuary (K1) reach (Table 3).

Comparatively, *P. minibicornis* prevalence of infection in natural Chinook salmon sampled above the Trinity River confluence was 47% (132/280, ci=41-53%) and 34% (24/70, ci=23-47%) below the confluence in the Trinity to Estuary (K1) reach.

Mixed-Origin Chinook Salmon

Prevalence of *C. shasta* infections in mixed-origin Chinook salmon, sampled above the Trinity confluence from 4 April to 16 August, was 16% (137/875, ci=13-18%). Below the confluence, *C. shasta* prevalence of infection was 25% (58/230, ci=20-31%) in the Trinity to Estuary (K1) reach and 26% (52/201, ci=20-33%) in the Klamath River Estuary (K0) (Table 3). Prevalence data for Coded-wire tagged Chinook salmon is discussed in further detail in a separate section of the report.

Table 3. Prevalence of *C. shasta* infection by reach in Mixed-Origin Chinook salmon (Natural, unmarked and CWT).

	IGD to Shasta (K5)	Shasta to Scott (K4)	Scott to Salmon (K3)	Salmon to TR (K2)	TR to Estuary (K1)	Estuary (K0)	Upper Trinity (PTT)	Lower Trinity (WCT)
Natural Chinook salmon – Sampled 4 April through 2 June[1]								
C. shasta+/ N	ND	16/131	14/79	1/70	4/70	ND	1/48	ND
***C. shasta* Percent Positive**	ND	**12%**	**18%**	**1%**	**6%**	ND	**2%**	ND
Unknown Origin Chinook salmon (Unmarked) – Sampled 3 June through 16 August								
C. shasta+/ N	0/11	14/92	53/154	20/175	8/59	ND	1/34	0/2
***C. shasta* Percent Positive**	Neg	**15%**	**34%**	**11%**	**14%**	ND	**3%**	Neg
CWT Chinook salmon – Sampled 3 June through 16 August[2]								
IGH-CWT *C. shasta*+/ N	0/44	5/68	1/17	11/20	41/81	47/152	ND	ND
***C. shasta* Percent Positive**	Neg	**7%**	**6%**	**55%**	**51%**	**31%**	ND	ND
TRH-CWT *C. shasta*+/ N	ND	ND	ND	0/3	0/9	1/36	0/85	0/131
C. shasta Percent Positive	ND	ND	ND	Neg	Neg	**3%**	Neg	Neg
Unreadable CWT[4]	0/1	0	2/6	0/4	5/11	4/13	0/1	0/6
ALL CWT[3] *C. shasta*+/ N	0/45	5/68	3/23	11/27	46/101	52/201	0/86	0/137
C. shasta Percent Positive	Neg	**7%**	**13%**	**46%**	**46%**	**26%**	Neg	Neg
All Mixed-Origin Chinook salmon Sampled (Natural, unknown/unmarked and CWT) – Sampled 4 April through 16 August[4]								
C. shasta+/ N	0/56	35/291	70/256	32/272	58/230	52/201	2/168	0/139
C. shasta Percent Positive	Neg	**12%**	**24%**	**12%**	**25%**	**26%**	**1%**	Neg

Key: N=Total sample number, ND=Not done (reach not sampled)

[1] Trinity River natural Chinook salmon were collected 13 May to 24 May in the upper basin at Pear Tree Trap (PTT).

[2] Trinity CWT Chinook salmon were collected 9 June through 16 August.

[3] Note: All CWT includes 42 CWT Chinook salmon which had unreadable tags (no tag, lost tag, or unreadable tag code). Therefore IGH and TRH CWT sample sizes (shown in gray highlighting) are slightly smaller than the All CWT figures given.

[4] Trinity River mixed-origin Chinook salmon were collected 9 June to 2 August at Pear Tree Trap (PTT), 9 June to 28 July at Willow Creek Trap (WCT), and 3 May to 24 May in the Trinity River confluence to Estuary (K1) reach.

Environmental Conditions

In 2010, river temperatures were lower in May and June, ranging from 10-17ºC. Only in late June and July did river temperatures reach over 18ºC at Seiad Valley (Figure 3). In previous study years, we typically observe temperatures above 18ºC (and often as high as 22ºC) approximately one month earlier than occurred in 2010.

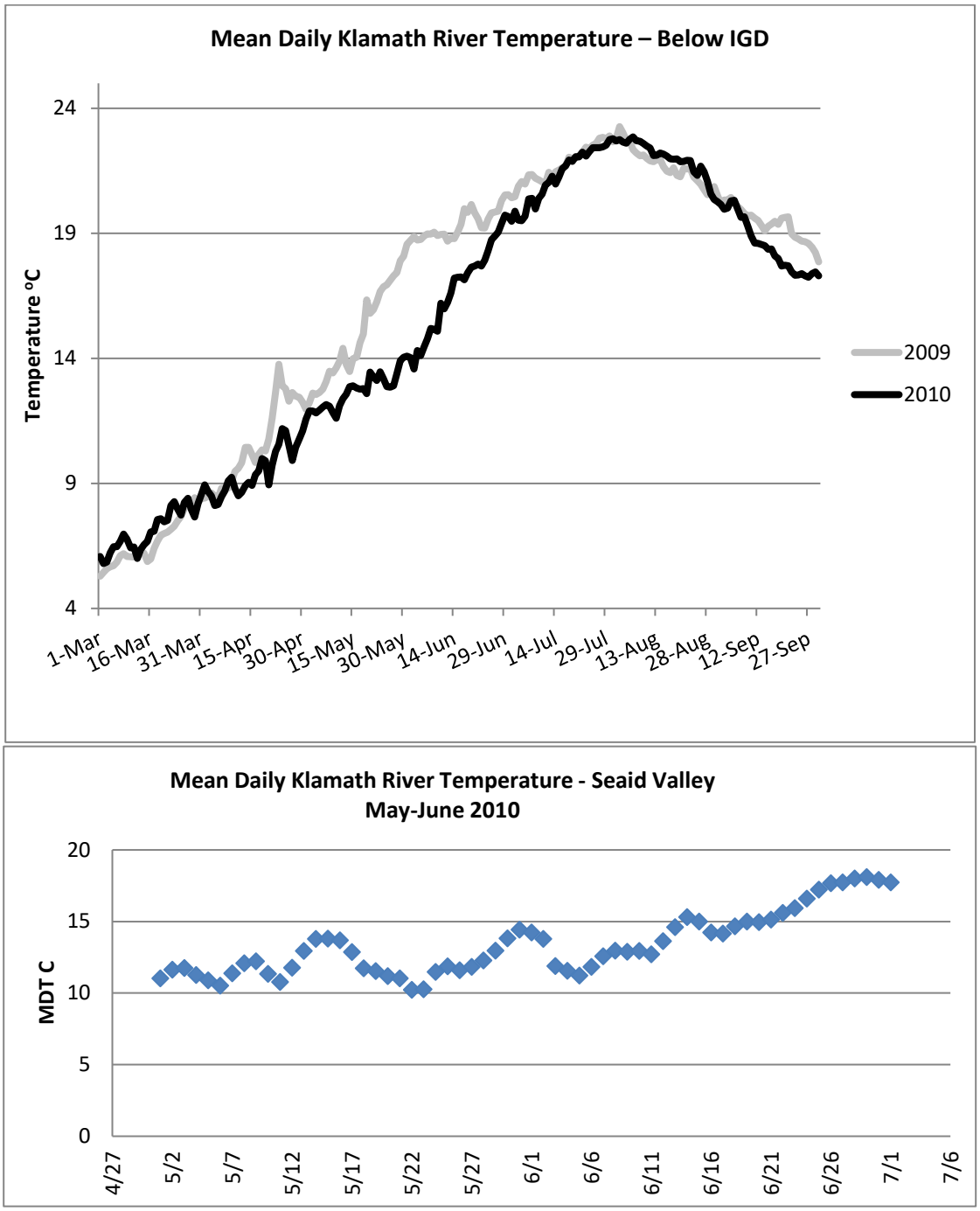

Figure 3. Mean Daily Temperature (MDT) below Iron Gate Dam for 2009 and 2010 (upper graph) and Mean Daily Temperature from May through June, at Seiad Valley (lower graph, USGS temperature gauge).

River flows below Iron Gate Dam in 2010 were fairly static and similar to previous study years (Figure 4).

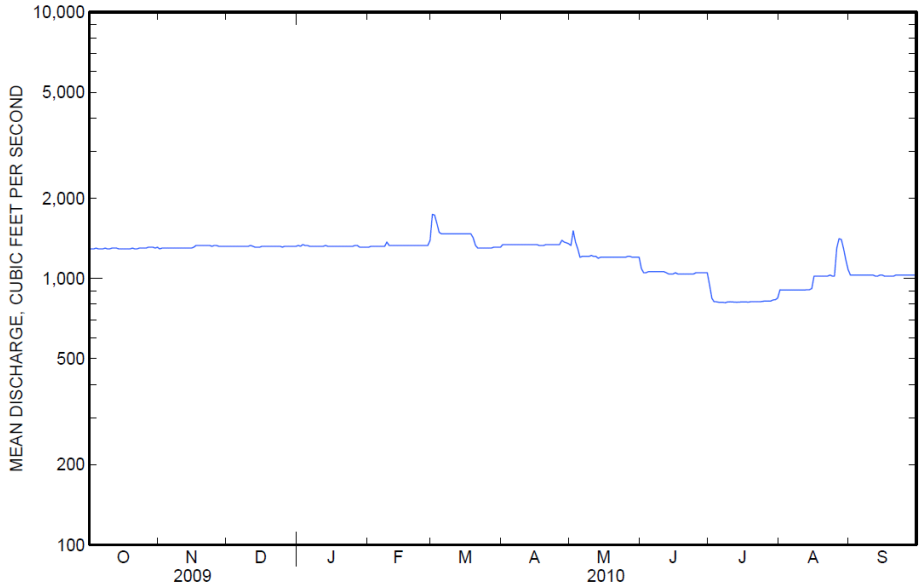

Figure 4. Mean Discharge (CFS) below Iron Gate Dam in 2010. USGS Gauge 11516530 located 0.5mi downstream from Iron Gate Dam, and 6 mi northeast of Hornbrook, CA. See Appendix V for the USGS 2010 Annual Water Year Report.

Bi-Weekly Prevalence of Infection by Sample Reach (QPCR and Histology)
As described in the methods section, the mixed-origin sample group consists of natural, unmarked fish of unknown origin, and CWT Chinook salmon. Histological assessments were performed on random, but separate, mixed-origin fish collected from the same reach and location (Appendix I, Tables 1A-4A).

Iron Gate Dam to Shasta R. (K5)
In the IGD to Shasta (K5) reach, *C. shasta* was not detected by QPCR (0%, 0/56, ci=0-6%) in mixed-origin Chinook salmon sampled from 31 May to 28 June. *P. minibicornis* was detected by QPCR in 27% (15/56, ci= 16-40%) of mixed-origin Chinook salmon. *P. minibicornis* was not detected in natural fish sampled during the week of 31 May. Infection prevalence was low (16%) in mid-June, and peaked at 60% in late June (Figure 5).

Histology sampling was not performed in this reach, due to the relatively low prevalence of *C. shasta* we have observed historically in this reach and the proximity to Iron Gate hatchery.

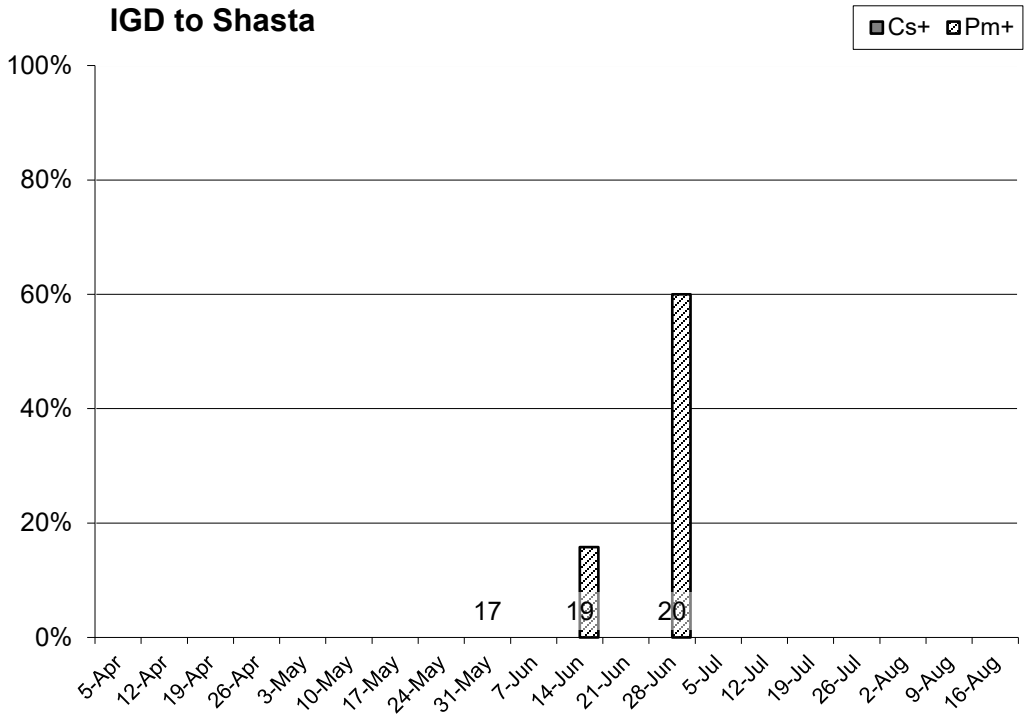

Figure 5. Bi-weekly prevalence of *Ceratomyxa shasta* (Cs +) and *Parvicapsula minibicornis* infection (Pm +) by QPCR in juvenile Klamath River Chinook salmon captured in K5 reach on the Klamath River (Iron Gate Dam to Shasta River). Sample numbers collected each week are displayed at the bottom of each column. *C. shasta* was not detected in K5, *P. minibicornis* was not detected on 31 May.

Shasta R. to Scott R. (K4)

In the Shasta to Scott reach (K4), *C. shasta* was detected by QPCR in 12% (35/291, ci=9-16%) of mixed-origin Chinook salmon. *C. shasta* was not detected late May when infection prevalence reached 26% (Figure 6). The prevalence decreased over a two week period, rose to 23% in early June and peaked at 41% in late June. In the natural fish subset collected in this reach (4 April to 2 June), *C. shasta* prevalence of infection was 12% (16/131, ci=7-19%) and similar to the mixed-origin group.

In the Shasta to Scott reach, *P. minibicornis* was detected by QPCR in 52% (150/291, ci=46-57%) of mixed-origin Chinook salmon. Infection prevalence reached 100% by the late May, decreased over a two week period, and peaked again between 87%-90% in mid to late June. *P. minibicornis* prevalence of infection in natural fish was 49% (78/163, ci=40-56%) and similar to the larger mixed-origin group.

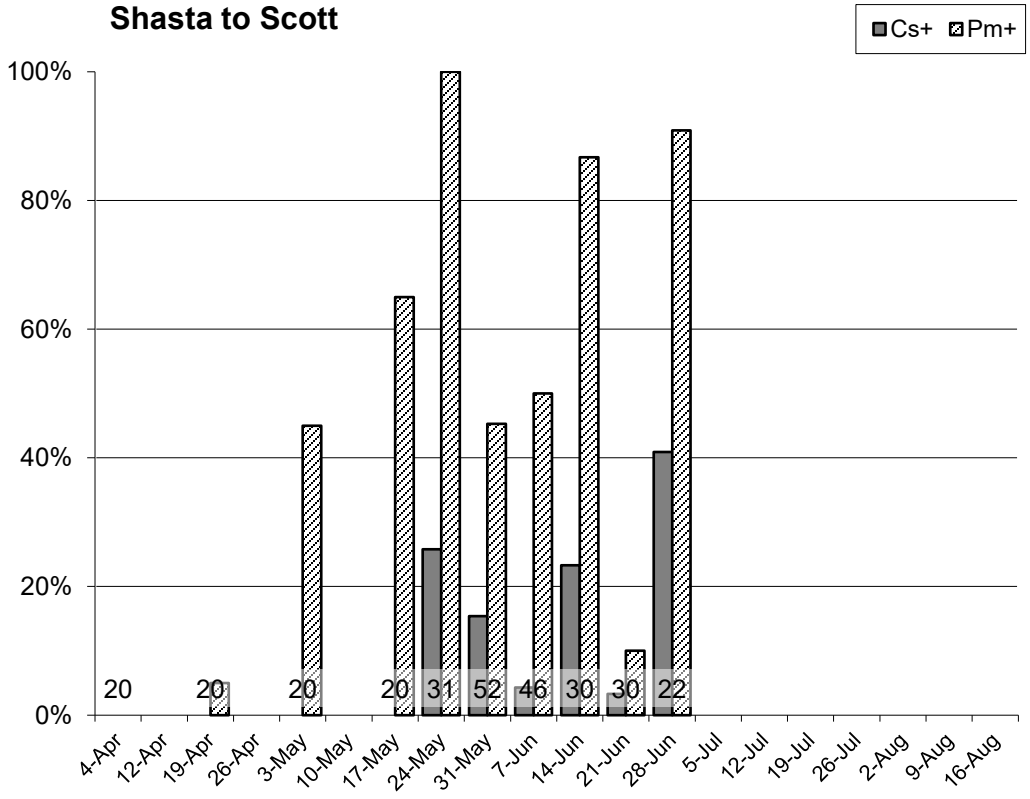

Figure 6. Bi-weekly prevalence of *Ceratomyxa shasta* (Cs+) and *Parvicapsula minibicornis* (Pm+) infection by QPCR in juvenile Klamath River Chinook salmon captured in K4 reach on the Klamath River (Shasta River to Scott River). Sample numbers collected each week are displayed at the bottom of each column; *C. shasta* was not detected on 4 Apr, 19 Apr, 3 May, and 17 May. *P. minibicornis* was not detected on 4 Apr.

C. shasta infectious load in this reach, as determined by the log mean parasite DNA copy number, was lower at the first detection (1.5 log) in 2010 compared to 2009 (3.1 log) (Figure 7).
In addition to lower *C. shasta* DNA copy number in Chinook salmon overall in 2010, the entire period of increasing prevalence of infection was shifted by approximately a three week period in the Shasta to Scott (K4) reach. In 2009, C. *shasta* bi-weekly prevalence of infection was 97% by QPCR when first detected in this reach in early May. Comparatively, *C. shasta* prevalence of infection in 2010 was 26% and did not occur until late May.

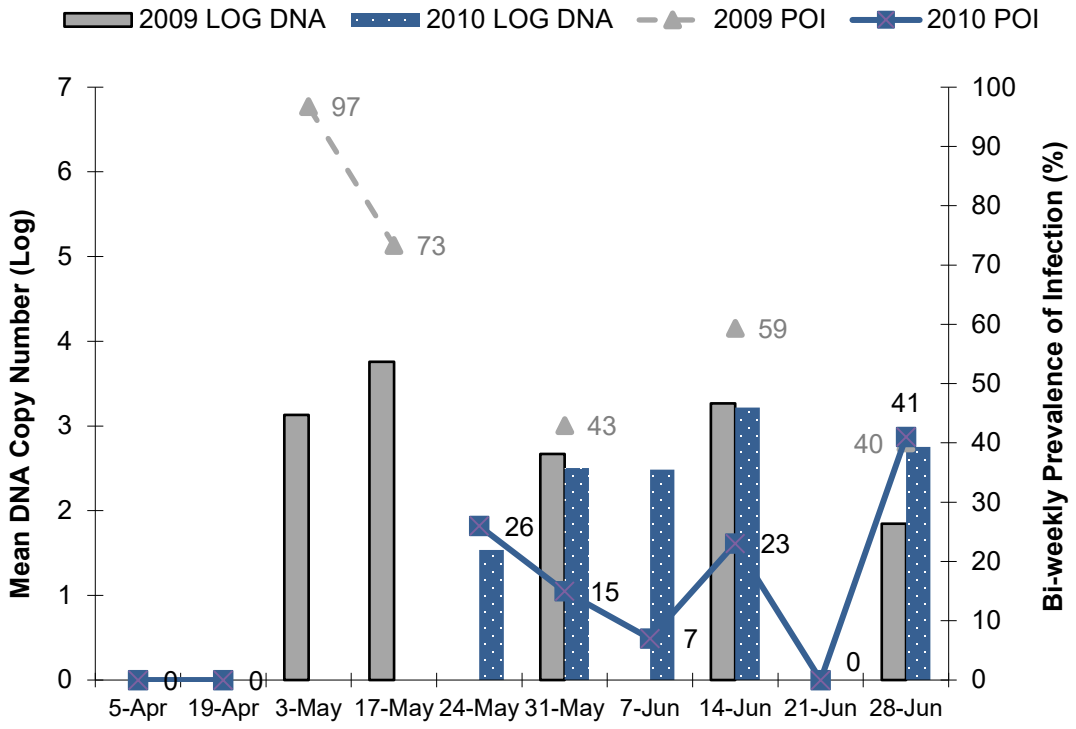

Figure 7. *Ceratomyxa shasta* mean log DNA copy number in 2009 (2009 LOG DNA) and 2010 (2010 LOG DNA) and bi-weekly prevalence of infection (2009 POI and 2010 POI) in the Shasta to Scott (K4) reach.

For histology, eight bi-weekly collections occurred between 5 April and 5 July for a total of 74 specimens (Appendix 1, Table 1A). Collection groups between 5 April and 31 May were natural origin given the 1 June initial Iron Gate Hatchery release. *C. shasta* was detected in 20% (15/74 ci=12-31%) of mixed-origin Chinook salmon (Figure 8).

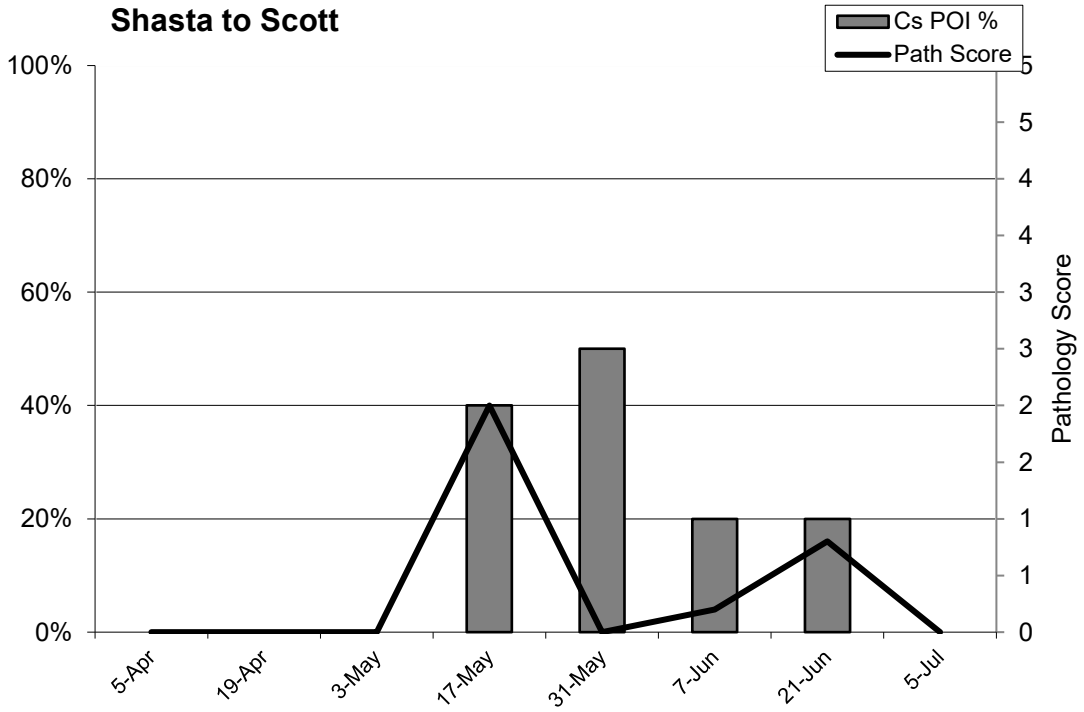

Figure 8. Bi-weekly prevalence of infection for *Ceratomyxa shasta* (Cs POI %) and mean pathology score (Path Score) by histology in juvenile Klamath River Chinook salmon captured in the Shasta to Scott (K4) reach.

Ceratomyxa shasta trophozoites were first observed within 40% the 17 May intestines with two peaks in prevalence and inflammation severity occurring on 31 May and 5 July (Figure 8). Presumptive *C. shasta* trophozoites were seen in 10% of gill sections beginning in the 7 June collection. Gill sections had a prevalence of 47% metacercaria cysts (34/72, ci=35-59%) with one *Ichthyophthirius multifiliis* trophozoite observed in one fish sampled 21 June. Multi-focal hyperplastic regions of gill epithelium without obvious parasite association were observed in 26% of the sections. A majority of the same sections had metacercaria cysts in other areas of the gill.

Histologically, *Parvicapsula minibicornis* trophozoites were seen in 51% of the fish collected (38/74 ci= 39-63%) with kidney inflammation scores increasing throughout the time period (Figure 9).

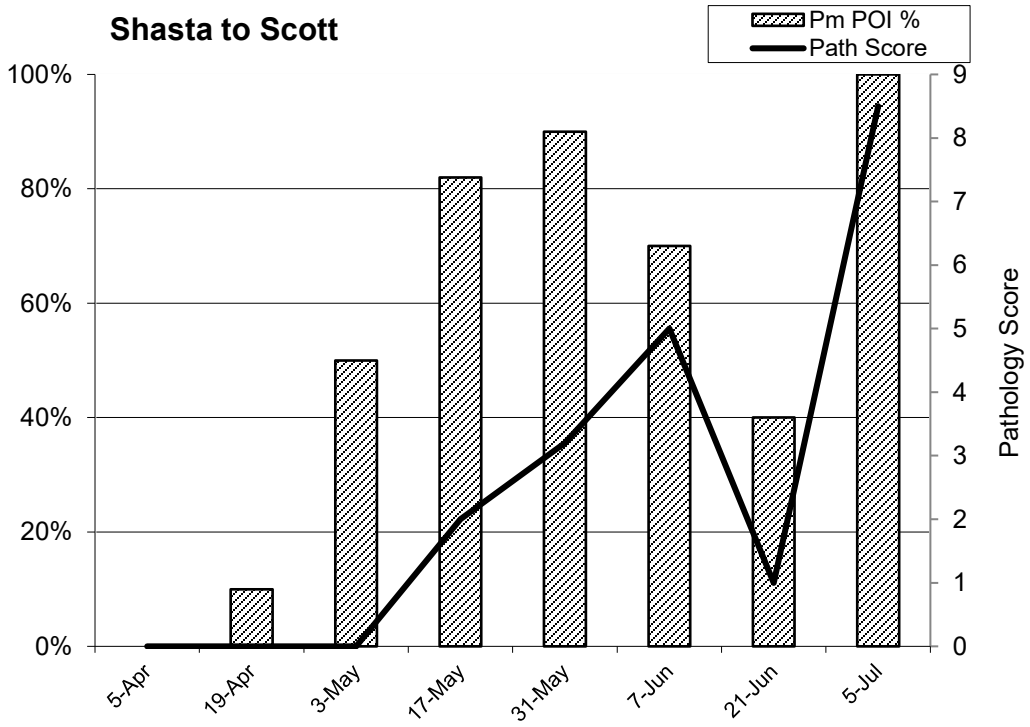

Figure 9. Bi-weekly prevalence of infection for *Parvicapsula minibicornis* (Pm POI %) and mean pathology score (Path Score) by histology in juvenile Klamath River Chinook salmon captured in the Shasta to Scott (K4) reach.

<u>Scott R. to Salmon R. reach (K3)</u>

In the Scott to Salmon reach (K3), *C. shasta* was detected by QPCR in 27% (70/256, ci=22-33%) of mixed-origin Chinook salmon. Infection prevalence peaked in early June at 50%, decreased over a two week period, and peaked again in late June at 43% before decreasing for the remainder of the sampling period (Figure 10). Comparatively, *C. shasta* prevalence of infection in natural fish (collected 3 May to 31 May) was 18% (14/79, ci= 10-28%) in this reach.

In this reach, *P. minibicornis* was detected by QPCR in 83% (213/256, ci=78-88%) of mixed-origin Chinook salmon. Infection prevalence peaked in early June at 90%, reached 100% the following week and remained high for the remainder of the sampling period (Figure 10). *P. minibicornis* prevalence of infection in natural fish sampled in this reach was 54% (43/79, ci=43-66%).

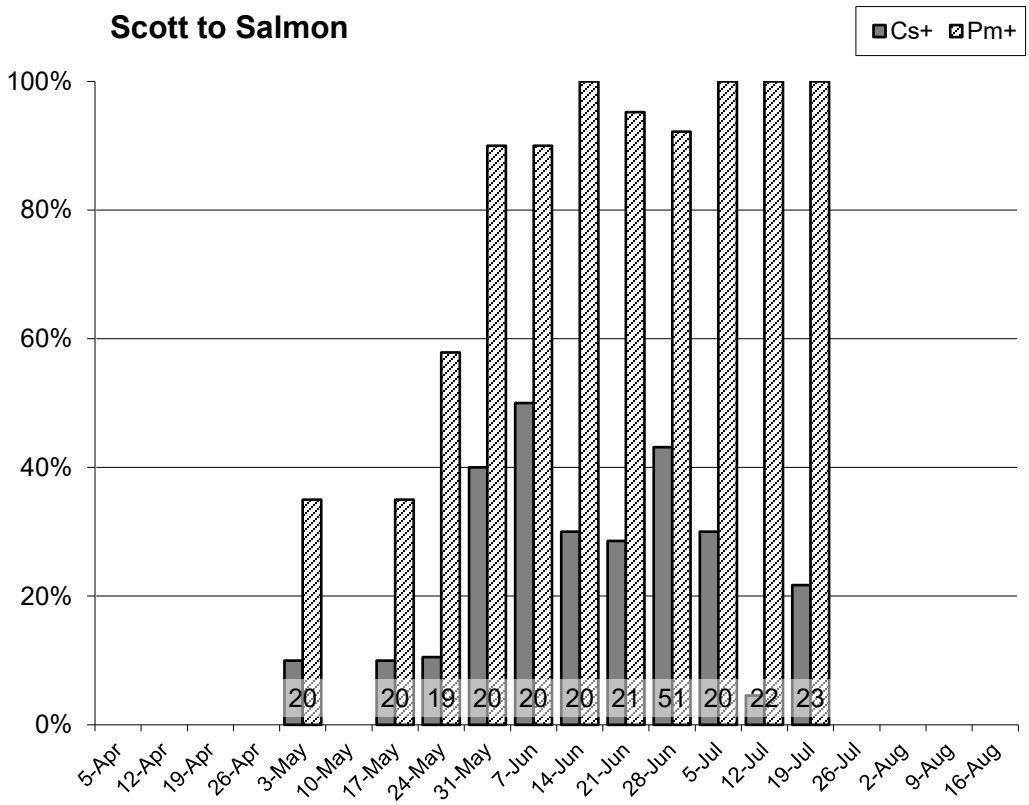

Figure 10. Bi-weekly prevalence of *Ceratomyxa shasta* (Cs+) and *Parvicapsula minibicornis* (Pm+) infection by QPCR in juvenile Klamath River Chinook salmon captured in the Scott to Salmon (K3) reach. Sample numbers collected each week are displayed at the bottom of each column.

An additional 40 Chinook salmon were sampled from the Salmon River RST on 2 Aug and tested negative for *C. shasta* and *P. minibicornis* by QPCR assay.

Histologically, *C. shasta* was detected in 20% (4/20, ci=6-45%) of mixed-origin Chinook salmon. *Parvicapsula minibicornis* trophozoites were seen in 65% (13/20, ci=41-85%) of the fish collected.

Twenty natural origin salmon were examined histologically, during the weeks of 3 May and 17 May (Appendix 1, Table 2A). *C. shasta* trophozoites were observed in 40% (4/10, ci=12-74%) of 17 May fish associated with only minor inflammation indicative of an early stage infection. Despite the relatively low water temperatures in May, inflammation of visceral adipose tissue (steatitis) was a common observation (prevalence 67%).

Parvicapsula minibicornis trophozoites were seen in collected fish with low kidney inflammation scores indicative of early stage infections. No parasites were seen in the gills.

Salmon R. to Trinity R. reach (K2)

In the Salmon to Trinity reach (K2), *C. shasta* was detected by QPCR in 12% (32/272, ci=8/16%) of mixed-origin Chinook salmon. Infection prevalence was low (0-13%) in the samples collected May through June, peaked in mid-July at 47% and decreased for the remainder of the sampling period (Figure 11). In contrast to the larger mixed-origin group, *C. shasta* prevalence of infection in natural Chinook salmon, collected 3 May to 24 May, was very low at 1% (1/70, ci=0-8%).

In the Scott to Salmon reach, *P. minibicornis* was detected by QPCR in 70% (191/272, ci=64-76%) of mixed-origin Chinook salmon. Bi-weekly infection prevalence steadily rose from mid-May (45%) to mid- August (97%), with the exception of two sample collections that occurred 24 May and 19 July (Figure 11). *P. minibicornis* prevalence of infection in natural fish, collected 3 May to 24 May, was 26% (18/70, ci=16-38%) in this reach.

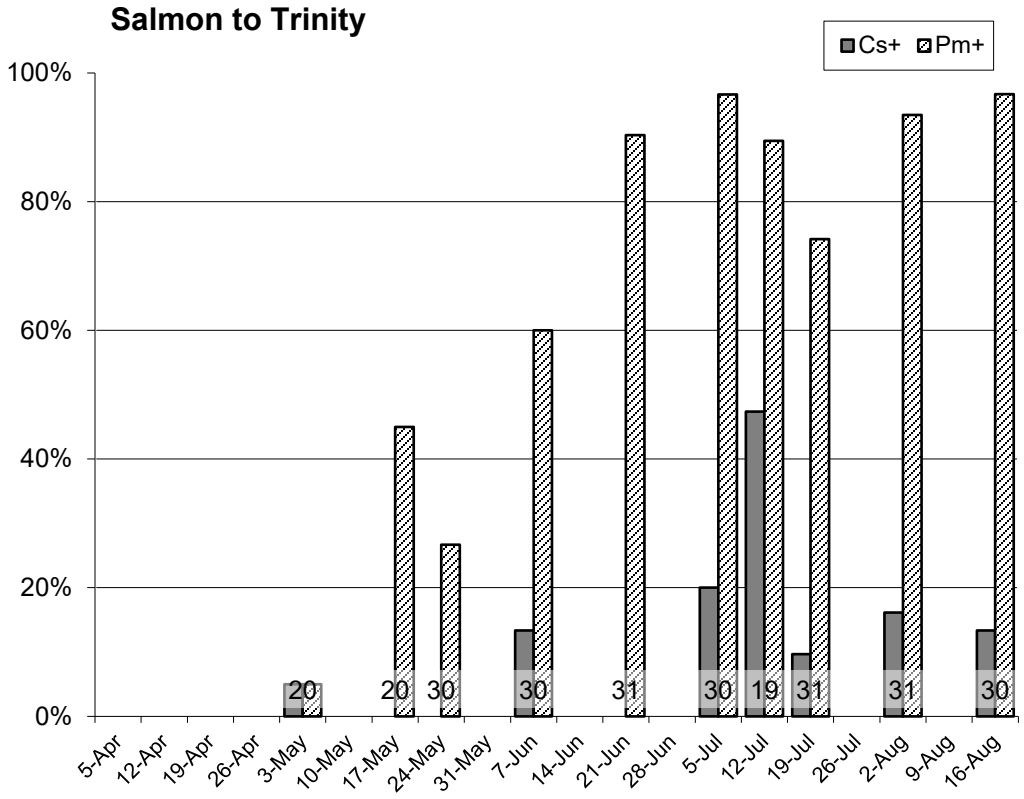

Figure 11. Bi-weekly prevalence of infection for *Ceratomyxa shasta* (Cs+) and *Parvicapsula minibicornis* (Pm+) by QPCR in juvenile Klamath River Chinook salmon captured in the Salmon to Trinity River confluence (K2) reach. Sample numbers collected each week are displayed at the bottom of each column; *C. shasta* was not detected on 17 May, and 24 May, and 21 Jun.

C. shasta infectious load in this reach, as determined by the log mean parasite DNA copy number, was lower at the first detection (<1 log, 4 May) in 2010 compared to 2009 (5 log, 18 May) (Figure 12). *Ceratomyxa shasta* DNA copy number in Chinook salmon was substantially lower in 2010 for the entire sampling period in this reach: the peak of 3.2 and 3.0 log DNA copy number occurred 7 June and 29 June. These peaks represent infection levels in natural Chinook salmon, and likely hatchery Chinook salmon (based on the June 1 release date for Iron Gate Hatchery, and the location of this reach).

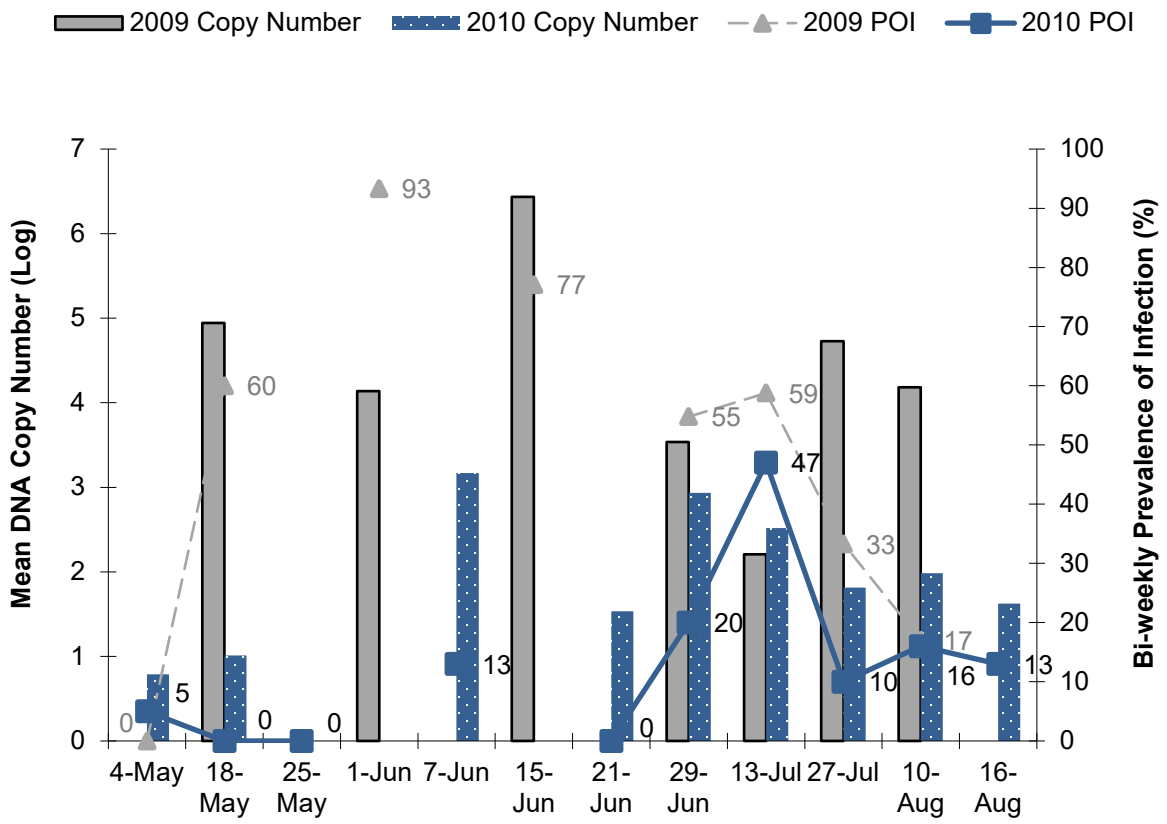

Figure 12. *Ceratomyxa shasta* mean log DNA copy number in 2009 (2009 LOG DNA) and 2010 (2010 LOG DNA) and bi-weekly prevalence of infection (2009 POI and 2010 POI) in the Salmon to Trinity River confluence (K2) reach.

Histologically, *C. shasta* was detected in 8% (7/93, ci=3-15 %) of mixed-origin Chinook salmon. Nine bi-weekly collections occurred between 3 May and 16 August for a total of 93 specimens (Figure 13 and Appendix I-Table 3A). Collection groups between 3 May and 31 May were considered of natural origin. *Ceratomyxa shasta* trophozoites were first observed within 40% the 7 June collection group. The infections were largely at an early stage with minimal inflammation. Trophozoites were associated with gill lamellar hyperplasia in 2 fish collected during August. In both cases, no other parasites were observed in the gill section suggesting the fish was responding to *C. shasta*. Inflammation of visceral adipose tissue (steatitis) was a common observation (prevalence 48%).

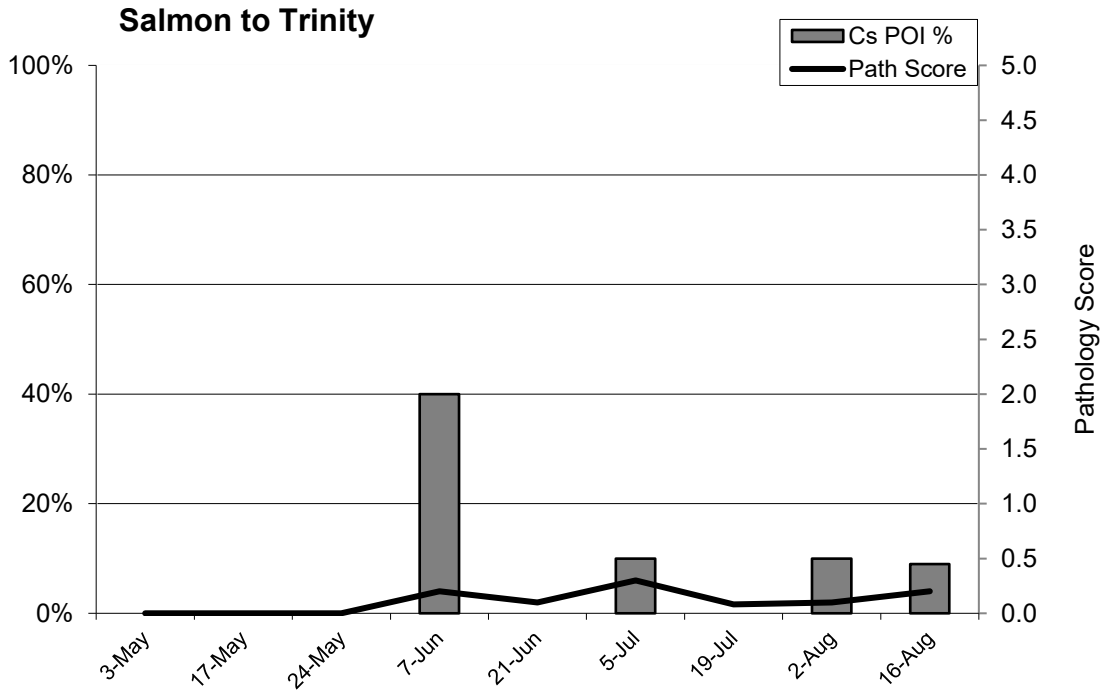

Figure 13. Bi-weekly prevalence of infection for *Ceratomyxa shasta* (Cs POI %) and mean pathology score (Path Score) by histology in juvenile Klamath River Chinook salmon captured in Salmon to Trinity confluence (K2) reach.

Parvicapsula minibicornis trophozoites were seen in 67% (62/93, ci=56-76%) of fish collected with moderately high kidney inflammation scores between 7 June and 19 July (Figure 14). Gill sections had a 68% prevalence of metacercaria cysts (63/92) with 51% having multi-focal hyperplastic regions of gill epithelium.

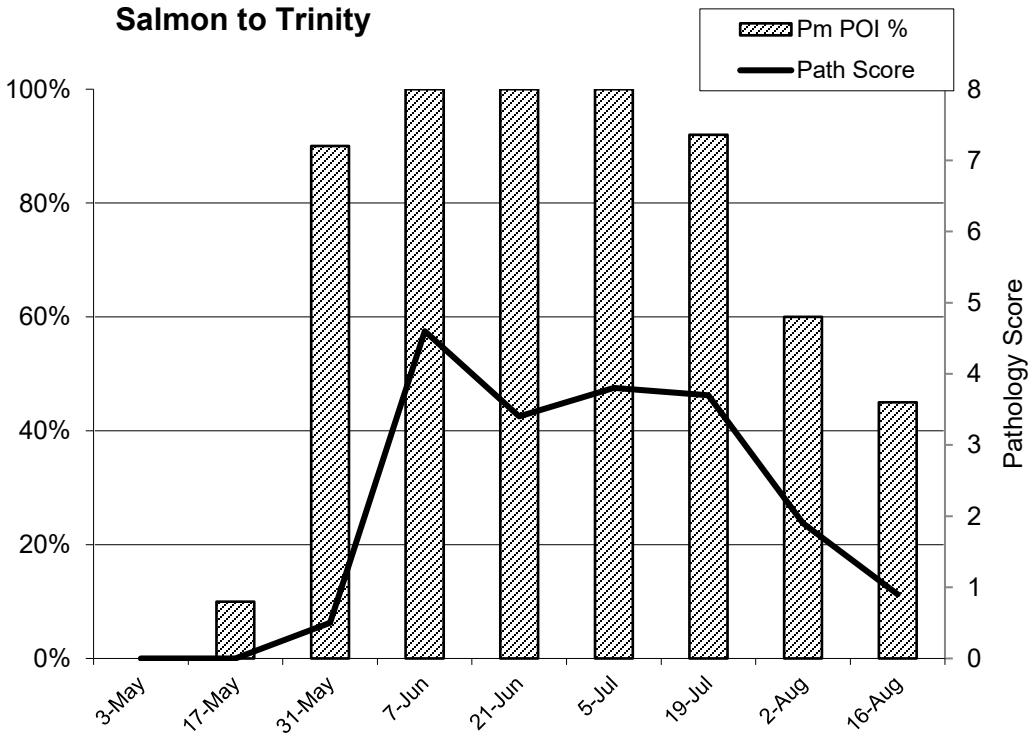

Figure 14. Bi-weekly prevalence of infection for *Parvicapsula minibicornis*(Pm POI %) and mean pathology score (Path Score) by histology in juvenile Klamath River Chinook salmon captured in the Salmon to Trinity River confluence (K2) reach.

Trinity R. to Estuary reach (K1)

In the Trinity to Estuary reach (K1), *C. shasta* was detected by QPCR in 25% (58/230, ci=20-31%) of mixed-origin Chinook salmon. Infection prevalence rose gradually (0%-86%) from 3 May to the peak prevalence on 12 July, generally decreased for three weeks, and peaked again at 75% on the last sample date of 16 August (Figure 15). *C. shasta* prevalence of infection in natural fish sampled in this reach (3 May to 31 May) was expectedly lower at 6% (4/70, ci=2-14%).

In the Trinity to Estuary reach, *P. minibicornis* was detected by QPCR in 73% (167/230, ci=66-78%) of mixed-origin Chinook salmon. Infection prevalence initially was 25% and increased gradually, except for the decrease to 5% during the second sample week. Prevalence peaked at 100% in early July and remained high for the remainder of the sampling period (83-100%). In natural fish, prevalence of infection was lower at 34% (24/70, ci=23-47%) in this reach.

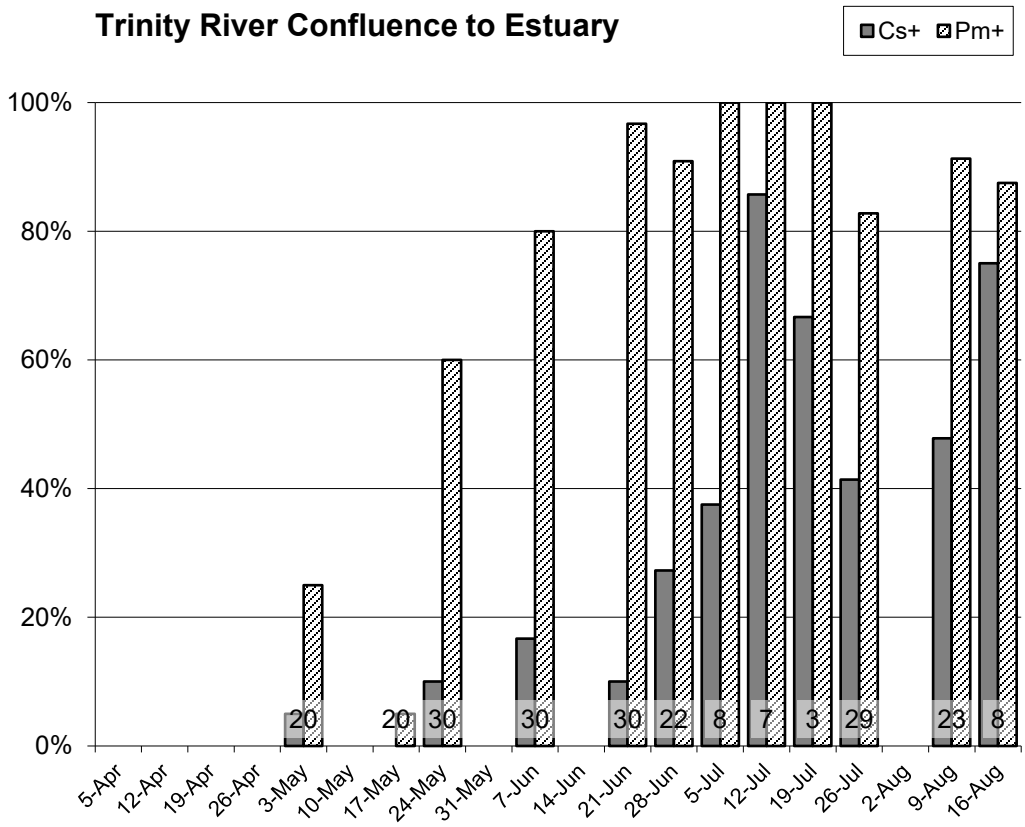

Figure 15. Bi-weekly prevalence of *Ceratomyxa shasta* (Cs+) and *Parvicapsula minibicornis* (Pm+) by QPCR in juvenile Klamath River Chinook salmon captured in the Trinity River confluence to Klamath River Estuary (K1) reach. Sample numbers collected each week are displayed at the bottom of each column; *C. shasta* was not detected on 17 May.

Histologically, eight bi-weekly collections occurred between 3 May and 26 July for a total of 80 specimens (Figure 16 and Appendix 1 – Table 4A). Collection groups between 3 May and 31 May were considered of natural origin. *Ceratomyxa shasta* trophozoites were first observed within 30% the 3 May collection group with an overall prevalence of only 18% (14/80, ci=10-28%). Half of these infected fish had intestinal inflammation indicative of a disease state. Inflammation of visceral adipose tissue (steatitis) was a common observation (prevalence 68%).

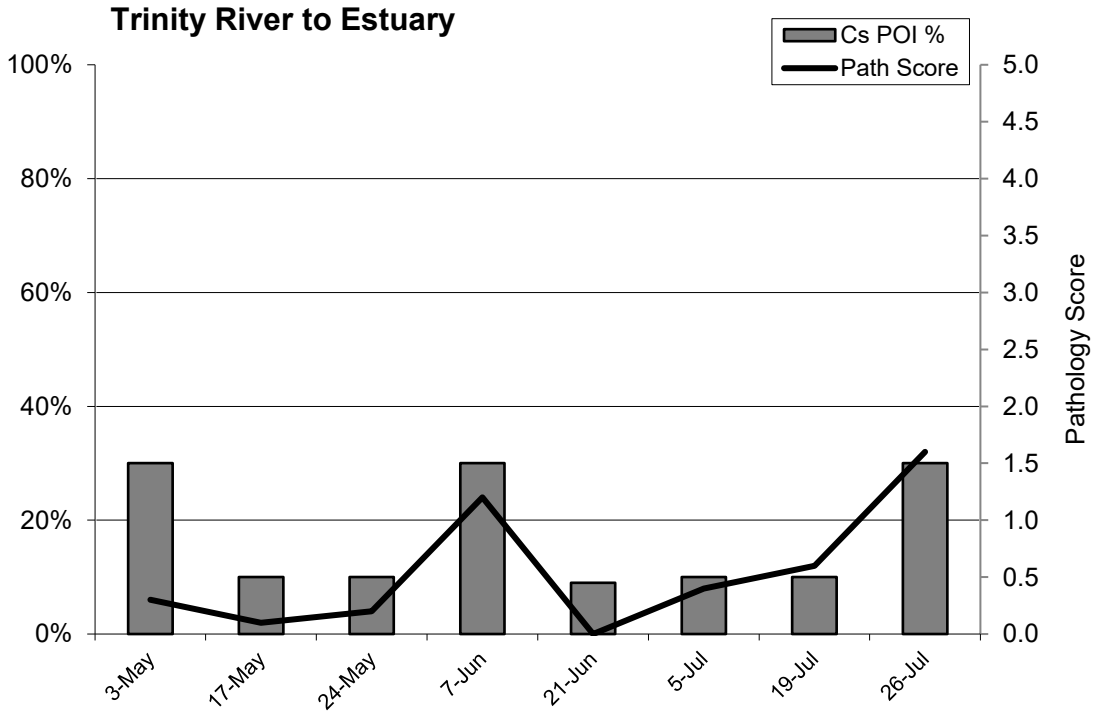

Figure 16. Bi-weekly prevalence of infection for *Ceratomyxa shasta* (Cs POI %) and mean pathology score (Path Score) by histology in juvenile Klamath River Chinook salmon captured in the Trinity River confluence to Estuary (K1) reach.

Histologically, *Parvicapsula minibicornis* trophozoites were seen in 54% (43/80, ci=42-65%) of fish collected with moderately high kidney inflammation scores between 7 June and 19 July (Figure 17). Gill sections had a 35% prevalence of metacercaria cysts (28/81, ci=24-46%) with 37% having multi-focal hyperplastic regions of gill epithelium.

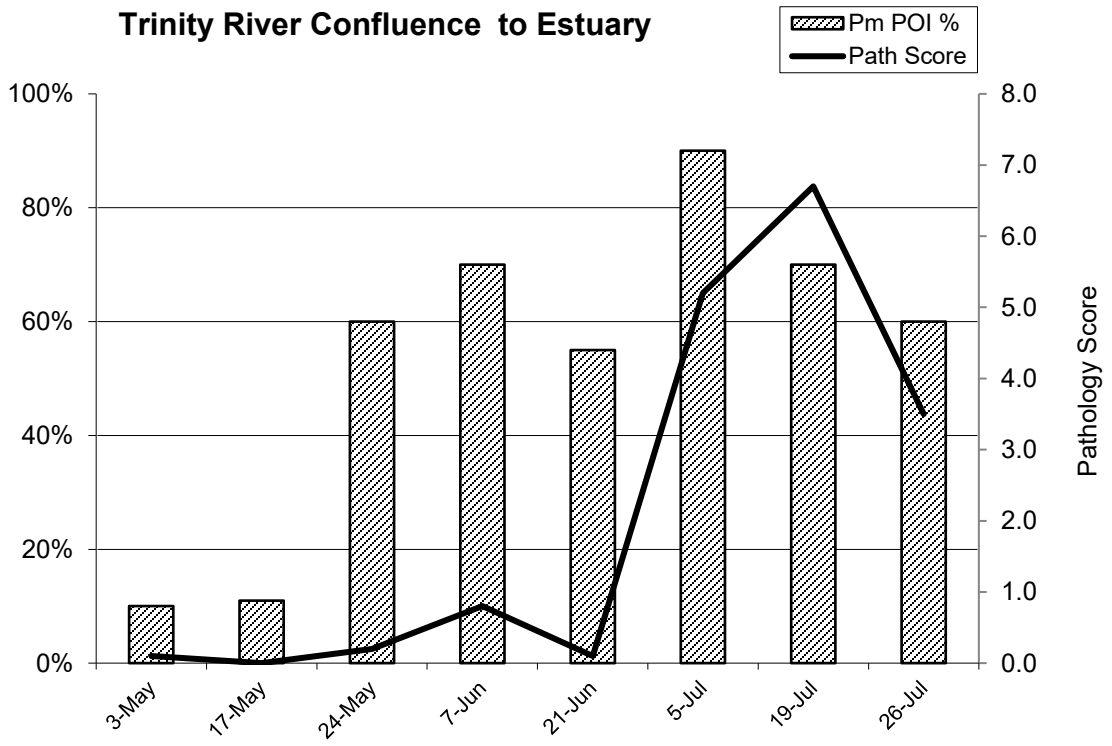

Figure 17. Bi-weekly prevalence of infection for *Parvicapsula minibicornis* (Pm POI %) by histology in juvenile Klamath River Chinook salmon captured in the Trinity River confluence to Estuary (K1) reach.

<u>Klamath River Estuary (K0)</u>

In the Klamath River Estuary (K0) reach *C. shasta* was detected by QPCR in 26% (52/201, ci=20-32%) of CWT Chinook salmon. The first estuary samples were collected the week of 21 June and had an initial infection prevalence of 10%. Prevalence peaked over a two week period to 50%, then decreased as in a normal bell shaped curve. A second rise in prevalence occurred in the 9 August sample set (Figure 18).

In the Klamath River Estuary reach, *P. minibicornis* was detected by QPCR in 87% (174/201, ci=81-91%) of CWT Chinook salmon. Prevalence of infection rose over a 5 week period (70%-93%), dropped the week of 26 July and resumed at high levels throughout the remainder of the sample period.

Histology sampling was not performed in this reach.

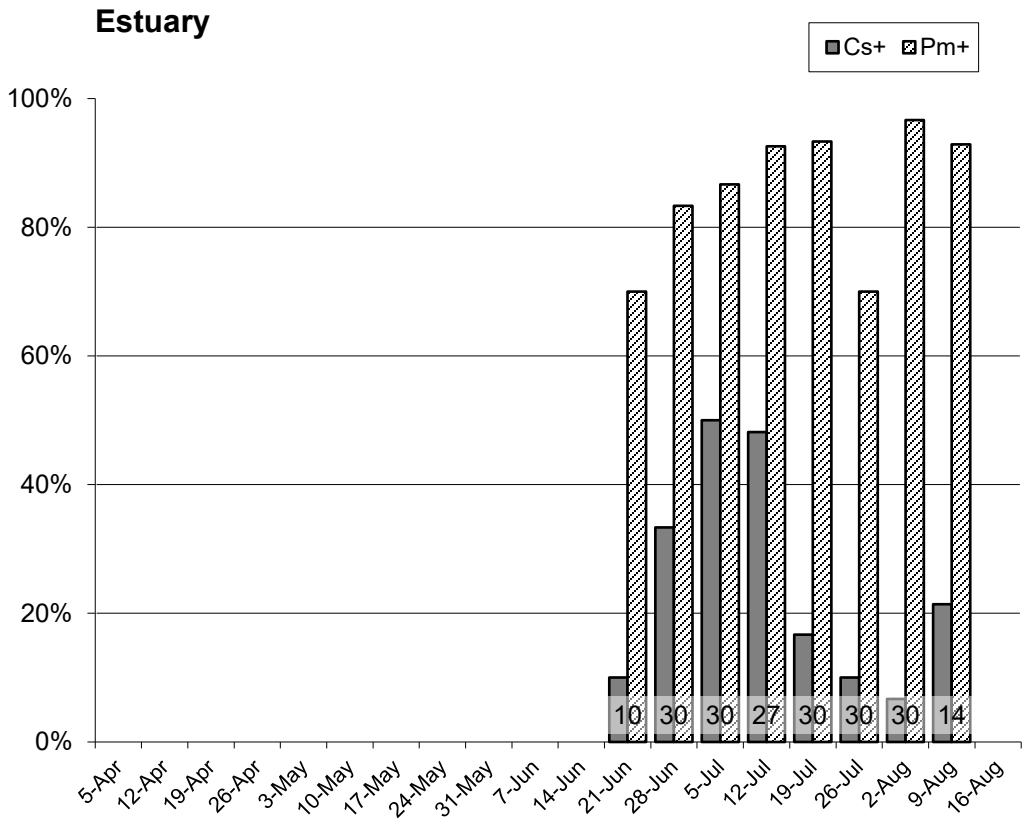

Figure 18. Bi-weekly prevalence of *Ceratomyxa shasta* (Cs+) and *Parvicapsula minibicornis* (Pm+) by QPCR in juvenile Klamath River Chinook salmon captured in the Klamath River Estuary (K0). Sample numbers collected each week are displayed at the bottom of each column.

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 salmon in the past two years of the monitoring study. The increased CWT mark provides a larger sample size and better assessment of myxozoan infection level at weeks post hatchery release. *Ceratomyxa shasta* was detected in 27% (105/382, 95% ci=23-32%) of all marked IGH Chinook salmon screened by QPCR and 11% (17/149, 95% ci=7-18%) of IGH-CWT collected above the confluence of the Trinity River. Historical data for QPCR and histology are given in Table 4.

Table 4. Historic annual prevalence 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 is shown in columns 4 & 5 for coded-wire tagged (CWT) fish from each hatchery: Iron Gate Hatchery Chinook salmon captured in reaches above the confluence of the Trinity River (K5, K4 and K2) and Trinity Hatchery Chinook salmon (positive/total, (percent positive)) collected below the Trinity R. confluence (K1) and estuary (K0).

Year	Chinook, May-July, Above TR Confluence (Percent Positive by Assay)		Iron Gate CWT-QPCR (Above TR confluence - reach K5, K4, and K2)	Trinity CWT- QPCR (Below TR confluence – reach K1/K0)
	Histology	QPCR		
1995	44	NS[1]	NS	NS
2002	19[2]	NS	NS	NS
2004	34	NS	NS	NS
2005	35	Not Included[3]	NS	NS
2006	21	34	6/18 (33%)	1/67 (1%)
2007	21	31	15/22 (68%)	46/332 (14%)
2008	37	49	9/13 (69%)	8/257 (3%)
2009	54	47	82/228 (36%)	13/100 (13%)
2010	**15**	**17**	**17/149 (11%)**	**1/45 (2%)**
Average (SE)	**33% (5)**	**36% (6)**	**30% (11)**	**9% (3)**

[1] NS= Not Sampled.
[2] Only TR CWT Chinook salmon were assayed in 2002 by histology, not included in average

Parvicapsula minibicornis was detected in 80% (302/382, ci=75-83%) of all marked IGH Chinook salmon screened by QPCR and 48% (72/149, 95% ci=40-57%) of IGH-CWT collected above the confluence of the Trinity River.

The largest proportions of Iron Gate Hatchery CWT Chinook salmon were recovered from the Estuary (K0) and the Trinity to Estuary (K1) reach (Figure 19). *C. shasta* prevalence of infection was highest in Chinook salmon recovered from the Salmon to Trinity (K2) reach at 54%. *C. shasta* was not detected in the 44 CWT Chinook salmon recovered from Iron Gate Dam to the Shasta (K5) reach. *C. shasta* prevalence of infection was notably low (<10%) in the upper reaches (Shasta to Scott and Scott to Salmon) in 2010.

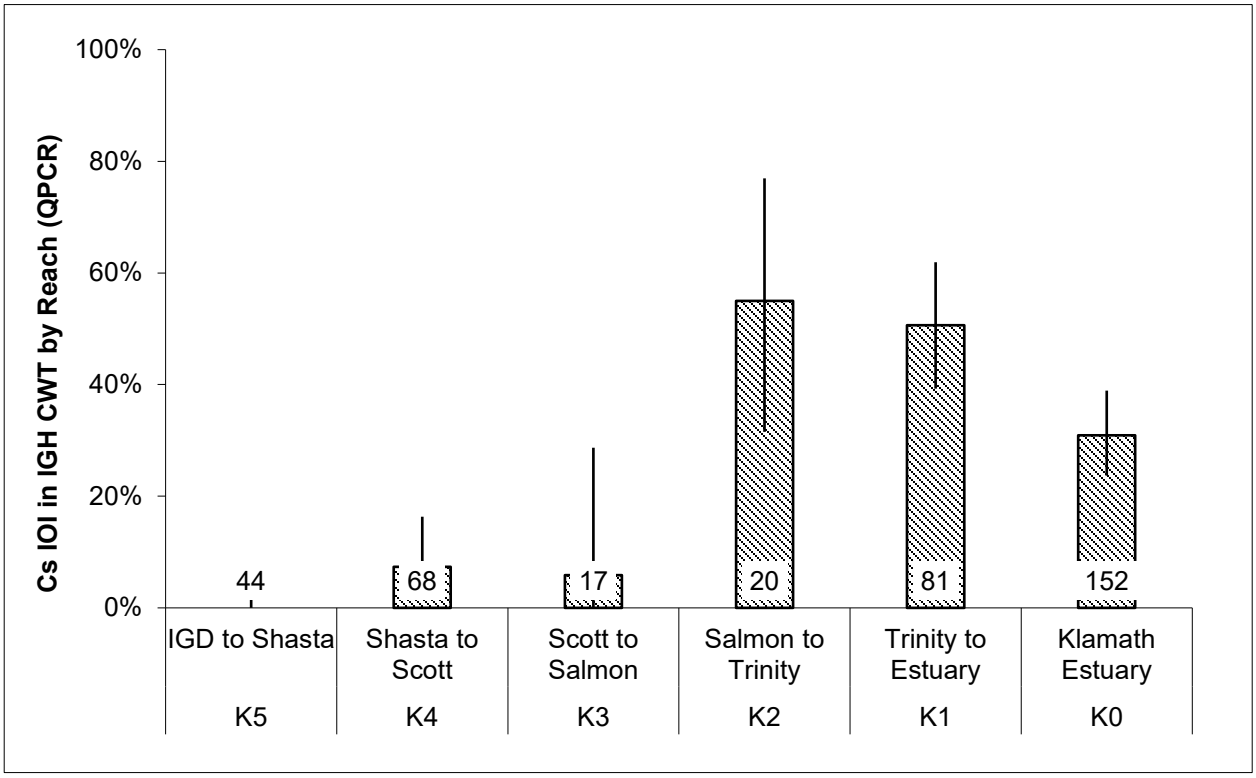

Figure 19. *Ceratomyxa shasta* prevalence of infection by QPCR in Iron Gate Hatchery CWT by reach in which marked Chinook salmon were recovered from. Whiskers indicate 95% confidence interval, sample number is at the base of each bar. No fish tested positive for *C. shasta* in the K5 reach.

C. shasta parasite load, as determined by parasite DNA copy number, was highest in IGH CWT Chinook salmon residing for 3 weeks post hatchery release (Figure 20). The average parasite copy number for infected fish was ~12,000 copies when prevalence of infection was over 40%. Parasite levels dropped dramatically in the 4 WAL group, while the prevalence of infection remained relatively similar to the 3 WAL group. The large rise in parasite number, followed by rapid decreases suggests that highly infected Chinook salmon are dropping out of the population between 3-4 WAL. However, the highest mean *C. shasta* DNA copy number of 12,000 is relatively low compared to levels obtained from clinically moribund fish: which correlates to ~96,000 *C. shasta* DNA copy number or a C_T value of approximately 25 (True et al. 2011).

A second rise in parasite numbers occurs in the 5-6 WAL groups, while prevalence of infection for these sample groups remains in the 25-30% range. Note that while *C. shasta* prevalence of infection remains moderate to high at 25-56% in the 7-8 WAL group, the parasite DNA copy number is negligible. This indicates that while *C. shasta* was present in Chinook salmon that resided for 7-8

weeks prior to recapture, the parasite load in these fish was minimal. Sample size for 9 WAL was notably small, and the single Chinook salmon recovered at 10 WAL was negative for *C. shasta*.

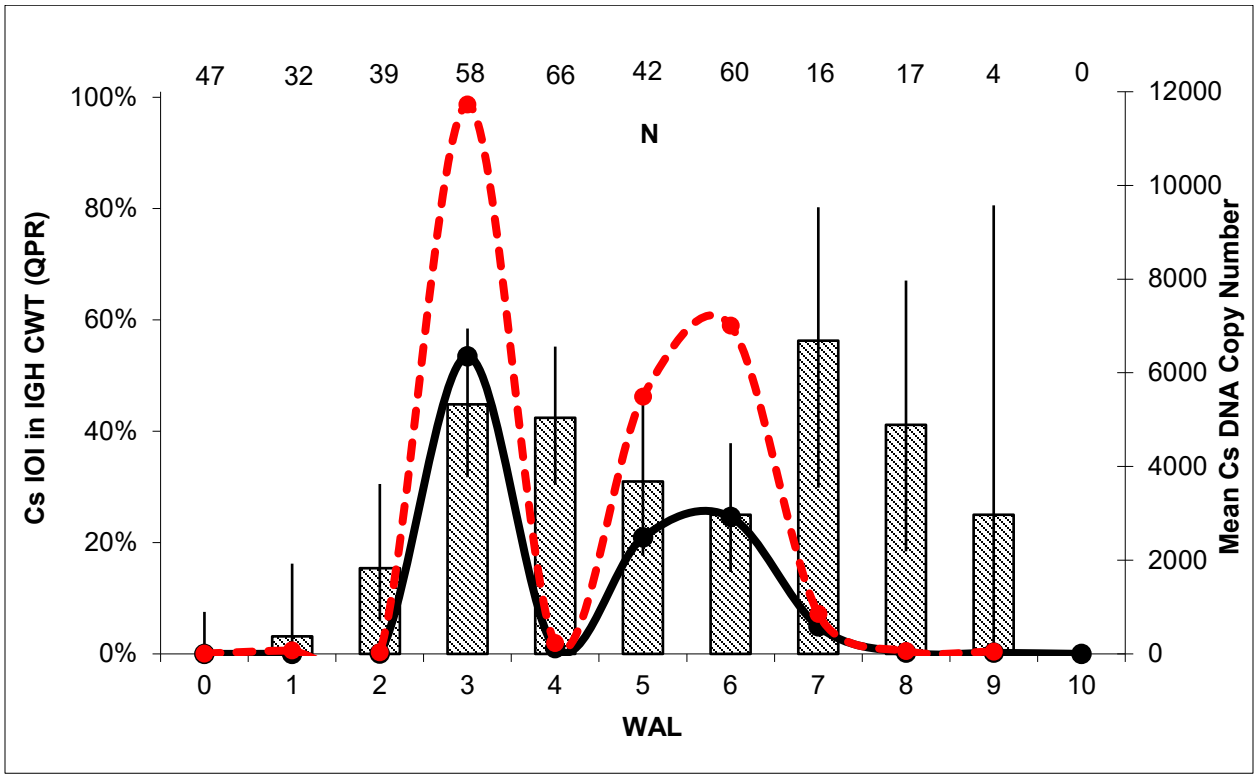

Figure 20. *C. shasta* prevalence of infection in IGH CWT by Weeks at Large (WAL) post hatchery release. Lines (dashed red) are the mean *C. shasta* DNA copy number for Chinook salmon testing positive by QPCR, and (solid black) mean DNA copy number for the entire population sampled.

The relatively low parasite infectious load observed for *C. shasta* in IGH CWT doesn't appear to hold true for *P. minibicornis* prevalence of infection at Weeks at Large. For *Parvicapsula minibicornis* in IGH-CWT Chinook salmon, parasite load is quite different, with a cyclic proliferation of parasites in the 3,6 and 8 WAL groups, and parasite DNA copy numbers above 300,000 copies (Figure 21). Prevalence of infection is high (90-100%) in all but the 1 and 2 WAL groups. We observe rapid cyclical proliferation of parasite numbers in the kidney tissue, which may explain why prevalence of infection in Chinook salmon rises rapidly in early Spring and remains high for this parasite throughout the sampling season.

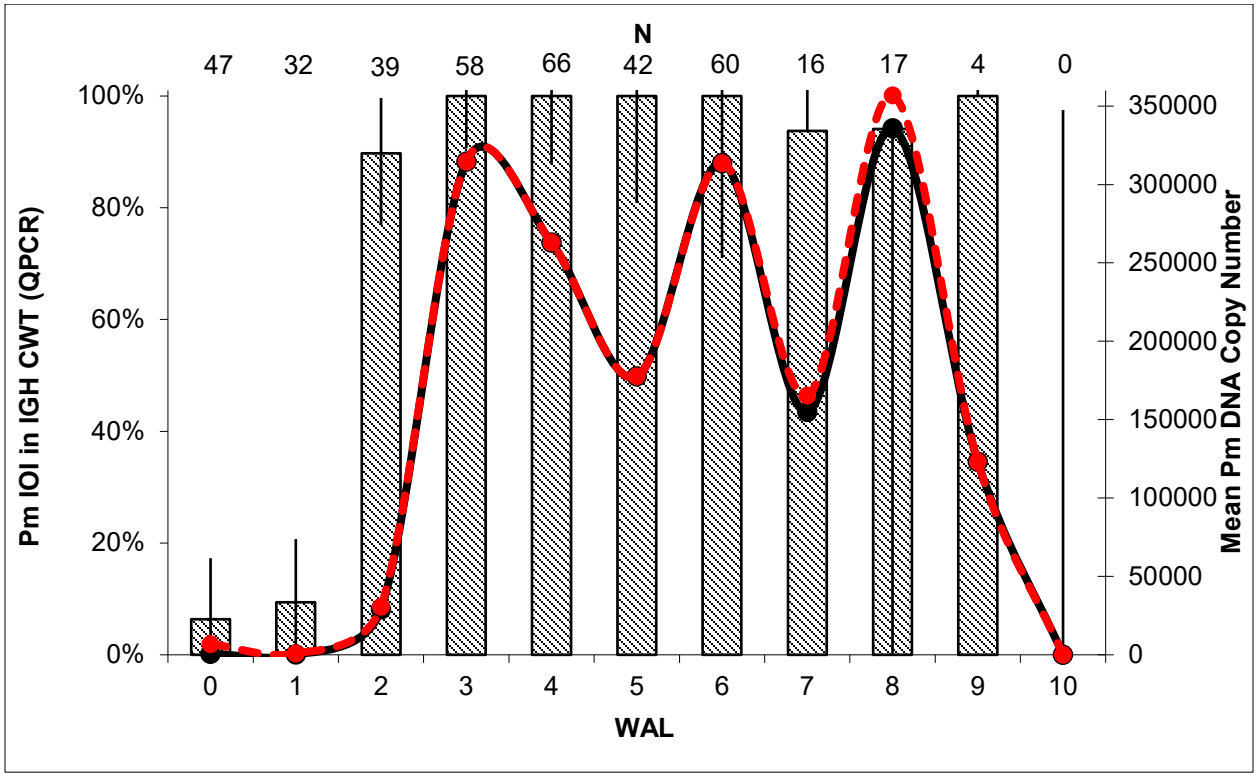

Figure 21. *P. minibicornis* prevalence of infection in IGH CWT by Weeks at Large (WAL) post hatchery release. Lines (dashed red) are the mean *P. minibicornis* DNA copy number for Chinook salmon testing positive by QPCR, and (solid black) mean DNA copy number for the entire population sampled.

Trinity River Hatchery

Ceratomyxa shasta was detected in 0.4% (1/264, 95%ci=0-2%) of the marked TRH Chinook salmon screened by QPCR (data not graphed). Parasite DNA levels were low (664 copies) in the single positive Chinook salmon that had been residing for 9 WAL upon recapture in the Estuary.

Parvicapsula minibicornis was detected in 7% (18/264, ci=4-11%) of all marked TRH Chinook salmon screened by QPCR. Similar to *C. shasta* in TRH CWT Chinook salmon, the only sample group with any significant level of *P. minibicornis* DNA occurred in fish captured 9 weeks post release (Figure 22).

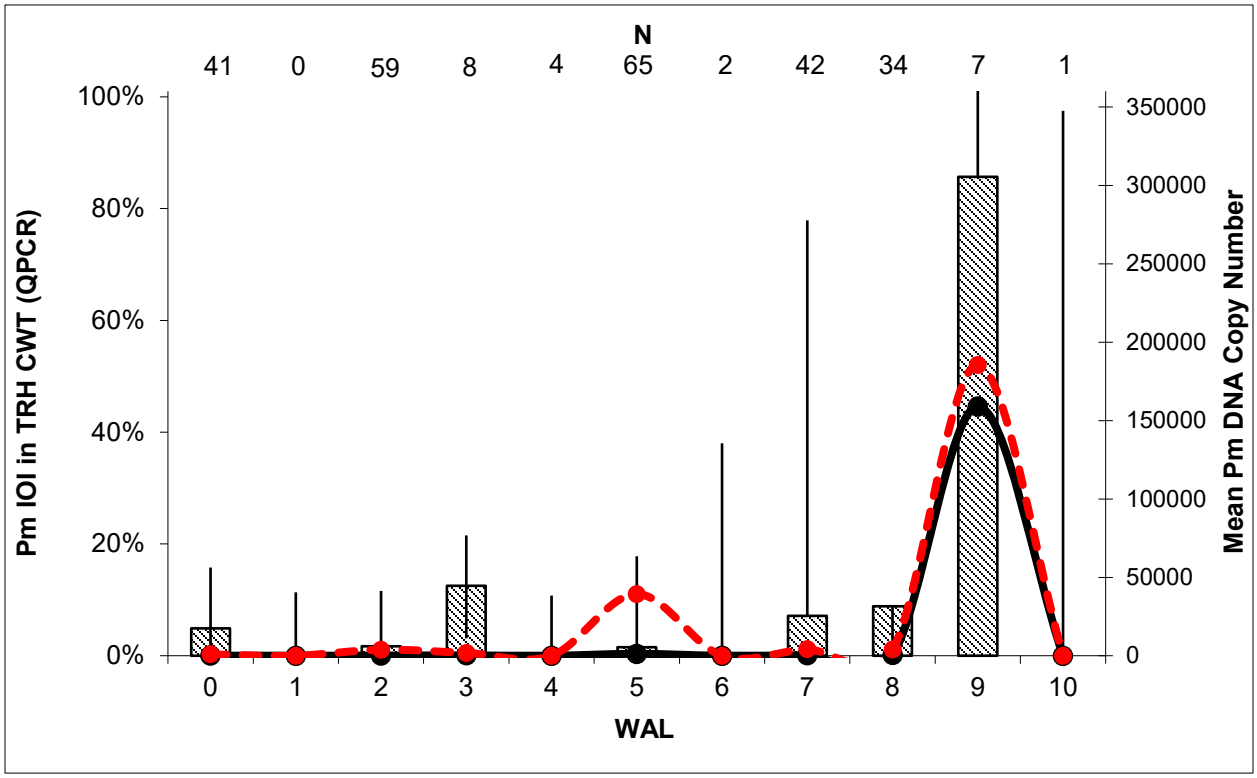

Figure 22. *P. minibicornis* prevalence of infection in TRH CWT by Weeks at Large (WAL) post hatchery release. Lines (dashed red) are the mean *P. minibicornis* (Pm) DNA copy number for Chinook salmon testing positive by QPCR, and (solid black) mean DNA copy number for the entire population sampled.

DISCUSSION

The prevalence of *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, True et al 2010). *C. shasta* prevalence of infection by histological assessment of Chinook salmon captured above the Trinity River confluence is the metric used to compare annual disease prevalence in the Klamath River. Annual comparisons are limited to May through July: the peak juvenile Chinook salmon migration period for Klamath River Chinook salmon (Leidy & Leidy 1984, Wallace & Collins 1997). Histological assessments, along with complimentary QPCR assays, provide a degree of temporal and spatial information for prevalence of infection over the 16 week study period, and for specific reaches of the Klamath River. Temporal data is also derived from coded wire tagged Chinook salmon, with known exposure periods based on hatchery release and in river recapture dates. Spatial data is provided as weekly prevalence of infection in the major reaches of the Klamath River, as juvenile Chinook salmon migrate towards the estuary.

Predictions for myxozoan disease impacts on Klamath River Chinook populations are limited by the nature of monitoring studies. The limitations are primarily due to difficulty in sorting out disease effects from broader environmental factors in a migratory Chinook salmon population. Myxozoan parasite exposure and subsequent disease progression is not a linear process in a riverine environment. Natural and hatchery juvenile Chinook salmon are likely to be exposed to infectious actinospores at multiple points in time and for variable durations once they enter the main stem Klamath River (Bartholomew 2007, Foott et al. 2004), particularly in the more infectious upper reaches above the Trinity River confluence. Past monitoring of tributary Chinook salmon, including a large hatchery component from Trinity River which has been monitored in this study since 2006, has shown negligible *C. shasta* infections in fish that do not rear in the Klamath main stem. This produces a complex epidemiological picture for the basin's Chinook salmon populations. We address these issues by discussing disease prevalence above and below the Trinity River confluence for natural, mixed-origin and CWT Chinook salmon.

For myxozoan fish diseases, the primary factors for the fish host include: species and individual fish susceptibility (Zinn 1977, Buchanan 1983, Ibarra et al. 1992, Bartholomew 1998,), parasite exposure dose (frequency and duration) (Ratcliff 1981, Bjork & Bartholomew 2009b, True et al 2011), and water temperature (Udey et al. 1975, Bartholow 2005). Temperature is extremely important in regulating fish metabolism (immune response and energy metabolism), as well as polychaete and parasite development (Ratcliff 1983, Foott et al. 2004, Bartholomew 2006, Meaders and Hedrickson 2009). Both biotic and abiotic factors are closely associated with complex ecological interactions between the polychaete and fish hosts. Main stem and tributary flows influence polychaete abundance, density and level of parasite infection within the worm host (Bjork and Bartholomew 2009b, Stocking et al. 2006,) as well as migration behavior of juvenile Chinook salmon (timing, rate, utilization of tributaries and/or thermal refugia)(Foott et al. 2004b, Harmon et al. 2001).

Infectivity patterns for *Ceratomyxa shasta* infections are fairly well defined for native Klamath basin salmonid species. At river temperatures (17-24ºC) commonly observed in the Klamath River during peak juvenile Chinook salmon migration, we generally see a three week cycle from initial parasite exposure to clinical disease that results in moderate to high levels of mortality. This

infectivity pattern has been established through sentinel susceptibility studies (Bartholomew 2010, Bjork and Bartholomew 2010, Bartholomew 2009, Stone et al. 2008, True et al. 2011) and annual monitoring of CWT Chinook salmon with known exposure periods in the main stem Klamath (Nichols and Foott 2006, Nichols et al. 2007, Nichols and True 2007, Nichols et al. 2009, True et al 2010). In 2010, this infectivity pattern was apparent in the majority of reaches as a bimodal distribution in bi-weekly prevalence of infection data: natural Chinook salmon sampled prior to hatchery releases, and in mixed-origin Chinook salmon collected after 1 June. We also observed this *C. shasta* infectivity pattern in Iron Gate Hatchery coded-wire tagged Chinook salmon (weeks at large data).

In 2010, *C. shasta* infection by histology (15%) was the lowest level observed during all previous Klamath River fish health monitoring studies conducted from 2005 to 2010. *C. shasta* prevalence of infection by the more sensitive QPCR assay (Bartholomew 2004, Hallett 2006, True et al. 2009) was also the lowest level (17%) observed to date. We observed a temporal shift, and lower magnitude of *C. shasta* bi-weekly prevalence and peak prevalence of infection, of approximately 1 month, depending on the specific reach, in this year's monitoring study. We observed a similar pattern in 2006, when a large precipitation and flow event shifted the disease onset and peak by approximately 2-3 weeks towards the later migration period. Petros and Dillon (2007) suggest that sentinel studies conducted in 2006 demonstrated reduced infection prevalence and mortality rates, as a consequence of lower infectious dose. We could speculate that this additional flow event may have diluted the infectious actinospore concentrations in the water column in 2010, thereby reducing infectivity. However, the precipitation event in 2006 only decreased *C. shasta* prevalence of infection temporarily and overall annual prevalence of infection was not substantially lower in 2006 compared 2007 when no significant flow events occurred. It is unlikely that a single rain event and the temporary increase in flows would decrease disease impacts substantially. An alternative argument could be made that higher flows may have promoted rapid emigration out of the most infectious upper reaches. However, we observed Iron Gate Hatchery coded-wire tagged Chinook salmon entering the Klamath River Estuary at approximately the same period as in previous years (3-5 weeks post hatchery release). So evidence that migration timing was affected in 2010 is lacking.

Either of the above hypotheses regarding reduced exposure dose due to dilution, or increased migration rate (indirectly reducing exposure period and therefore dose) do not appear supported by the juvenile Chinook salmon infectivity data collected in 2010. The primary differences observed in 2010 relate to later infectivity in the upper reaches and lower magnitude of infection in the lower reaches. It should also be noted that Iron Gate Chinook salmon were released approximately two weeks later in 2010 (1 June) compared to 2009 (19 May). In typical monitoring years, later hatchery releases are generally less favorable for juveniles, due to rising river temperatures. But despite this later release date in 2010, juvenile Chinook salmon experienced lower *C. shasta* infection levels in all reaches sampled and lower annual prevalence of infection than previously observed.

In past monitoring studies, *C. shasta* weekly prevalence of infection increases in the upper sample reaches during May and June, when we typically observe 35-80% of juvenile Chinook salmon infected. The peak prevalence of ceratomyxosis disease typically occurs in mid-June (Nichols and True 2007, Nichols et al. 2008, Nichols et al. 2009, True et al. 2010). By contrast, in 2010 *C. shasta* was not detected at all in the uppermost Iron Gate Dam to Shasta (K5) reach, immediately below Iron Gate Hatchery. Furthermore, *C. shasta* was detected in only 12% of both natural Chinook

salmon sampled in May and in hatchery Chinook salmon sampled in June in the next lower reach (Shasta to Scott). *C. shasta* was not detected in 2 of 4 sample weeks in June, after Iron Gate Hatchery Chinook salmon releases, and the highest peak prevalence of 41% did not occur until 28 June. This delayed onset and peak of infection by approximately 2 weeks, in one of the most infectious reaches, indicates that Chinook salmon either experienced a substantially lower infectious dose, a delayed progression of disease, or a compounded beneficial effect attributable to both of these factors.

In the two most infectious reaches historically (Shasta to Scott (K4) and Salmon to Trinity (K2)) the parasite infectious load, as determined by *C. shasta* DNA copy number, was delayed in the upper reach, and of lower magnitude in the lower reach. In the Shasta to Scott reach, the magnitude of the temporal distribution of log mean DNA copy number was not remarkably lower (~ 1 log) in 2010; however the distribution was shifted approximately 3 weeks into late May, compared to 2009 data. In the lower Salmon to Trinity (K2) reach, the frequency distribution of *C. shasta* DNA copy number was flattened in magnitude (2-3 logs lower) compared to 2009. These patterns of infectivity levels in juvenile Chinook salmon suggest that fish were infected at somewhat lower levels in 2010 in the upper reaches, compared to 2009. However the onset of infection and progression to clinical disease was delayed by several weeks. Subsequently, infectious load in Chinook salmon sampled in the lower reach (Salmon to Trinity) was several DNA copy number logs lower compared to the same period in 2009.

In Iron Gate Hatchery coded-wire tagged Chinook salmon, *C. shasta* prevalence of infection in the Klamath River Estuary in 2010 (26%) was lower than observed in 2009 and the majority of study years: comparable to 2008 (27%), lower than 2006 (65%), 2007 (69%) and 2009 (65%). For IGH CWT Chinook salmon collected above the Trinity River confluence, *C. shasta* annual prevalence of infection was also lower in 2010 (11%) compared to previous monitoring years where it ranged from 33%-69% in 2006-2009. In the Klamath River Estuary reach specifically, *C. shasta* annual prevalence of infection was 26% in 2010, and less than half the prevalence level (65%) observed in 2009. Trinity Hatchery coded-wire tagged Chinook salmon were not infected in the Trinity River in 2010; the only *C. shasta* positive coded-wire tagged Chinook salmon was captured in the Estuary, indicating that exposure and infection occurred after entering the main stem Klamath River, below the Trinity River confluence.

River temperatures and flows are both important considerations in assessing disease impacts on juvenile Chinook salmon in a given study year. River flows below Iron Gate Dam were not substantially different in 2010 compared to previous study years. Mean monthly discharges were relatively static (May = 1225cfs, June =1050cfs and July = 825cfs) and therefore flows do not appear to account for the differences in *C. shasta* infectivity that we observed in 2010. Water temperature however influences both hosts in the parasite's life cycle. Water temperature affects polychaete development, sexual maturation and production of infectious actinospores in infected worm populations. In the fish host, temperature plays a key role in immune function and energy metabolism (Wedemyer 1996, Jobling 1995). Immune function is particularly important in resistance to parasite invasion and/or containment (Bartholomew 1998), and more generally in terms of parasite proliferation and disease progression (Ibarra 1992b, Foott et al. 2004, True 2011). Cooler Spring and early summer temperatures appear to have played a more significant role in disease dynamics in 2010. Udey (1975) demonstrated that post-exposure temperature is inversely related to mortality rates: 22% when fish were held at 15ºC compared to 84% at 20ºC. We

hypothesis that cooler Spring temperatures (below 13ºC) below Iron Gate Dam (in the infectious zone) followed by 3-5ºC cooler temperatures in late May and early June at Seiad Valley (the lower boundary of the infectious zone) likely delayed both the maturation of polychaetes and the progression of disease in out-migrating juvenile Chinook salmon in the Klamath River basin.

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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.
- Anne Bolick - Necropsy, DNA extraction, QPCR assay, assistance with pivot tables, report tables and figures.
- Scott Foott – Histology, diagnostic assessments, and histological report sections.

REVISION
April 2018 – This report was revised due to errors found post publication, as follows:

1) Average prevalence value for Histology was recalculated in Table 4, page 29. Annual value for 2002 (19%) was excluded from the average for consistency with the previous report. The average value changed from 31% to 33%, and the standard error changed from 3 to 5.

2) Miscalculation of the average value for both Iron Gate and Trinity CWT by QPCR, again in Table 4, page 29. Miscalculation resulted in POI average of 43% for Iron Gate CWT when the accurate POI was 30%; it also resulted in 7% average POI for Trinity CWTs when the accurate POI was 9%.

3) Revision to 2009 report resulted in corrections to Table 4 in this report, page 29, for the 2009 QPCR value (see 2009 revision note #1 for details).

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APPENDIX I – Histological Summary Table

Table 1A. Prevalence of parasite infection and tissue (no. positive / total (%)) and pathology score for kidney and intestine observed in histological sections of juvenile Klamath River Chinook salmon collected from the Shasta to Scott reach (K4).

	4/5	4/19	5/3	5/17	5/31	6/7	6/21	7/5	Prevalence
Kidney									
Pm Troph.	0 / 10 (0)	1 / 10 (10)	5 / 10 (50)	8 / 10 (80)	9 / 10 (90)	7 / 10 (70)	4 / 10 (40)	0 / 4 (0)	34 / 74 (46)
Pm Myxosp.	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 4 (0)	0 / 74 (0)
Metacercaria	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	2 / 10 (20)	0 / 10 (0)	2 / 10 (20)	4 / 10 (40)	0 / 4 (0)	8 / 74 (11)
C. shasta troph.	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 4 (0)	0 / 74 (0)
Chloromyxum sp.	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 4 (0)	0 / 74 (0)
Pathology Score	0.00	0.00	0.00	2.00	3.20	5.00	1.00	0.00	
Intestinal tract									
C. shasta troph.	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	4 / 10 (40)	5 / 10 (50)	2 / 10 (20)	2 / 10 (20)	0 / 4 (0)	13 / 74 (18)
C. shasta myxosp.	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 4 (0)	0 / 74 (0)
Helminth	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	1 / 10 (10)	0 / 10 (0)	1 / 10 (10)	0 / 4 (0)	2 / 74 (3)
Pathology Score	0.00	0.00	0.00	2.00	0.00	0.20	0.80	0.00	
Adipose steatitis	0 / 10 (0)	0 / 2 (0)	7 / 7 (100)	1 / 3 (33)	4 / 5 (80)	5 / 7 (71)	5 / 7 (71)	0 / 4 (0)	22 / 45 (49)
Adipose lipofuscin	0 / 10 (0)	0 / 1 (0)	0 / 7 (0)	0 / 3 (0)	0 / 5 (0)	0 / 7 (0)	0 / 7 (0)	0 / 4 (0)	0 / 44 (0)
Liver *C. shasta*	ND	ND	ND	ND	ND	ND	ND	ND	
Gill									
Ich	0 / 10 (0)	0 / 10 (0)	0 / 8 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	1 / 10 (10)	0 / 4 (0)	1 / 72 (1)
Glochidia	0 / 10 (0)	0 / 10 (0)	0 / 8 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	2 / 10 (20)	0 / 4 (0)	2 / 72 (3)
Miricidia	0 / 10 (0)	0 / 10 (0)	0 / 8 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 4 (0)	0 / 72 (0)
Metacercaria	0 / 10 (0)	1 / 10 (10)	0 / 8 (0)	1 / 10 (10)	7 / 10 (70)	9 / 10 (90)	8 / 10 (80)	0 / 4 (0)	26 / 72 (36)
Invasive *C. shasta*	0 / 10 (0)	0 / 10 (0)	0 / 8 (0)	5 / 10 (50)	0 / 10 (0)	1 / 10 (10)	1 / 10 (10)	0 / 4 (0)	7 / 72 (10)
Multif. Hyperplasia	0 / 10 (0)	0 / 10 (0)	0 / 8 (0)	0 / 10 (0)	2 / 10 (20)	4 / 10 (40)	10 / 10 (100)	0 / 4 (0)	16 / 72 (22)

Table 2A. Prevalence of parasite infection (no. positive / total (%)) and pathology score for kidney and intestine observed in histological sections of juvenile Klamath River Chinook salmon collected from the Scott to Salmon reach (K3). Overall prevalence of infection for the bi-weekly collections (date reported as Monday of given week) also reported.

	5/3	5/17	Prevalence
Kidney			
Pm Troph.	4 / 10 (40)	9 / 10 (90)	13 / 20 (65)
Pm Myxosp.	0 / 10 (0)	0 / 10 (0)	0 / 20 (0)
Metacercaria	0 / 10 (0)	0 / 10 (0)	0 / 20 (0)
C. shasta troph.	0 / 10 (0)	0 / 10 (0)	0 / 20 (0)
Chloromyxum sp.	0 / 10 (0)	0 / 10 (0)	0 / 20 (0)
Pathology Score	0.00	1.2	
Intestinal tract			
C. shasta troph.	0 / 10 (0)	4 / 10 (40)	4 / 20 (20)
C. shasta myxosp.	0 / 10 (0)	0 / 10 (0)	0 / 20 (0)
Helminth	0 / 10 (0)	0 / 10 (0)	0 / 20 (0)
Pathology Score	0.00	0.6	
Adipose steatitis	3 / 5 (60)	7 / 10 (70)	10 / 15 (67)
Adipose lipofuscin	0 / 5 (0)	0 / 10 (0)	0 / 15 (0)
Gill Ich	0 / 10 (0)	0 / 10 (0)	0 / 20 (0)
Glochidia	0 / 10 (0)	0 / 10 (0)	0 / 20 (0)
Miricidia	0 / 10 (0)	0 / 10 (0)	0 / 20 (0)
Metacercaria	0 / 10 (0)	0 / 10 (0)	0 / 20 (0)
Invasive *C. shasta*	0 / 10 (0)	0 / 10 (0)	0 / 20 (0)
MF- Hyperplasia	0 / 10 (0)	6 / 10 (60)	6 / 20 (30)

Table 3A. Prevalence of parasite infection (no. positive / total (%)) and pathology score for kidney and intestine observed in histological sections of salmon collected from the Salmon to Trinity reach (K2). Overall prevalence of infection for the bi-weekly collections (date reported as Monday of given week) also reported.

	5/3	5/17	5/24	6/7	6/21	7/5	7/19	8/2	8/16	Prevalence
Kidney										
Pm Troph.	0 / 10 (0)	1 / 10 (10)	9 / 10 (90)	10/ 10 (100)	10 / 10 (100)	10 / 10 (100)	11 / 12 (92)	6 / 10 (60)	5 / 11 (45)	62 / 93 (67)
Pm Myxosp.	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)		0 / 10 (0)	0 / 12 (0)	0 / 10 (0)	0 / 11 (0)	0 / 93 (0)
Metacercaria	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	1 / 10 (10)	0 / 10 (0)	3 / 10 (30)	3 / 12 (25)	3 / 10 (30)	2 / 11 (18)	15 / 93(16)
C. shasta troph.	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	3 / 10 (30)	1 / 10 (10)	0 / 12 (0)	0 / 10 (0)	0 / 11 (0)	1 /93 (1)
Chloromyxum sp.	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 12 (0)	0 / 10 (0)	1 / 11 (9)	1 /93(1)
					0 / 10 (0)					
Pathology Score	0.00	0.00	0.50	4.60	3.40	3.80	3.67	1.90	0.91	
Intestinal tract										
C. shasta troph.	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	4 / 10 (40)	0 / 10 (0)	1 / 10 (10)	0 / 12 (0)	1 / 10 (10)	1 / 11 (9)	7 / 93(8)
C. shasta myxo.	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 12 (0)	0 / 10 (0)	0 / 11 (0)	0 / 93 (0)
Helminth	0 / 10 (0)	1 / 10 (10)	2 / 10 (20)	1 / 10 (10)	3 / 10 (30)	0 / 10 (0)	0 / 12 (0)	0 / 10 (0)	1 / 11 (9)	8/ 93 (9)
Pathology Score	0.00	0.00	0.00	0.20	0.10	0.30	0.08	0.10	0.20	
Adipose steatitis	0 / 10 (0)	2 / 7 (29)	2 / 5 (40)	5 / 5 (100)	8 / 9 (89)	7 / 9 (78)	1 / 5 (20)	3 / 7 (43)	3 / 8 (38)	31 / 65 (48)
Adipose lipofuscin	0 / 10 (0)	0 / 7 (0)	0 / 5 (0)	0 / 5 (0)	1 / 9 (11)	0 / 9 (0)	1 / 5 (20)	0 / 7 (0)	0 / 8 (0)	1 / 65 (2)
Gill										
Ich	0 / 9 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 12 (0)	0 / 10 (0)	0 / 11 (0)	0 / 92 (0)
Glochidia	0 / 9 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	1 / 10 (10)	0 / 12 (0)	0 / 10 (0)	0 / 11 (0)	0 / 92 (0)
Miricidia	0 / 9 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (10)	0 / 10 (0)	0 / 10 (0)	1 / 12 (8)	0 / 10 (0)	0 / 11 (0)	0 / 92 (0)
Metacercaria	1 / 9 (11)	1 / 10 (10)	5 / 10 (50)	7 / 10 (70)	8 / 10 (80)	10 / 10 (100)	12 / 12 (100)	9 / 10 (90)	10 / 11 (91)	63 / 92 (68)
Invasive *C. shasta*	0 / 9 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 12 (0)	1 / 10 (10)	1 / 11 (9)	2 / 92 (2)
MF-Hyperplasia	0 / 9 (0)	1 / 10 (10)	2 / 10 (20)	5 / 10 (50)	6 / 10 (0)	9 / 10 (90)	9 / 12 (75)	7 / 10 (70)	8 / 11 (73)	47 / 92 (51)

Table 4A. Prevalence of parasite infection (no. positive / total (%)) and pathology score for kidney and intestine observed in histological sections of salmon collected from the Trinity R. to Klamath estuary reach (K1). Overall prevalence of infection for the bi-weekly collections (date reported as Monday of given week) also reported.

	5/3	5/17	5/24	6/7	6/21	7/5	7/19	7/26	Prevalence
Kidney									
Pm Troph.	1 / 10 (10)	1 / 9 (11)	6 / 10 (60)	7 / 10 (70)	6 / 11 (55)	9 / 10 (90)	7 / 10 (70)	6 / 10 (60)	43 / 80 (54)
Pm Myxosp.	0 / 10 (0)	0 / 9 (0)	0 / 10 (0)	0 / 10 (0)	0 / 11 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 80 (0)
Metacercaria	0 / 10 (0)	0 / 9 (0)	1 / 10 (10)	1 / 10 (10)	0 / 11 (0)	2 / 10 (20)	3 / 10 (30)	6 / 10 (60)	13 / 80 (16)
C. shasta troph.	0 / 10 (0)	0 / 9 (0)	0 / 10 (0)	0 / 10 (0)	0 / 11 (0)	0 / 10 (0)	0 / 10 (0)	3 / 10 (30)	3 / 80 (4)
Chloromyxum sp.	0 / 10 (0)	0 / 9 (0)	1 / 10 (10)	1 / 10 (10)	2 / 11 (18)	0 / 10 (0)	1 / 10 (10)	1 / 10 (10)	6 / 80 (8)
Pathology Score	0.10	0.00	0.20	0.8	0.09	5.20	6.70	3.5	
Intestinal tract									
C. shasta troph.	3 / 10 (30)	1 / 10 (10)	1 / 10 (10)	3 / 10 (30)	1 / 11 (9)	1 / 10 (10)	1 / 10 (10)	3 / 10 (30)	14 / 80 (18)
C. shasta myxosp.	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 11 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 80 (0)
Helminth	0 / 10 (0)	1 / 10 (10)	1 / 10 (10)	1 / 10 (10)	2 / 11 (8)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	5 / 80 (6)
Pathology Score	0.30	0.10	0.20	1.20	0.00	0.40	0.60	1.60	
Adipose steatitis	2 / 10 (20)	4 / 5 (80)	4 / 6 (67)	6 / 7 (86)	3 / 5 (60)	5 / 5 (100)	2 / 2 (100)	6 / 7 (86)	32 / 47 (68)
Adipose lipofuscin	0 / 10 (0)	0 / 5 (0)	0 / 6 (0)	0 / 7 (0)	0 / 5 (0)	0 / 5 (0)	1 / 2 (50)	0 / 7 (0)	1 / 47 (2)
Gill									
Ich	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	1 / 10 (10)	0 / 11 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	1 / 81 (1)
Glochidia	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	1 / 10 (10)	0 / 11 (00)	2 / 10 (20)	0 / 10 (0)	1 / 10 (10)	3 / 81 (4)
Miricidia	0 / 10 (0)	0 / 10 (0)	0 / 10 (10)	0 / 10 (0)	0 / 11 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 81 (0)
Metacercaria	0 / 10 (0)	0 / 10 (0)	2 / 10 (20)	1 / 10 (10)	3 / 11 (27)	10 / 10 (100)	6 / 10 (60)	6 / 10 (60)	28 / 81 (35)
Invasive *C. shasta*	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 10 (0)	0 / 11 (0)	0 / 10 (0)	0 / 10 (0)	1 / 10 (10)	1 / 81 (1)
Multif. Hyperplasia	1 / 10 (10)	2 / 10 (20)	4 / 10 (40)	2 / 10 (20)	1 / 11 (9)	9 / 10 (90)	7 / 10 (70)	4 / 10 (40)	30 / 81 (37)

APPENDIX II - Validation and Sensitivity of *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.

APPENDIX III – Water Data Report for Iron Gate Dam Discharges in 2010
Statistical daily mean averages are given for years 1996-2010.

Water-Data Report 2010

11516530 Klamath River below Iron Gate Dam, CA

Klamath River Basin

LOCATION.--Lat 41°55′41″, long 122°26′35″ referenced to North American Datum of 1927, in SE ¼ NE ¼ sec.17, T.47 N., R.5 W., Siskiyou County, CA, Hydrologic Unit 18010206, Mt. Diablo Meridian, on left bank, 0.1 mi downstream from Bogus Creek, 0.5 mi downstream from Iron Gate Dam, and 6 mi northeast of Hornbrook.

DRAINAGE AREA.--4,630 mi², approximately (not including Lost River, Butte Creek, or Lower Klamath Lake Basins).

SURFACE-WATER RECORDS

PERIOD OF RECORD.--October 1960 to current year.
PRECIPITATION: Water years 1999-2001
CHEMICAL DATA: Water years 1962-81, 2002-04 (seasonal records only).
DISSOLVED OXYGEN: Water years 1999-2001, 2002-03 (seasonal records only).
pH: Water years 1999-2001, 2002-03 (seasonal records only).
SPECIFIC CONDUCTANCE: Water years 1999-2001, 2002-03 (seasonal records only).
AIR TEMPERATURE: Water years 1999-2001.
WATER TEMPERATURE: Water years 1962-80, 1999-2001, 2002-03 (seasonal records only).

GAGE.--Water-stage recorder. Datum of gage is 2,162.44 ft above NGVD of 1929 (levels by PacifiCorp, formerly Pacific Power and Light Co.).

REMARKS.--Records good. Flow regulated by Upper Klamath Lake, capacity, 523,700 acre-ft; Iron Gate Reservoir (station 11516510), other smaller reservoirs and diversions upstream from station. Records collected in connection with Federal Energy Regulatory Commission (FERC) project no. 2083. See schematic diagram of Klamath River and Trinity River Basins available from the California Water Science Center.

EXTREMES FOR PERIOD OF RECORD.--Maximum discharge, 29,400 ft³/s, Dec. 22, 1964, gage height, 13.63 ft, from rating curve extended above 15,000 ft³/s on basis of slope-area measurement of peak flow; minimum daily, 389 ft³/s, Aug. 25-28, 1992.

U.S. Department of the Interior
U.S. Geological Survey

11516530 Klamath River below Iron Gate Dam, CA—Continued

DISCHARGE, CUBIC FEET PER SECOND
WATER YEAR OCTOBER 2009 TO SEPTEMBER 2010
DAILY MEAN VALUES

Day	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep
1	1,290	1,290	1,320	1,330	1,310	1,740	1,340	1,330	1,090	945	906	1,030
2	1,290	1,300	1,320	1,320	1,320	1,730	1,340	1,510	1,050	843	905	1,030
3	1,290	1,300	1,320	1,340	1,320	1,610	1,340	1,370	1,050	818	905	1,030
4	1,300	1,300	1,320	1,330	1,320	1,490	1,340	1,290	1,060	817	904	1,030
5	1,290	1,300	1,320	1,330	1,320	1,470	1,340	1,200	1,060	814	906	1,030
6	1,290	1,300	1,320	1,320	1,320	1,470	1,340	1,210	1,060	814	906	1,030
7	1,290	1,300	1,320	1,320	1,320	1,470	1,340	1,210	1,060	813	905	1,030
8	1,300	1,300	1,320	1,320	1,320	1,470	1,340	1,210	1,060	811	905	1,030
9	1,290	1,300	1,320	1,320	1,370	1,470	1,340	1,210	1,060	816	906	1,030
10	1,290	1,300	1,320	1,320	1,330	1,470	1,340	1,220	1,060	817	907	1,030
11	1,300	1,300	1,330	1,320	1,330	1,470	1,340	1,210	1,060	816	905	1,030
12	1,300	1,300	1,320	1,320	1,330	1,470	1,340	1,210	1,050	815	904	1,020
13	1,300	1,300	1,310	1,330	1,330	1,470	1,340	1,190	1,040	814	905	1,020
14	1,290	1,300	1,310	1,320	1,330	1,470	1,340	1,200	1,040	815	907	1,030
15	1,290	1,300	1,310	1,320	1,330	1,470	1,340	1,200	1,040	816	916	1,030
16	1,290	1,310	1,320	1,320	1,330	1,470	1,340	1,200	1,050	816	1,020	1,020
17	1,290	1,330	1,320	1,320	1,330	1,470	1,330	1,200	1,040	816	1,020	1,020
18	1,290	1,330	1,320	1,320	1,330	1,470	1,330	1,200	1,040	815	1,020	1,020
19	1,290	1,330	1,320	1,320	1,330	1,420	1,330	1,200	1,040	817	1,020	1,020
20	1,300	1,330	1,320	1,320	1,330	1,330	1,340	1,200	1,040	818	1,020	1,020
21	1,290	1,330	1,320	1,320	1,330	1,300	1,340	1,200	1,040	817	1,020	1,030
22	1,290	1,330	1,320	1,320	1,330	1,300	1,340	1,200	1,040	818	1,020	1,030
23	1,300	1,330	1,320	1,320	1,330	1,300	1,340	1,200	1,040	818	1,030	1,030
24	1,300	1,320	1,320	1,320	1,330	1,300	1,340	1,200	1,040	819	1,020	1,030
25	1,300	1,330	1,310	1,320	1,330	1,300	1,340	1,200	1,050	822	1,020	1,030
26	1,300	1,330	1,320	1,330	1,330	1,300	1,340	1,210	1,050	822	1,300	1,030
27	1,310	1,320	1,320	1,330	1,330	1,300	1,390	1,210	1,050	822	1,410	1,030
28	1,310	1,320	1,320	1,310	1,390	1,310	1,370	1,200	1,050	823	1,400	1,030
29	1,310	1,320	1,320	1,310	---	1,310	1,360	1,200	1,050	830	1,290	1,030
30	1,300	1,320	1,320	1,310	---	1,310	1,350	1,200	1,050	832	1,170	1,030
31	1,310	---	1,320	1,310	---	1,310	---	1,200	---	844	1,080	---
Total	40,180	39,370	40,890	40,960	37,250	44,240	40,280	37,990	31,510	25,533	31,452	30,830
Mean	1,296	1,312	1,319	1,321	1,330	1,427	1,343	1,225	1,050	824	1,015	1,028
Max	1,310	1,330	1,330	1,340	1,390	1,740	1,390	1,510	1,090	945	1,410	1,030
Min	1,290	1,290	1,310	1,310	1,310	1,300	1,330	1,190	1,040	811	904	1,020
Ac-ft	79,700	78,090	81,110	81,240	73,890	87,750	79,900	75,350	62,500	50,640	62,390	61,150

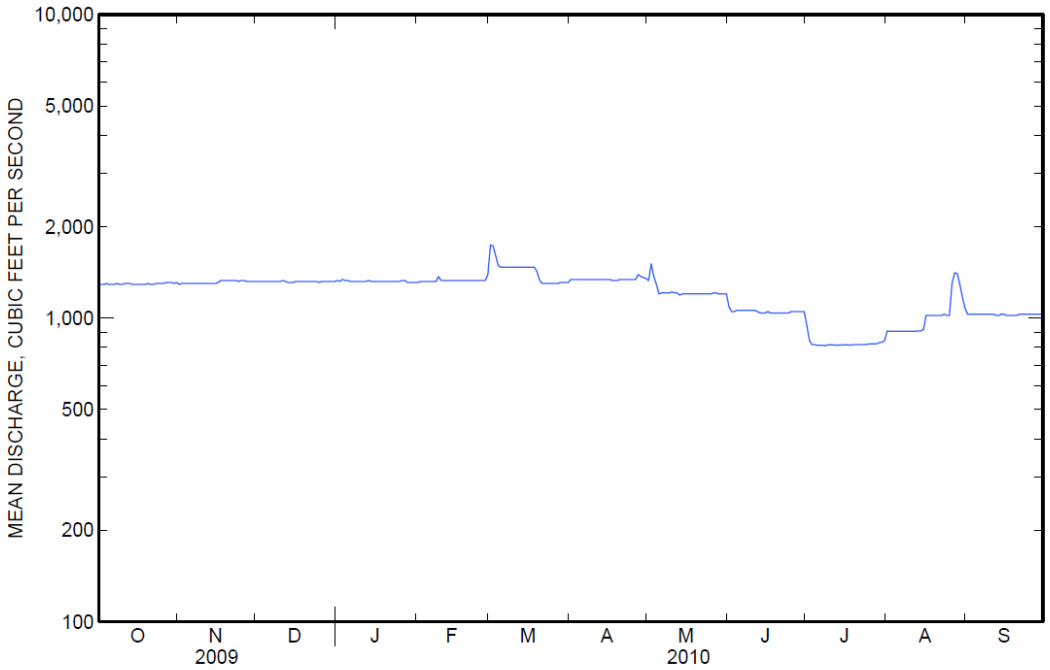
STATISTICS OF MONTHLY MEAN DATA FOR WATER YEARS 1961 - 2010, BY WATER YEAR (WY)

	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep
Mean	1,563	1,935	2,471	2,801	2,907	3,388	2,920	2,151	1,222	825	978	1,245
Max	3,353	5,254	6,735	9,553	9,150	10,780	6,922	5,559	3,289	1,429	1,208	2,052
(WY)	(1985)	(1985)	(1984)	(1997)	(1965)	(1972)	(1971)	(1998)	(1998)	(1982)	(1965)	(1965)
Min	852	873	889	888	525	511	572	512	506	428	398	538
(WY)	(1982)	(1992)	(1992)	(1992)	(1992)	(1992)	(1994)	(1992)	(1992)	(1992)	(1992)	(1992)

11516530 Klamath River below Iron Gate Dam, CA—Continued

SUMMARY STATISTICS

	Calendar Year 2009		Water Year 2010		Water Years 1961 - 2010	
Annual total	485,447		440,485			
Annual mean	1,330		1,207		2,029	
Highest annual mean					3,657	1965
Lowest annual mean					641	1992
Highest daily mean	1,780	Mar 5	1,740	Mar 1	25,000	Dec 22, 1964
Lowest daily mean	988	Sep 21	811	Jul 8	389	Aug 25, 1992
Annual seven-day minimum	990	Sep 17	814	Jul 5	390	Aug 24, 1992
Maximum peak flow			1,760	Mar 1	29,400	Dec 22, 1964
Maximum peak stage			3.39	Mar 1	13.63	Dec 22, 1964
Annual runoff (ac-ft)	962,900		873,700		1,470,000	
10 percent exceeds	1,610		1,340		3,860	
50 percent exceeds	1,320		1,300		1,360	
90 percent exceeds	995		905		741	



APPENDIX IV – Summary Table of *Ceratomyxa shasta* and *Parvicapsula minibicornis* bi-weekly prevalence of infection (POI) and prevalence by Sample Reach

River / Reach Name	Reach Code	Sample Week	Weekly Start Date	Total Sampled	*C. shasta* +	Cs POI	*P. minibicornis*+	Pm POI
KLAMATH RIVER IGD to Shasta	**K5**	9	7-Jun	17	0	0%	0	0%
		11	14-Jun	19	0	0%	3	16%
		13	28-Jun	20	0	0%	12	60%
K5 Total				**56**	**0**	**0%**	**15**	**27%**
Shasta to Scott	**K4**	1	5-Apr	20	0	0%	0	0%
		3	19-Apr	20	0	0%	1	5%
		5	3-May	20	0	0%	9	45%
		7	17-May	20	0	0%	13	65%
		8	24-May	31	8	26%	31	100%
		9	31-May	52	8	15%	24	46%
		10	7-Jun	46	3	7%	23	50%
		11	14-Jun	30	7	23%	26	87%
		12	21-Jun	30	0	0%	3	10%
		13	28-Jun	22	9	41%	20	91%
K4 Total				**291**	**35**	**12%**	**150**	**52%**

River / Reach Name	Reach Code	Sample Week	Weekly Start Date	Total Sampled	*C. shasta* +	Cs POI	*P. minibicornis*+	Pm POI
KLAMATH RIVER Scott to Salmon	**K3**	5	3-May	20	2	10%	7	35%
		7	17-May	20	2	10%	7	35%
		8	24-May	19	2	11%	11	57.9%
		9	31-May	20	8	40%	18	90%
		10	7-Jun	20	10	50%	18	90%
		11	14-Jun	20	6	30%	20	100%
		12	21-Jun	21	6	29%	20	95.2%
		13	28-Jun	51	22	43%	47	92.2%
		14	5-Jul	20	6	30%	20	100%
		15	12-Jul	22	1	5%	22	100%
		16	19-Jul	23	5	22%	23	100%
Salmon River RST (These tributary Chinook were not included in reach POI summary)		18	2-Aug	40	0	0%	0	0%
K3 Total				**256**	**70**	**27%**	**213**	**83%**
Salmon to Trinity	**K2**	5	3-May	20	1	5%	1	5%
		7	17-May	20	0	0%	9	45%
		8	24-May	30	0	0%	8	27%
		10	7-Jun	30	4	13%	18	60%
		12	21-Jun	31	0	0%	28	90%
		14	5-Jul	30	6	20%	29	97%
		15	12-Jul	19	9	47%	17	89%
		16	26-Jul	31	3	10%	23	74%
		18	2-Aug	31	5	16%	29	94%
		20	16-Aug	30	4	13%	29	97%
K2 Total				**272**	**32**	**12%**	**191**	**70%**

River / Reach Name	Reach Code	Sample Week	Weekly Start Date	Total Sampled	*C. shasta* +	Cs POI	*P. minibicornis*+	Pm POI
KLAMATH RIVER Trinity to Estuary	**K1**	5	3-May	20	1	5%	5	25%
		7	17-May	20	0	0%	1	5%
		8	24-May	30	3	10%	18	60%
		10	7-Jun	30	5	17%	24	80%
		12	21-Jun	30	3	10%	29	97%
		13	28-Jun	22	6	27%	20	91%
		14	5-Jul	8	3	38%	8	100%
		15	12-Jul	7	6	86%	7	100%
		16	19-Jul	3	2	67%	3	100%
		17	26-Jul	29	12	41%	24	83%
		19	9-Aug	23	11	48%	21	91%
		20	16-Aug	8	6	75%	7	88%
K1 Total				**230**	**58**	**25%**	**167**	**73%**
Klamath River Estuary	**K0**	12	21-Jun	10	1	10%	7	70%
		13	28-Jun	30	10	33%	25	83%
		14	5-Jul	30	15	50%	26	87%
		15	12-Jul	27	13	48%	25	93%
		16	19-Jul	30	5	17%	28	93%
		17	26-Jul	30	3	10%	21	70%
		18	2-Aug	30	2	7%	29	97%
		19	9-Aug	14	3	21%	13	93%
K0 Total				**201**	**52**	**26%**	**174**	**87%**

River / Reach Name	Reach Code	Sample Week	Weekly Start Date	Total Sampled	*C. shasta* +	Cs POI	*P. minibicornis*+	Pm POI
TRINITY RIVER Pear Tree RST	**T2**	6	10-May	28	0	0%	1	4%
		8	24-May	20	1	5%	2	10%
		10	7-Jun	30	1	3%	0	0%
		12	21-Jun	30	0	0%	1	3%
		15	12-Jul	30	0	0%	1	3%
		17	26-Jul	30	0	0%	0	0%
T2 Total				**168**	**2**	**1%**	**5**	**3%**
Willow Creek RST	**T1**	10	7-Jun	19	0	0%	1	5%
		12	21-Jun	29	0	0%	0	0%
		15	12-Jul	60	0	0%	0	0%
		17	26-Jul	31	0	0%	0	0%
T2 Total				**139**	**0**	**0%**	1	**1%**
Cs and Pm IOI - All Reaches, All Weeks				**1653**	**249**	**15%**	**773**	**47%**

APPENDIX V - Reviewers' comments

Listed below are verbatim (in quotes) or paraphrased comments provided by reviewers of a draft of this report. The primary author's reply is given unless noted otherwise (additional authors name and responses are provided for specific sections of this report).

Reviewer #1

Pg 2 - Summary: Reviewer requested clarification on 3 statements in the summary that discuss the lowest prevalence of *C. shasta* observed in the monitoring study conducted from 2005-2010.
Response: The following edits were done:
Annual metric was defined for the first instance of *C. shasta* prevalence data given. The second reference to low *C. shasta* prevalence discussed this trend in all Chinook salmon groups sampled (natural, IGH CWT, and TRH CWT Chinook salmon). The overall trend of lower *C. shasta* prevalence of infection, for all study years of the monitoring program, is meant to be a concluding statement at the end of the summary paragraph for overall results.

Pg 3 – Introduction: In reference to the opening sentence which reads, "Juvenile Klamath River Chinook salmon (*Oncorhynchus tshawytscha*) often experience high prevalence and severity of infection with the myxosporean parasites *Ceratomyxa shasta* and *Parvicapsula minibicornis*." , reviewer commented, "How high, what is the range?"
Response: The following sentence was added.
The annual metric for *C. shasta* prevalence of infection by QPCR in study years 2006-2010 has ranged from 19-45% (Table 4) and
P. minibicornis prevalence has ranged from 66-91% (data not shown in Table 4).

Pg 3 – Reviewer requested clarification on the name of the metric of parasite "DNA copy number".
Response: "DNA copy number" is the name of the metric, and refers to the quantity of parasite DNA present in target tissues. The previous term used in prior reports was Cycle Threshold (C_T) but this unit is inversely related to parasite DNA copy number in fish tissues (higher C_T values mean less DNA, whereas lower C_T values represent higher levels of parasite DNA). The C_T term does not provide a direct, or meaningful measure of parasite DNA levels in fish tissue and is confusing for the average reader.

Pg 7 Figure 2: Reviewer requested larger type and axis labels for this figure, and suggested using full parasite names versus abbreviations (Cs and Pm).
Response: The graph is exported from the QPCR instrument and editing ability is limited. The graph was moved to a new page and enlarged. The primary point of the graph was to demonstrate that we use 6 plasmid dilutions to determine the standard curve of the assay and that both QPCR assays have similar standard curves and therefore direct comparisons of parasite quantities can be made between *C. shasta* and *P. minibicornis* test results for Chinook salmon. The methods section notes that a further technical discussion of assay sensitivity and threshold values for the QPCR assays are given in Appendix II.

Pg 8 – Reviewer comment follows regarding the statement, "Histological rankings of 'clinical disease' included 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.
"In my discussions with Scott on a separate mesocosm study, I brought up the possibility of indexing disease in a manner similar to this one. Scott was concerned with indexing disease, if I remember correctly, because fish would likely have most or all of the abnormalities/parasites, so we would have only been comparing groups 2 and 3, since we'd see few group 0 or 1 fish. Do you feel you have a sufficiently broad range of scores to overcome this concern and make this analysis work?"
Response (Scott Foott): I believe he is discussing a single rating system (i.e., individual fish is deemed diseased in a ordered manner based on multiple organ evaluations). The kidney and intestine pathology index is a single organ metric to describe abnormalities in that organ by an ordered (progressive scoring) method.

Pg 8 – Reviewer comment follows regarding the statement, "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."
"Does a mean really make sense here, since your data isn't continuous but it isn't truly categorical either. For example, if you can apply a mean, what about a standard deviation? This could be helpful to understand the diversity of each group's pathology. However, in this situation standard deviation may not make much sense. Perhaps a mean doesn't make sense either? Is this a standard technique, or did you develop it. If standard, who developed it?"
Response (Scott Foott): One can argue that that median can be used to report central tendency in a categorical scoring system (Scott Foott developed the scoring system).

Pg 8 – Reviewer comment regarding mean pathology score in the statement above, " So...you have 5 scores for kidney, three for intestine, and six for gills. When you sum them for each fish, then average them, they won't be weighted the same and thus won't be comparable to each other. Should they be comparable?"
Response (Scott Foott): tissue specific, see above responses.

Pg 8 – Table 2. Parasite abbreviations and tissue abnormalities listed in the histological results table. Reviewer comment regarding the table title, "It would help the reader to understand how the path score is calculated if you put some numbers (0-3?) next to where you're actually scoring them."
Response (Scott Foott): I believe the methods section description is sufficient.

Pg 16 – Figure 7. Reviewer comment regarding term 'bi-weekly prevalence' in the chart caption, "You seem to use "prevalence" and "prevalence" (y2-axis) interchangeably. My understanding: prevalence is total number of disease cases, and prevalence is frequency of occurrence. I suggest deciding which one you're talking about here, and then be consistent throughout the document.
Response: Definitions used for prevalence of infection and period prevalence were added to the methods section for clarification. While annual prevalence of infection is an appropriate term for the overall annual infection rate, this term was removed from the report due to confusion caused by the use of both terms. The report was reviewed for consistency of terms and corrected where appropriate.

Pg 25 & 26 – Figures 16 and 17. Reviewer asked, "What's the difference between the path score in this figure [Figure 17] and the path score in Figure 17?"
Response: Figure 16 is *C. shasta* pathology score for intestinal tissue, and Figure 17 is *P. minibicornis* pathology score for kidney tissue.

Pg 34 – Reviewer comment follows regarding the statement, "We can speculate that this additional flow event may have diluted the infectious actinospore concentrations in the water column in 2010, thereby reducing infectivity. "
"Unfortunately, data has been collected for seven years, and we're still speculating. I encourage the authors to consider methods of quantifying underlying mechanisms of disease severity, i.e. build a quantitative, predictive model. It is easy to list the big factors: susceptibility, exposure, temperature, migration pattern. It is harder to understand and predict how they affect disease processes.
I realize a large modeling effort is outside the scope of this document, but an attempt to quantify the drivers and interactions of disease factors may well be the next step in determining how to reduce disease in the Klamath River. As the experts, would a paragraph in your discussion section on "next steps" or "future research" be appropriate? I would be curious to read your opinions on how to use the large amount of quality data you've collected to improve the disease situation for these fish."
Response: The statement is speculative only and not intended to be interpreted as a strong hypothesis to explain lower *C. shasta* infectivity in 2010. An alternate statement follows regarding flow as a possible factor in promoting rapid juvenile migration and thereby reducing parasite exposure dose. Cooler and sustained Spring temperatures are given as the primary hyphothesis for decreased *C. shasta* infection levels in the discussion section.
In response to the reviewer's larger question regarding "quantifying underlying mechanisms of disease severity", the author believes this task is outside of the scope and stated objectives of the juvenile fish health monitoring program. To 'quantify the drivers and interactions of disease factors' would require a synthesis report from all groups collecting and analyzing data including the following disease factors: flow- temperature – sediment, salmon abundance – size-movement, actinospore concentration, polychaete prevalence of infection, adult return – myxospore prevalence of infection – range of input, and winter DNA levels above the infectious zone. A large multi-agency effort is underway to synthesize all existing data from the Klamath basin, primarily to assess the potential change in disease impacts under a proposed 'dams out' scenario. The California-Nevada Fish Health Center has assisted with that effort by providing data from the fish health monitoring studies conducted from 2004-2010.

Pg 35 – Reviewer comment follows regarding the paragraph, "River temperatures and flows are both important considerations in assessing disease impacts on juvenile Chinook salmon in a given study year. Water temperature affects polychaete development, sexual maturation and production of infectious actinospores in infected worm populations. In the fish host, temperature plays a key role in immune function and energy metabolism (Wedemyer 1996, Jobling 1995). Immune function is particularly important in resistance to parasite invasion and/or containment (Bartholomew 1998), and more generally in terms of parasite proliferation and disease progression (Ibarra 1992b, Foott et al. 2004, True 2011). River flows below Iron Gate Dam were not substantially different in 2010 compared to previous study years. Mean monthly discharges were relatively static (May = 1225cfs, June =1050cfs and July = 825cfs) therefore temperature appears to be the more important environmental factor associated with disease prevalence in

2010. This is not to say that river flows are not very important to broader environmental conditions that support juvenile Chinook salmon survival at the population level."
"You seem to indicate it's either discharge or temperature, but other factors could be at play. The evidence is that discharge was similar to previous years but temperature was different. However, other unmeasured factors could also have been different. So discharge did not appear to be a factor in 2010, but temperature *may* have been. Again, a quantitative model would help here."
Response: Other factors associated with disease impacts were acknowledged in the discussion. Temperature is the strongest hypothesis based on the prevalence of infection data for 2010 (i.e., low levels of *C. shasta* in natural fish, delayed infection and lower magnitude of infection in hatchery Chinook salmon sampled in lower reaches, low *C. shasta* prevalence of infection in CWT Chinook salmon). Temperature is also believed to be a strongest single factor because it plays an important role in both hosts in the parasite's life cycle: the biology of the invertebrate host, *Manayunkia speciosa* and response in the vertebrate Chinook salmon host.
See comments above regarding what would be required to develop a quantitative model of disease factors.

Additional reviewer comments regarding spelling errors, unnecessary page break, and consistency in section formatting. Reviewer asked that all time series graphs include an x axis label.
Response: All spelling and formatting inconsistencies were corrected. The x axis for all bi-weekly graphs include the sample date and the author believes these axis units are self-explanatory. The caption accompanying each figure also describes the information as bi-weekly data.

Reviewer#2
Pg 3 – Reviewer comment: " Does natural mean unmarked hatchery or wild?"
Response: Natural Chinook salmon are those that are captured prior to hatchery releases. Sentence was changed to clarify this point, "Sampling effort in 2009 and this year focused on capturing fish of known origin (*natural Chinook salmon collected before hatchery releases* and hatchery CWT Chinook salmon).

Pg 4 – Sample Sites, Reviewer asked that RM be defined. Sentence was changed to define River Mile, "Fish were collected in the Klamath River from below Iron Gate Dam (Klamath River Mile [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)."

Pg 15 – Figure 7, Reviewer comment: "The last two data points on Figure 7 are overlapping.".
Response: Figure 7 was modified by moving the data labels to make them more visible and 2009 data points were changed to a gray scale color to differentiate 2009 *C. shasta* prevalence of infection from 2010 data.

Pg 19 – Figure 11, Reviewer comment: "Prevalence of infection or incidence of infection?.
Response: Caption for Figure 11 was corrected to read Bi-weekly *prevalence* of infection for Ceratomyxa shasta and Parvicapsula……".

Pg 29, Figure 20, Reviewer comment regarding sentence, "However, the highest mean *C. shasta* DNA copy number of 12, 000 is relatively low (Figure 20).". Asked "Relative to what? Other years?"
Response: Relative to infectious load (DNA copy number) observed in clinically moribund Chinook salmon with ceratomyxosis. Sentence changed to read, "However, the highest mean *C. shasta* DNA copy number of 12,000 is relatively low (Figure 20) *compared to levels obtained from moribund fish: CT 25, which correlates to ~96,000 C. shasta DNA copy number (True et al. 2011).*

Pg 32 – Reviewer comment to the sentence, "Predictions for myxozoan disease impacts on Klamath River Chinook salmon populations are limited by the nature of monitoring studies. The limitations are primarily due to difficulty in sorting out disease effects from broader environmental factors in a migratory Chinook salmon population.", suggested a list of these factors even though they are discussed below.
Response: The factors are discussed below in detail, listing them twice may be redundant.

Pg 32 – Reviewer asked in regard to the following sentence, "Tributary Chinook salmon, including a large hatchery component from the Trinity River, have negligible *C. shasta* infections.","Aside from Trinity River hatchery fish, how are the origins of these 'tributary Chinook salmon' determined and how are they distinguished from non-tributary Chinook salmon?"
Response: The sentence was modified to clarify that previous monitoring of tributary Chinook salmon showed negligible *C. shasta* infections in fish that do not rear in the main stem. The sentence was modified to read, "*Past monitoring of tributary Chinook salmon, including a large hatchery component form Trinity River which has been monitored in this study since 2006, has shown negligible C. shasta infections in fish that do not rear in the Klamath main stem.*"

Pg 32 – Reviewer commented on the sentence, "For myxozoan fish diseases, the primary factors for the fish host include: species and individual fish susceptibility (Zinn 1977, Buchanan 1983, Ibarra et al. 1992, Bartholomew 1998), parasite exposure dose (frequency and duration) (Ratliff 1981, Bjork & Bartholomew 2009b, True et al. 2011), and water temperature (Udey et al. 1975, Bartholow 2005). Reviewer asked, "What about parasite genotype?"
Response: Identifying parasite genotypes is not within the scope of the juvenile fish health monitoring study. We do not sample coho or steelhead/rainbow trout and the predominant genotype in the reaches below IGD is reported to be specific to Chinook salmon (genotype I). Our objectives are to monitor disease (regardless of genotype) and assess inter-annual variation of *C. shasta* and *P. minibicornis* disease prevalence in Chinook salmon.

Additional reviewer comments regarding spelling errors, and graph formatting.
Response: All spelling and formatting inconsistencies were corrected.

Reviewer#3
No comments were received from the 3rd requested reviewer.