U.S. Fish & Wildlife Service

California-Nevada Fish Health Center
FY 2016 Investigational Report:

Myxosporean Parasite (*Ceratonova shasta* and *Parvicapsula minibicornis*) Prevalence of Infection in Klamath River Basin Juvenile Chinook Salmon, March – August 2016

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Summary

Juvenile Klamath River Chinook salmon (*Oncorhynchus tshawytscha*) were assayed from March to August 2016 by quantitative polymerase chain reaction (QPCR) and histology for myxosporean parasite infections, *Ceratonova shasta* and *Parvicapsula minibicornis*. The seasonal *C. shasta* prevalence of infection by QPCR in Chinook salmon collected above the Trinity River confluence during the peak out-migration period (May-July) was 48%, considerably lower than 91% observed in 2015, and 81% in 2014. *Parvicapsula minibicornis* in Chinook salmon above the Trinity River confluence for the same period was 89% (compared to 99% and 92% in 2015 and 2014, respectively).

Among the various fish groups tested, naturally produced Chinook salmon had a 27% prevalence of *C. shasta* infection by QPCR, considerably lower than 75-76% observed in 2015 and 2014. The onset of infection (first detection) in 2016 occurred on April 25 when mean daily river temperature below Iron Gate Dam was 13.0°C and at Seiad Valley was 15.0°C. By histology, natural fish sampled from the Shasta to Scott and Scott to Salmon (K4 and K3 reaches) in April-May had low *C. shasta* POI and pathology scores indicating infection levels were not causing clinical disease in natural Chinook juvenile salmon in upper reaches in late Spring.

In coded-wire tagged (CWT) juvenile Chinook salmon released from Iron Gate Hatchery from May 17 – June 9, *C. shasta* was detected in 45% of fish screened by QPCR (no pre-release fish tested positive). The highest *C. shasta* prevalence of infection in marked Chinook juvenile salmon ranged from 51-72% in fish residing 2-4 Weeks At Large (WAL) at time of recapture. The infection pattern (infection within 2-3 weeks post-exposure) is commonly observed for juvenile Chinook salmon entering the main stem Klamath River, under typical temperature regimes (15-18°C) at time of release (mid-May to early June).

In summary, 2016 was a relatively typical year for myxozoan infections in out-migrating juvenile Chinook, and *C. shasta* prevalence of infection was considerably lower than that observed during the past two severe drought years.

The correct citation for this report is:

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Introduction

The Klamath River drainage is approximately 30,000 km^2 located in southern Oregon and northern California. It consists of an upper basin which extends northeast from Iron Gate Dam (IGD) on the main stem Klamath River, and a lower basin extending southwest to the Pacific Ocean.

The lower Klamath River supports 19 species of native fishes including Chinook salmon (*Oncorhynchus tshawytscha)*, which continues to be the most abundant anadromous fish in the river (Council 2004). Also present in the Klamath River are two myxozoan parasites, *Ceratonova shasta* (*syn. Ceratomyxa shasta*, Atkinson et al., 2014) and *Parvicapsula minibicornis*. The parasites share both vertebrate and invertebrate hosts (Bartholomew et al., 1997; Jones et al., 2004; Bartholomew et al., 2007). The parasites life cycles include the invertebrate polychaete host, *Manayunkia speciosa*, which (if infected) releases the actinospore stage into the water column which can subsequently infect the vertebrate salmon host. The actinospore develops within the vertebrate host, salmon or trout species, into a myxospore. Once shed from an infected fish, the myxospore can infect the polychaete host to complete the life cycle (Bartholomew et al., 1997).

The two myxozoan parasites have overlapping distributions throughout the Pacific Northwest, where they are present in many of the larger river systems (Ching et al., 1984; Hoffmaster et al., 1988; Hendrickson et al., 1989; Bartholomew et al., 1997; Jones et al., 2004; Bartholomew et al., 2006; Stocking et al., 2006). *Ceratonova shasta* and *P. minibicornis* are distributed throughout the main stem Klamath River system including the lower reaches of the Williamson and Sprague Rivers, Agency Lake, Klamath Lake, Copco Reservoir, and the Klamath River from Iron Gate Dam to the estuary (Hendrickson et al., 1989; Stocking et al., 2006; Bartholomew et al., 2007). A 2006 study monitoring the waterborne stages showed that *C. shasta* abundance was low at the outflow of Iron Gate Reservoir (RM 190), but increased in the main stem Klamath River between the interstate five bridge crossing (RM 177) and the confluence of the Scott River (RM 144; Hallett et al., 2006). This section of the Klamath River has been termed the "infectious zone" and this general pattern of parasite abundance remains steady, but the size of the infectious zone and the magnitude of parasite densities change seasonally and annually (Bartholomew et al., 2010).

Ceratonova shasta causes enteronecrosis and is a significant contributor to mortality in juvenile fish that migrate through the region (Hoffmaster et al., 1988; Bartholomew et al., 1997; Stocking et al., 2006). Infectivity patterns of enteronecrosis are well defined for native Klamath basin salmonid species. At river temperatures commonly observed in the Klamath River during peak juvenile Chinook salmon migration of April to August (17-24ºC), clinical disease occurs within three weeks of initial exposure resulting in moderate to high levels of mortality. This infectivity pattern has been established through sentinel susceptibility studies (Stone et al., 2008; Bjork et al., 2009; Bartholomew et al., 2010; True et al., 2012) and annual monitoring of coded-wire tagged (CWT) Chinook salmon with known exposure periods in the main stem Klamath (Nichols et al., 2009; Bolick et al., 2013; True et al., 2013).

Klamath River juvenile Chinook salmon can experience high prevalence and severity of infection with these two myxosporean parasites, particularly when river temperatures promote earlier reproduction and expansion of the polychaete host population (Bartholomew et al. 2010) which can lead to earlier infection and proliferation of the parasite within the fish host (True et al., 2011). For

salmonids, mortality from enteronecrosis is temperature dependent as demonstrated by Udey et al. (1975), but water discharge can also play an important role. Bjork et al., (2009) found prevalence of *C. shasta* infection was higher in a smaller volume of water when fish were exposed to the same number of parasites. Therefore, parasite concentration affects infection prevalence. Higher flows may not only dilute the infectious spore stages, but transmission efficiency may also be decreased (Hallett et al., 2012; Ray et al., 2013).

The primary objectives of this study were: 1) examine parasite prevalence in Klamath River juvenile Chinook salmon during the spring outmigration period; and 2) compare parasite prevalence in 2016 to previous years.

Methods

Pre-Release Examination

Prior to the Iron Gate Hatchery releases (May 17 through June 9, 2016) of approximately 4 million fall Chinook salmon, a fish health examination of 32 hatchery fish was conducted at the hatchery on May 12 to determine infection levels of *C. shasta* and *P. minibicornis*.

Sample Sites, Fish Groups and Number Sampled

Fish were collected in the main stem Klamath River between the Shasta River confluence and the Klamath River estuary. The middle and lower Klamath River is divided into five sample reaches at major tributaries, with study cooperators collecting fish in each reach (Figure 1, Table 1). When possible, existing salmonid downstream migrant traps were used for collection, but beach seining was also performed to collect fish in some weeks/reaches. Field crews collected weekly samples within a 1-2 day period to preclude protraction of the sampling period within the sample week. The date reported for fish collection is the start date (Sunday) of the sampling week. Specific dates are given for hatchery releases, and first pathogen detections, and histology collection dates.

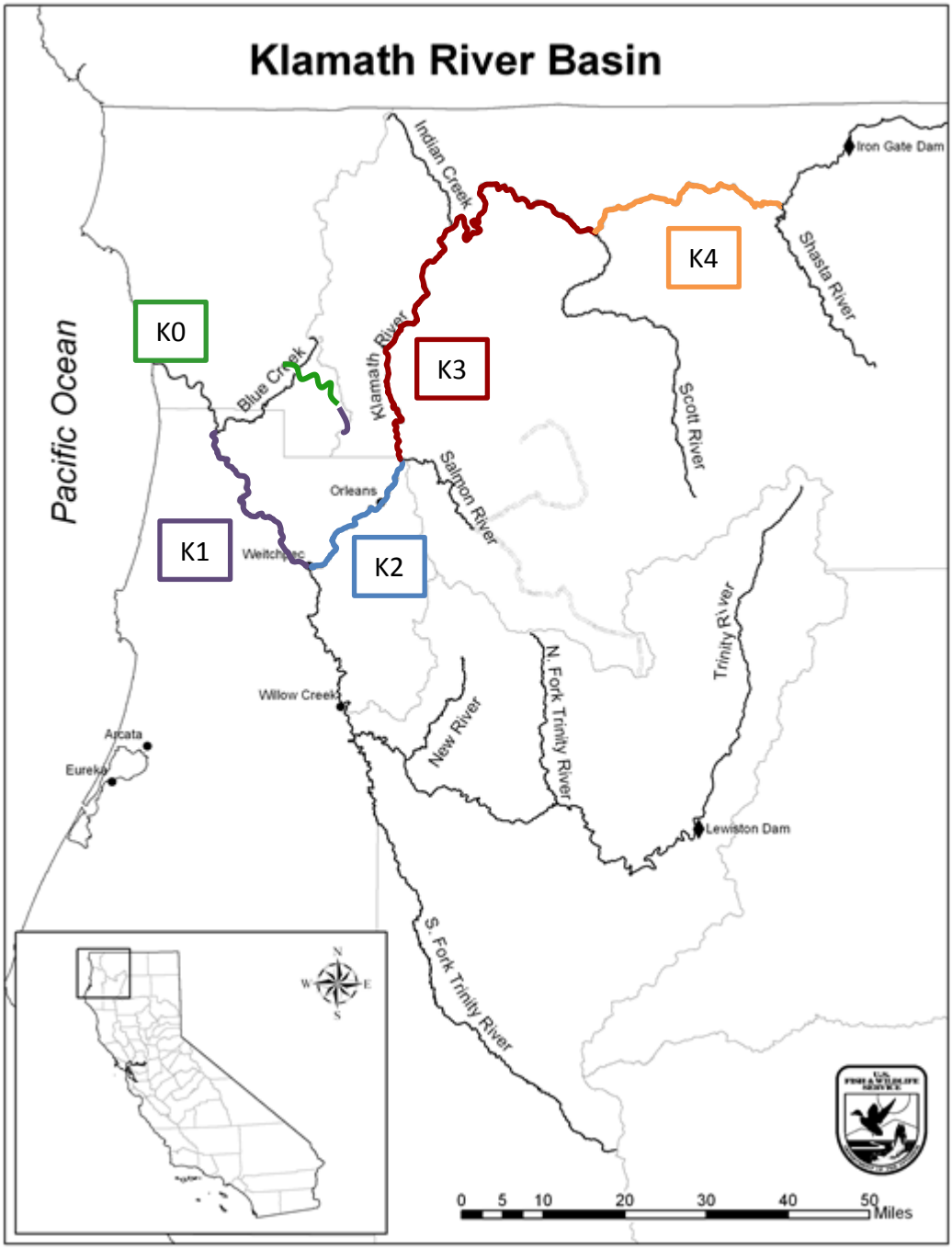


Figure 1. Klamath River watershed, major tributaries, and sample reaches: Shasta River to Scott River (K4), Scott River to Salmon River (K3), Salmon River to Trinity River confluence (K2), Trinity River to upper Estuary (K1), and Klamath River Estuary (K0). Map provided by the Arcata Fish and Wildlife Office.

Table 1. Sample reach locations, distances, and cooperating agencies performing fish collection on the main stem Klamath River.

Sample Reach	Reach Code	River miles (Upstream – Downstream)	Primary Collector
Klamath River main stem			
Shasta R. to Scott R.	K4	177-144	USFWS
Scott R. to Salmon R.	K3	144-66	Karuk Tribe
Salmon R. to Trinity R.	K2	66-44	Karuk Tribe
Trinity R. to Estuary R.	K1	44-4	Yurok Tribe
Estuary	K0	4-0	Yurok Tribe

Fish were sampled, according to True et al. (2013), from the Shasta River confluence to the Klamath River estuary. Fish were collected in the upper reaches, K4 and K3, early in the sampling season (the week of March 27-July 24). Lower reaches were sampled later in the season (June 5-August 14) as fish were migrating downstream (Appendix A – Table 1).

All fish sampled were categorized into three group types based on their origin: natural (before hatchery release), unknown (adipose fin present after hatchery release), and CWT (coded-wire tagged and adipose fin clipped). Fish numbers tested in the Klamath River varied by reach, with emphasis on natural fish in the reaches below IGD initially, then hatchery CWT fish for the remainder of the spring/summer migration.

Historical comparison between monitoring years restricts data to the peak migration period (May to end of July) and to reaches above the Trinity confluence.

Both quantitative polymerase chain reaction (QPCR) and histological assays were used to identify and quantify infectivity patterns for both *C. shasta* and *P. minibicornis* in juvenile Chinook salmon tissues (Hallett et al., 2006; True et al., 2009).

Parasite Infection Levels by Quantitative PCR Assays

Fish collection, necropsy, and DNA extraction were done according to True et al. (2013). The *C. shasta* reference standard curve was obtained using synthesized DNA (Gene Block, IDT, Coralville Iowa) containing the 18S ribosomal DNA target sequence. Specifically, 1 ng of DNA, corresponding to 6.83×10^9 copies of *C. shasta* DNA was serially diluted over 8 orders of magnitude in molecular grade water. Using QPCR analysis software, the cycle threshold (C_T) values for each standard concentration were calculated (SDS software 7300 SDS v 1.3.1, Applied Biosystems). The standard curve was used to evaluate PCR amplification efficiency (slope of the standard curve, efficiency was 93%), fit to the curve (R^2 value = 0.997) and the y-intercept (C_T value for a single copy of parasite DNA).

Quantification of fish tissue (*C. shasta* DNA copy number) was determined using 5 µL of DNA template in a 30 µL reaction. Each assay plate included a standard curve with three concentrations of reference standards (two replicates each) at known DNA copy number, and two negative control wells. Each assay was evaluated for expected C_T values of the reference standards, and assay efficiency. Any

plates with more than 3% decrease in assay efficiency were retested and reevaluated. A total of two plates were re-run over the 2016 field season.

The *P. minibicornis* reference standard curve was obtained in a similar manner by using plasmid DNA containing the 18S ribosomal DNA target sequence. Specifically, 1 ng of DNA, corresponding to 2.41 $x10^8$ copies of *P. minibicornis* DNA was serially diluted over 8 orders of magnitude in molecular grade water. Using QPCR analysis software, the cycle threshold (C_T) values for each standard concentration were calculated (SDS software 7300 SDS v 1.3.1, Applied Biosystems). The standard curve was used to evaluate PCR amplification efficiency (slope of the standard curve, efficiency was 90%), fit to the curve (R^2 value = 0.998) and the y-intercept (C_T value for a single copy of parasite DNA).

Quantification of fish tissue (*P. minibicornis* DNA copy number) was determined using the same reaction volume of 5 µL. Each assay plate included a standard curve with three concentrations of reference standards (two replicates each) at known DNA copy number, and two negative control wells. Each assay was evaluated for expected C_T values of the reference standards, and assay efficiency. No plates required retesting over the 2016 field season.

In the results section, QPCR data are presented first for each group of fish or type of spatial analysis, followed by histology data in a separate paragraph.

Parasite Infection Levels by Histology

Histological assays were done according to True et al. (2013). In 2016, histology samples were collected in the Shasta to Scott reach (K4) and the Scott to Salmon reach (K3) between the week of April 17 and May 29 (Appendix A -Table 1). Histology results are presented in a separate paragraph in appropriate sections.

Histological assays were assigned a pathology score: a numeric index of disease severity for kidney and intestine. The pathology was based on the degree of specific tissue abnormalities and parasite distribution (Appendix B -Table 1), but did not affect the overall prevalence of infection reported for histological assessments. Pathology scores are reported for fish grouped by collection date, not pathology scores for individual fish.

Statistical Analysis

Point prevalence of infection and annual prevalence (defined by Durfee, 1978; USFWS, 2004) for *C. shasta and P. minibicornis* were reported with 95% confidence intervals (denoted ci) for each sample reach. Prevalence of infection (POI) was used to describe the proportion of infected Chinook salmon (numerator) in the sample (number of animals examined) for a particular calendar week. Annual prevalence was used to describe the overall prevalence of infection in the sampled population during the entire sampling period that year. Annual prevalence estimate is not an estimate of the annual proportion of the population that is infected, because weekly estimates are not weighted by abundance values.

Results

Pre-Release Examination of IGH Chinook Salmon

Juvenile Chinook salmon reared at Iron Gate Hatchery were screened for infections of *C. shasta* and *P. minibicornis* by QPCR prior to release (May 17 through June 9, 2016) on May 12th. *Ceratonova shasta* was not detected in the 32 juvenile Chinook salmon tested whereas *Parvicapsula minibicornis* was detected in 3% (1/32, ci = 0-16%). River temperatures on initial release of May 17 were 16.0ºC (60.8ºF), and 18.8ºC (65.8ºF) on final release date of June 9 (data from Karuk Tribe, below Iron Gate Dam).

Number of All Fish Collected by Origin

In 2016 we examined 934 juvenile Chinook salmon collected from the main stem Klamath River. The sample consisted of 277 natural fish, and 657 fish collected after hatchery release which included 600 CWTs. Coded-wire tagged Chinook salmon accounted for 64% (600/934) of all fish sampled in 2016 (Figure 2). Natural fish accounted for 30% (277/934) and 6% (57/934) of the fish are of unknown origin (unmarked hatchery fish or natural).

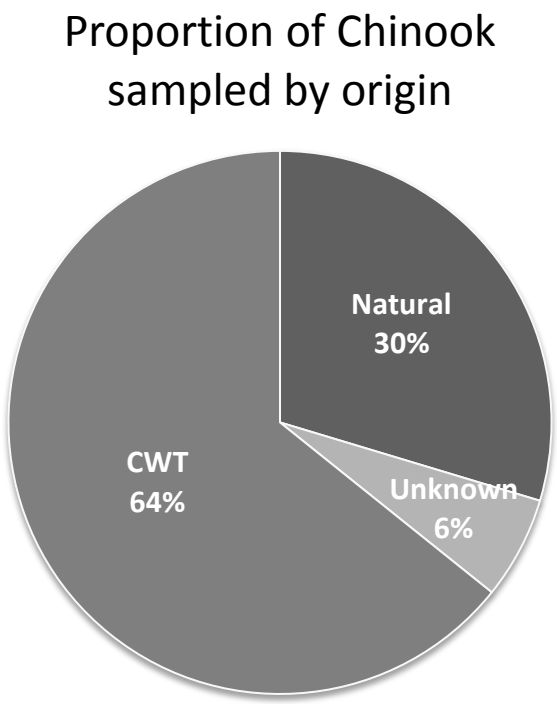


Figure 2. Proportion and origin of Chinook salmon collected (N = 934) in 2016.

After removing unreadable and/or Trinity River Hatchery (TRH) coded-wire tags, the total number of juvenile Chinook salmon analyzed for prevalence of infection in this report was 861: this consisted of 277 natural fish (32%), 57 unknown fish (7%), and 527 IGH CWT (61%, Figure 3).

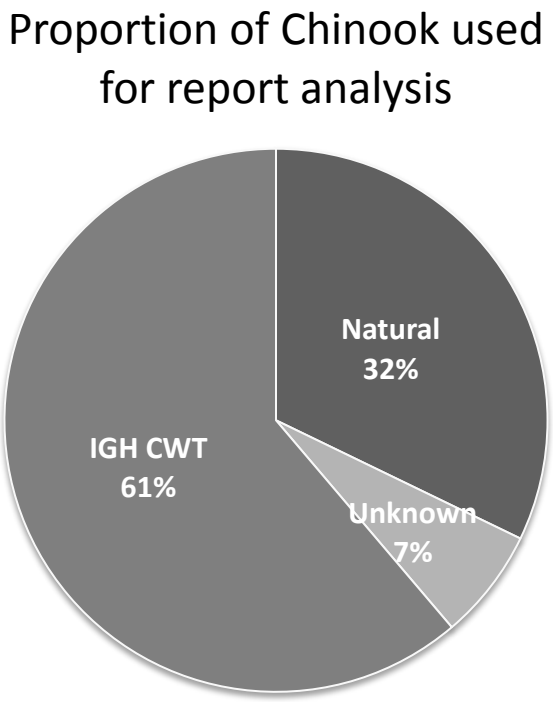
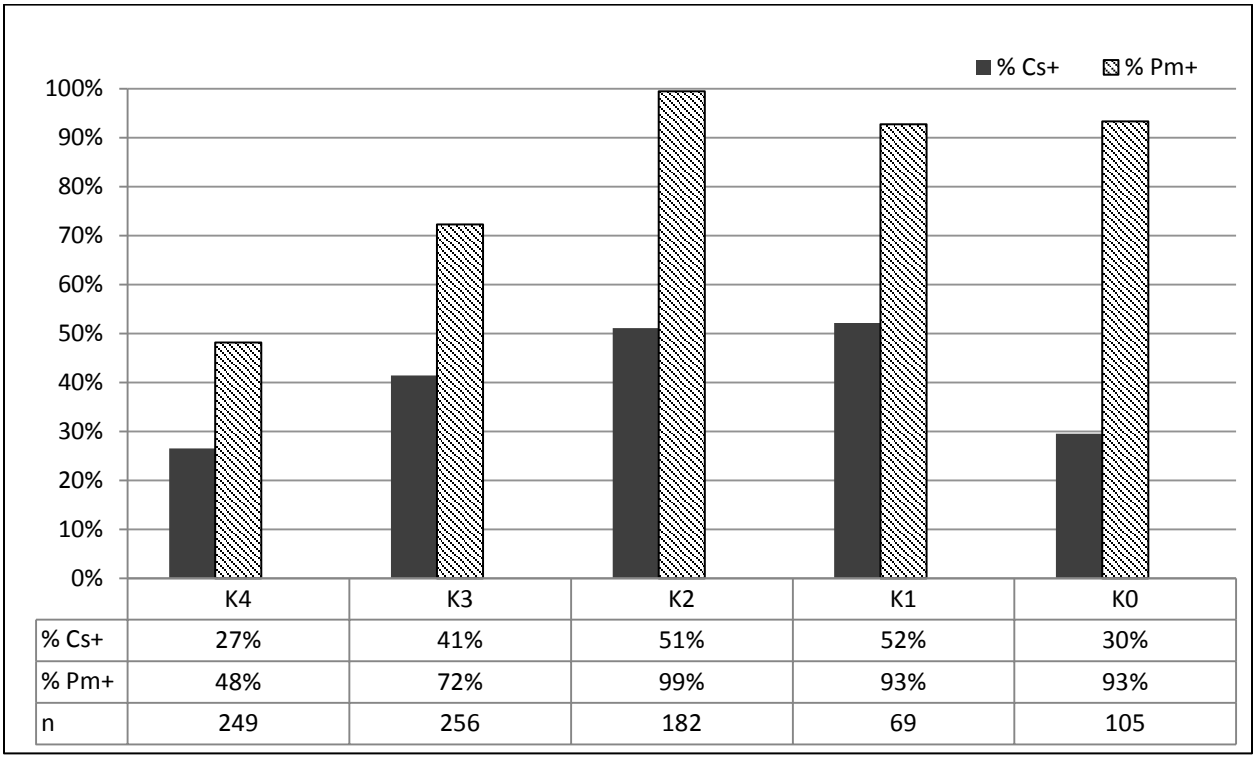


Figure 3. Proportion and origin of Chinook salmon used for prevalence of infection analysis (N = 861). Unreadable tag codes or lost tags, and TRH tags have been removed from total number of fish collected.

Annual Prevalence of Infection by Klamath River Reach

The annual prevalence of *C. shasta* infection in all Chinook salmon collected in 2016 by QPCR was 39% (332/861, ci = 35-42 %%). *Ceratonova shasta* was first detected on April 25 in the Scott to Salmon reach (K3). *Ceratonova shasta* POI was highest in the Trinity River to Estuary (K1) reach at 52%, followed by 51% in the Salmon to Trinity (K2) reach. The lowest prevalence of 27% was observed in the Shasta to Scott reach (K4, Figure 4).

The annual *P. minibicornis* POI in all Chinook salmon by QPCR was 75% (648/861, ci = 72-78 %). *Parvicapsula minibicornis* was detected on April 25 in the Scott to Salmon reach (K3). Prevalence was highest in the Salmon to Trinity reach (K2) at 99%, followed closely by the Trinity to Estuary (K1) and Estuary (K0) reaches, both at 93% (Figure 4). The lowest prevalence of 48% was observed in the Shasta to Scott reach (K4).



	K4	K3	K2	K1	K0
% Cs+	27%	41%	51%	52%	30%
% Pm+	48%	72%	99%	93%	93%
n	249	256	182	69	105

Figure 4. Prevalence of *Ceratonova shasta* (Cs+) and *Parvicapsula minibicornis* (Pm+) infection in juvenile Klamath River Chinook salmon by collection reach in 2016. Shasta River to Scott River (K4), Scott River to Salmon River (K3), Salmon River to Trinity River confluence (K2), Trinity River to upper Estuary (K1), and Klamath River Estuary (K0). Sample numbers collected (N) are displayed in the table below and were the same for both pathogens.

As described in methods, histology sampling occurred during the week of April 17 to May 29 in the K4 reach and K3 reaches (Appendix B, Table 2 and Table 3). The annual *C. shasta* POI in 2016 by histology for all fish tested was 13% (10/78, ci = 6-22%) and for *P. minibicornis* was 46% (36/78, ci = 35-58%).

Prevalence of Infection by Fish Origin

Naturally produced Chinook salmon

Naturally produced Chinook salmon represent early infection status by these two myxozoan parasites in the Klamath River, as river temperatures are generally 8-10ºC cooler in the collection months of late March to late May compared to hatchery fish sampled during the peak salmon migration period of late May to end of July. A total of 277 natural fish were collected in the Klamath River above the Trinity River confluence (K4 and K3) for testing by QPCR. Natural fish were collected from March 31 through May 19 in Shasta to Scott (K4) reach and from April 12 through May 16 in the Scott to Salmon (K3) reach. Mean daily river temperature was 13.3°C (data from Karuk Tribe, below IGD) at first detection of *C. shasta* in natural fish collected in K4 on April 25, two weeks later than the first detection on Apr 9 in 2015.

Ceratonova shasta was detected by QPCR in 27% (74/277, ci = 22-32%) of natural fish. *Ceratonova shasta* POI was highest (38%, ci = 29-47%) in the Scott to Salmon (K3) reach compared to 18% (ci = 13-25%) in the K4 reach above. Field crews reported distended abdomens in 15% of fish collected May 16. Histology also indicated *C. shasta* POI of 30% for this collection week. Comparatively, *P. minibicornis* was detected in 39% (109/277 ci = 34-45%) of naturally produced Chinook salmon by QPCR. The highest *P. minibicornis* prevalence of 49% (ci = 39-58%) was detected in Scott to Salmon reach (K3) and the lowest prevalence 32% (ci = 25-40%) was observed in the upper Shasta to Scott reach (K4).

Natural fish were collected for histology prior to first IGH releases on May 17. One histological sample set was collected post hatchery release on May 29, and these unmarked fish were considered to be of unknown origin. The prevalence of *C. shasta* infection by histology, by collection location, was similar in natural fish at 13.3% (4/30, ci = 4-31%) in the Shasta to Scott (K4) reach and 12.9% (4/31, ci = 4-30%) in the Scott to Salmon (K3) reach. In the Shasta to Scott (K4) reach, *C. shasta* prevalence of infection by histology ranged from undetected to 30% over the three sample dates. The pathology scores were low overall, with the highest score of 1.0 occurring May 15 (Appendix B, Table 2). In the Scott to Salmon (K3) reach, the highest *C. shasta* prevalence (18%) occurred May 15 and the highest pathology score (0.3) occurred two weeks prior on May 1. For comparison, clinically infected salmon generally have *C. shasta* intestine pathology scores between 3 and 4 (True et al., 2010).

Natural fish had an overall *P. minibicornis* POI by histology of 33% (20/59, ci = 22-47%). Prevalence was highest in the upper Shasta to Scott (K4) reach at 57% (17/30, ci = 37-75%) compared to 10% (3/29, ci = 2-27%) in the Scott to Salmon (K3) reach. The kidney pathology scores were zero for two collection dates in the Shasta to Scott (K4) reach, and zero for all three collection dates for the Scott to Salmon (K3) reach (Appendix B, Table 3). For reference, pathology scores of 6-8 have been observed in clinical disease, in previous monitoring years (True et al., 2010).

In an analysis completed this year, to determine the disease threshold by QPCR, *C. shasta* DNA copy number for natural Chinook juveniles was assessed for fish sampled from the upper reaches (infection zone) over the past four years. This 4-year period represents a moderate year (2013), the two severe drought years (2014-2015) and the most recent data from 2016, a relatively low infection year. QPCR data from previous studies (True et al. 2012), where daily parasite DNA levels were tracked in high resolution, corresponded with histological assessment (parasite numbers with histological rankings of clinical disease) associated with disease and impending mortality.

A QPCR disease threshold of parasite levels (log DNA copy number) was determined for fish that were not expected to recover (by histological assessment) and therefore considered to have a high likelihood of eventual mortality as the parasite infection continued to proliferate over time (Figure 5). The infection level corresponding to 2-4 logs of *C. shasta* DNA in juvenile Chinook salmon intestine is highly likely to lead to mortality as the infection progresses at temperatures (15-18 ºC) that are common during natural juvenile Chinook migration period.

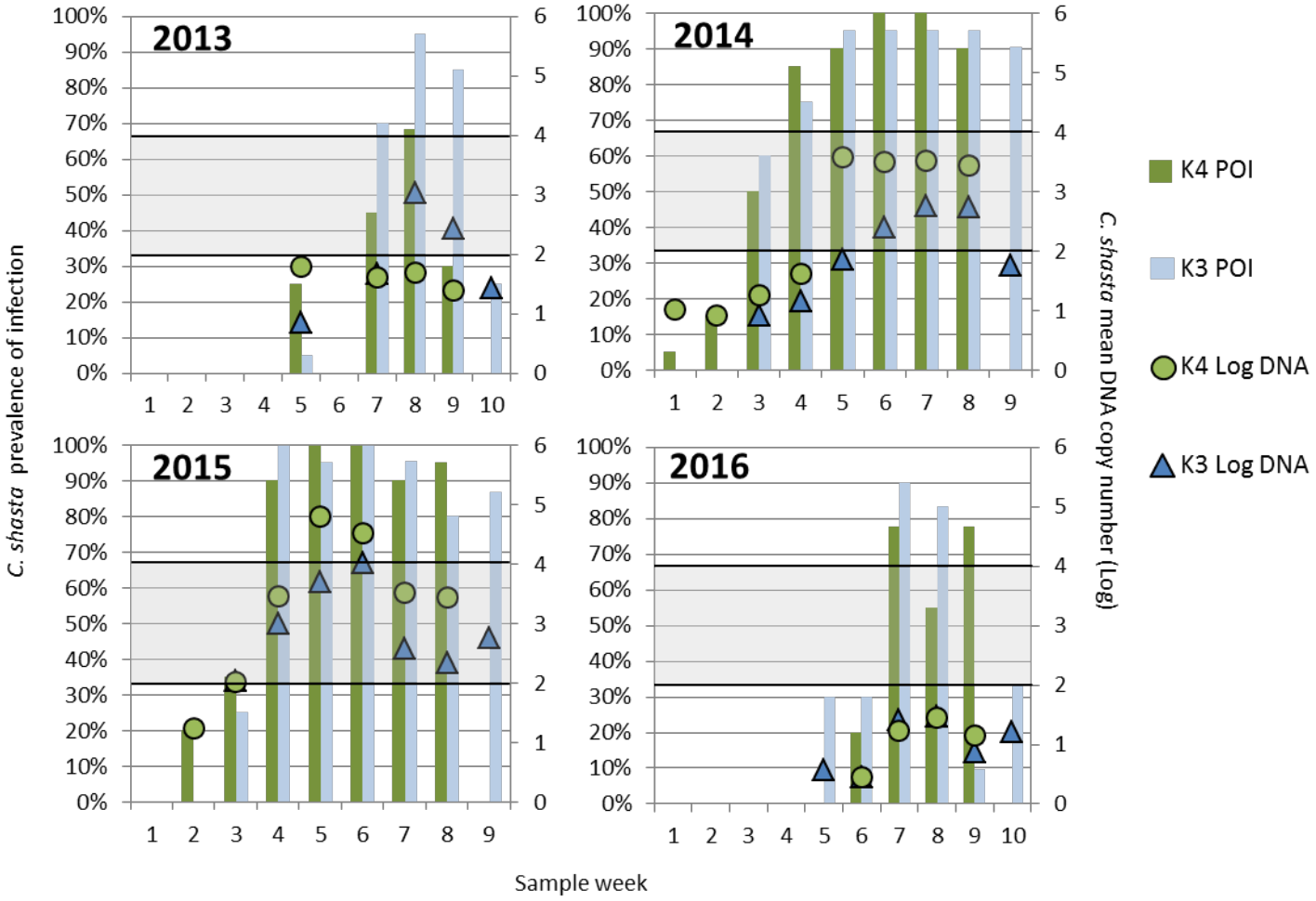


Figure 5. *Ceratonova shasta* prevalence of infection in natural Chinook juveniles, captured in Shasta to Scott (K4) reach and Scott to Salmon (K3). Prevalence of infection shown in columns (Y axis) and *C. shasta* mean DNA copy number (log) shown in circles and triangles (secondary Y axis). *Ceratonova shasta* mean DNA range of 2-4 logs determined to correlate with unrecoverable infection levels by histology, likely resulting in mortality as parasite proliferation continues. Sample Week shown on X axis, beginning with natural fish collected the end of March-first week of April (Week 1), through end of May (Week 10).

Unknown Chinook salmon

Unknown origin Chinook salmon are unmarked fish (adipose fin present) collected after hatchery release that could not be differentiated from either natural fish or unmarked hatchery fish. A total of 57 fish of unknown origin were collected from May 23 to June 3 from the Kinsman RST in the Shasta to Scott reach (N=18) and Presido Bar in the Scott to Salmon reach (N=39). *Ceratonova shasta* was detected by QPCR in 39% (22/57, ci = 26-52%) of unknown origin Chinook salmon. *Parvicapsula minibicornis* POI in was 91% (52/57, ci = 81-97%).

Iron Gate Hatchery (CWT) Chinook salmon

The 25% constant fractional mark rate at IGH since 2009 has facilitated the capture of a large proportion of IGH CWT Chinook salmon in the past seven years of the monitoring program (Buttars et al., 2009). A total of 600 CWT Chinook salmon were collected this season from the Klamath River. Iron Gate Hatchery CWT fish accounted for 88% (527/600) and TRH CWT fish accounted for 9% (55/600) of CWT fish tested. Additionally, 18 fish (3%) had lost or unreadable tags, which meant their release date is unknown. Release dates, numbers and environmental conditions, provided by Iron Gate Hatchery, are given in Table 2.

Coded-wire tagged salmon originating from IGH were collected in the Klamath River from June 1 to August 18. *Ceratonova shasta* was detected in 45% (236/527, ci = 40-49%) of all IGH CWT screened by QPCR. The largest proportion of IGH CWT (N=182 fish), were collected in the Salmon to Trinity reach (K2) where *C. shasta* prevalence of infection was 51% (93/182, ci = 44-59%). Prevalence of infection for *C. shasta* was highest in the Scott to Salmon reach (K3) at 54% (53/98, ci = 44-64%) followed closely by the Trinity to Estuary (K1) reach at 52% (36/69, ci = 40-64%). Field crews had difficulty collecting fish in K1 during July, because fish were not present in thermal refugia (Appendix A). Crews observed fish primarily in the thalweg and attributed this to active migration. The lowest prevalence was in the Estuary reach (K0) at 30% (31/105, ci = 21-39%).

Parvicapsula minibicornis was detected by QPCR in 92% (487/527, ci = 90-95%) of IGH CWT. Prevalence of infection for *P. minibicornis* was highest at 99% (181/182, ci = 97-100%) in the Salmon to Trinity reach (K2) and lowest at 70% (51/73, ci = 58-80%) in the Shasta to Scott reach (K4).

Table 2. Iron Gate Hatchery Chinook BY2015 Release Groups (Data provided by IGH).

Release Date	Approximate number of Chinook smolts released	Fish per pound	Hatchery temperature	Klamath River temperature	Klamath River flow (cfs)
5/17/16	870,000	87	13°C	16°C	1550
5/27/16	860,000	90	13°C	15°C	1180
6/3/16	989,000	90	14°C	17°C	1050
6/9/16	1,010,000	90	14°C	18°C	1070

IGH CWT Weeks At Large

In the monitoring program, temporal data is derived from IGH CWT codes obtained from juvenile Chinook salmon with known exposure period (hatchery release to in-river recapture date). The period of how long fish reside in the Klamath River post hatchery release is Weeks At Large (WAL). *Ceratonova shasta* POI in IGH CWT Chinook salmon by WAL analysis shows the highest prevalence followed a more typical pattern of infectivity in 2016, when contrasted with 2015 where prevalence was high across all WAL groups.

The highest *C. shasta* prevalence of infection occurred in groups residing 2-4 WAL (51-72%, Figure 6). Intermediate POI ranges occurred for WAL 1, 7, and 10 at 41%, 42%, and 40% respectfully. In 2016 the lowest *C. shasta* prevalence of infection occurs in fish residing at 9 WAL (15%) and 5 and 6 WAL (25% for both groups).

As stated in the methods, the QPCR assay can quantify parasite DNA copies within fish tissue and therefore describe infection level at specific exposure periods. In IGH CWT Chinook salmon, the highest mean DNA copy number (5,000 to 38,000; 2.2 to 2.3 logs) was observed in groups residing 2-3 WAL (Figure 6). Mean DNA copy number was low across the remaining weeks (under 300 copies). Mean DNA copy number for all IGH CWTs in 2016 was 8,567 copies (1.6 logs) compared to 53,000 copies (2.8 logs) in 2015 (True et al., 2016). Sample size was small (1-5 fish) for 10 and 11WAL groups (sample size shown at base of each column in Figure 6).

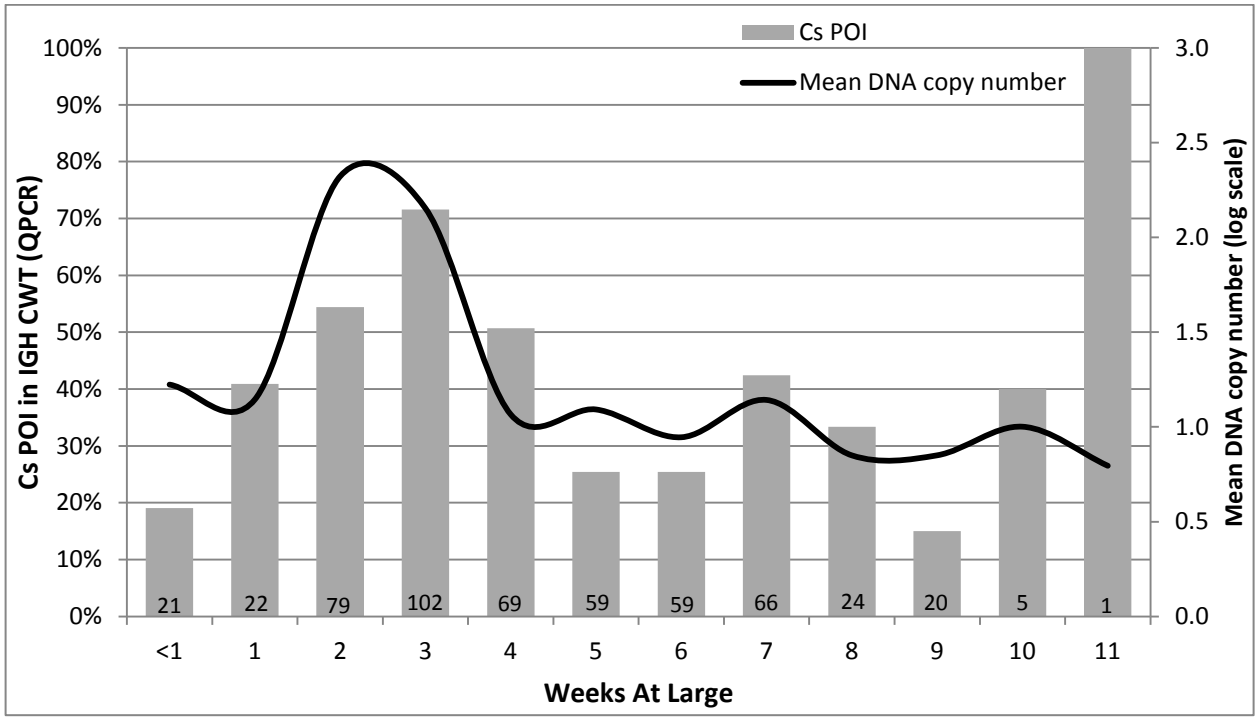


Figure 6. *Ceratonova shasta* prevalence of infection in IGH CWT by Weeks At Large (WAL) post hatchery release. The bar graph is prevalence of infection (%) on the primary y-axis and the line graph is the mean *C. shasta* DNA copy number (log scale) on the secondary y-axis for Chinook salmon tested by QPCR. The number of fish collected is listed inside the base of each bar.

Historical Comparison

Prevalence of infection by QPCR is the metric that been used for historical comparisons of disease prevalence in the monitoring program since 2009. Data is confined to the peak migration period of May 1 to July 31 and fish collected above the Trinity confluence. Supplemental histology continues to be performed annually for select reaches to assess tissue damage associated with clinical disease and to detect other pathogens that may be present in out-migrating juvenile Chinook salmon.

Prevalence of *C. shasta* infection by QPCR during the peak outmigration period was moderate at 48% (243/504, ci = 44-53%) in 2016, and similar to the average of 44% for the past decade (2006-2015, Table 3). *Parvicapsula minibicornis* prevalence of infection by QPCR in Chinook salmon above the Trinity River confluence for the same period was 89% (451/504, ci = 86-92%) compared to 99% in 2015 and 92% in 2014.

Prevalence of *C. shasta* infection by histology was very low in 2016, compared to the most severe drought years (2014-2015) and corresponded with low to moderate disease years such as 2010 and 2012. Histology sampling is limited to the upper reaches from mid-April and late May, so later periods are assessed by parasite DNA copy number determined by QPCR.

Table 3. Historic annual prevalence of *Ceratonova shasta* infection (% positive by assay) in all juvenile Chinook salmon collected from the main stem Klamath River between Iron Gate Dam and Trinity River confluence during May through July, 2006-2016.

Year	Histology (% Positive)	QPCR (% Positive)
2006	21	34
2007	21	31
2008	37	49
2009	54	45
2010	15	17
2011	2[1]	17
2012	9[1]	30
2013	16[1]	46
2014	42[1]	81
2015	62[1]	91
2016	14[1]	48
Mean	**27**	**44**

[1] Histology limited to two reaches in 2011 (K4 and K1); and two reaches in 2012-2016 (K4 and K3).

Environmental Conditions

In previous study years, we typically observed mean daily water temperatures of approximately 18ºC, and often as high as 22ºC, during the peak juvenile migration period of May through July. The exception was 2010 and 2011 in which mean daily water temperatures were cooler for an extended period in May and June (2011 graphed in Figure 7). These cooler temperatures coincided with the lowest *C. shasta* POI observed to date for the juvenile fish health monitoring program (17% by QPCR for both 2010 and 2011). In the severe drought years of 2014 and 2015, mean daily water temperatures were approximately 2°C higher in March when compared to 2013.

In 2016, the mean river temperature profile was unique in comparison to all previous years: temperatures increased in early Spring in a similar pattern observed in past years, however a cooling trend occurred from mid-June to mid-July with temperatures holding at or slightly above 18°C (Figure 7).

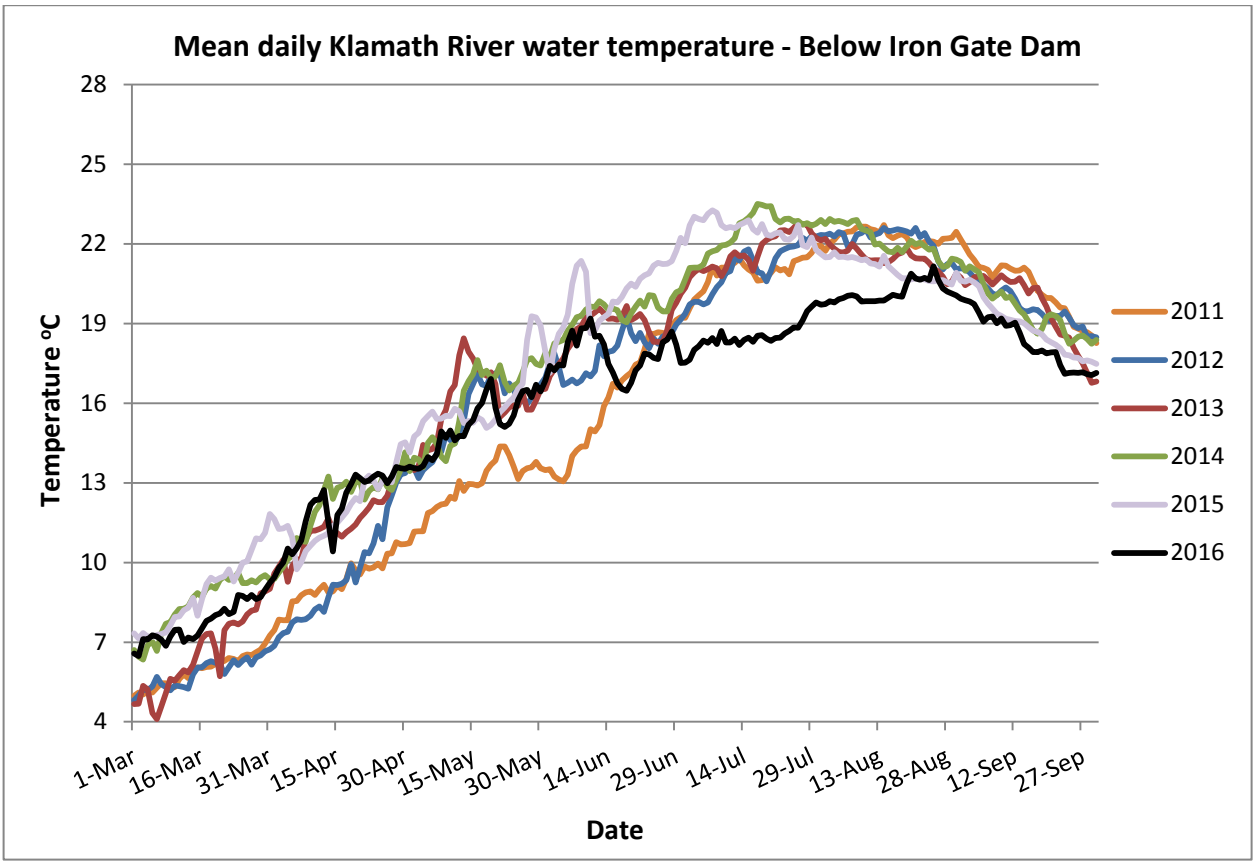


Figure 7. Mean daily Klamath River water temperature below Iron Gate Dam for 2011-2016. Temperature data for 2011 and 2013-2015 acquired from Arcata Fish and Wildlife Field Office. Temperature data for 2012 acquired from Iron Gate Hatchery and taken from the main stem Klamath River, not the hatchery facility. Temperature data for 2016 was acquired from the Karuk Tribe.

At the Seiad Valley temperature gauge, mean daily Klamath River temperatures were more variable than below Iron Gate Dam and intermediate compared to all other years (Figure 8). The decrease in temperature observed for below Iron Gate Dam can also be observed at Seiad Valley where mean daily temperature decreased over a week period from 21°C on June 6 to 16°C on June 16. Temperature rose back to 21°C by June 22 (Figure 8).

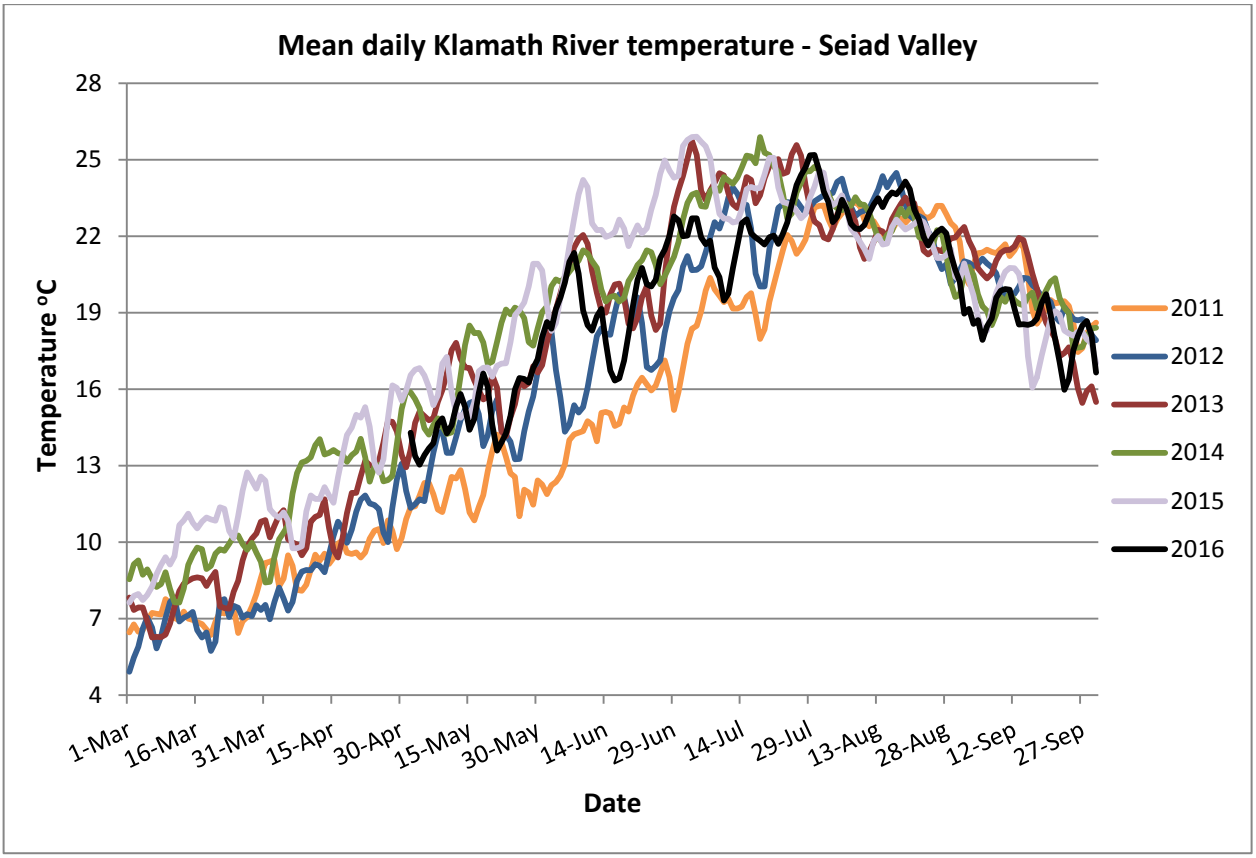


Figure 8. Mean daily Klamath River temperature from March through September 2011-2016 at Seiad Valley. Data from 2011 to 2015 were provided by the Arcata Fish and Wildlife Field Office, with the exception of 2014 temperature data that were provided by Arcata FWO and Karuk Tribe. Temperature data for 2016 was acquired from the Karuk Tribe.

Three marked decreases in temperature occurred at Seiad Valley in 2016, compared to 2014-2015. Decreases in mean daily temperature occurred on May 21 (13.6 ^{o}C), June 16 (16.4 ^{o}C) and July 11 (19.8 ^{o}C) relative to the general trend for the 2016 season, and the previous drought years of 2014 and 2015 (Figure 9).

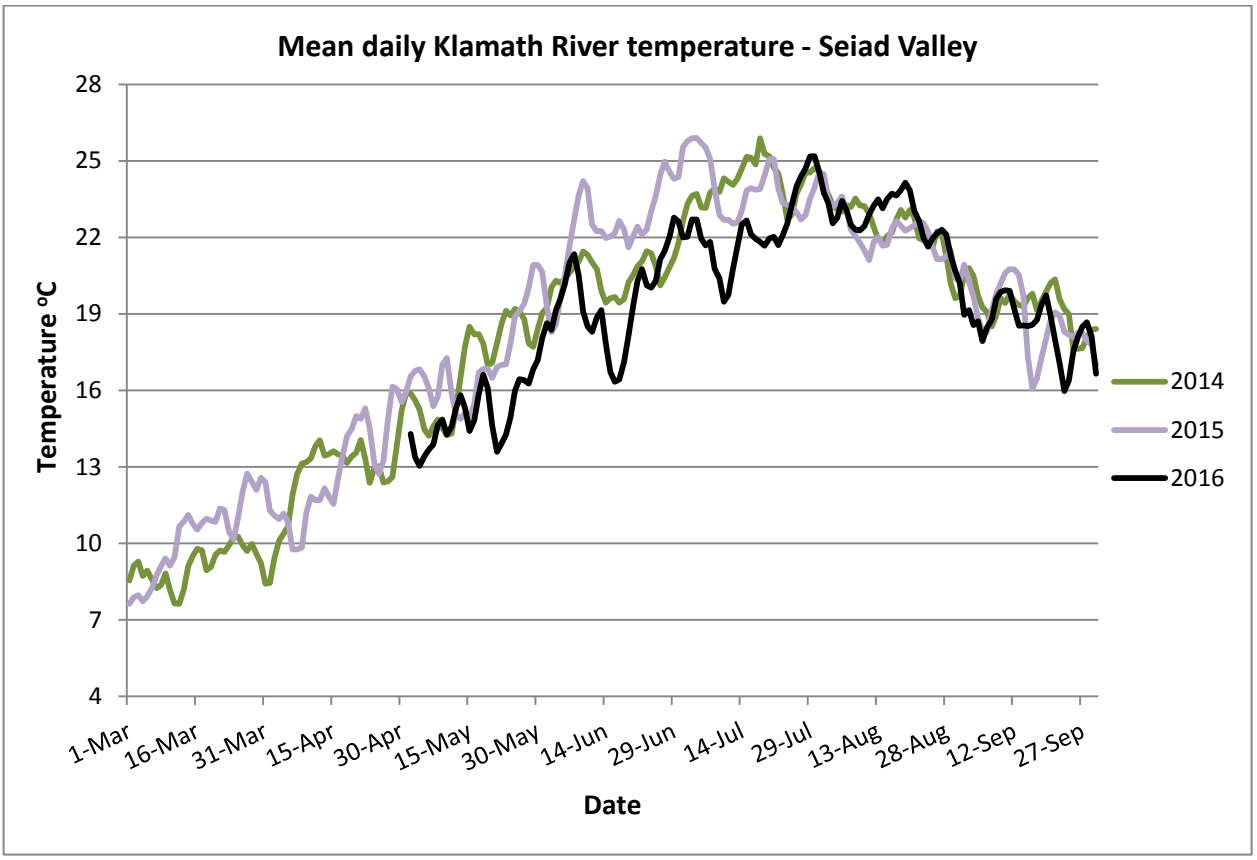


Figure 9. Mean daily Klamath River temperature from March through September 2014-2016 at Seiad Valley. Temperature data were provided by Arcata FWO and the Karuk Tribe.

River Flows

On March 16 2016, a large precipitation event occurred on the Klamath River when flow below Iron Gate dam peaked at 11,100 cfs. The first juvenile fish sample was collected on March 31 and discharge below Iron Gate dam was 3,400 cfs that day. This was the maximum discharge recorded during the sampling season.

River discharge continued to drop in early April to 1,330 cfs on April 10th and then increased to 2,370 on April 25th. River discharge gradually decreased for the remainder of the sampling season. The minimum discharge observed during the sampling season at 862 cfs on July 31 (Figure 10).

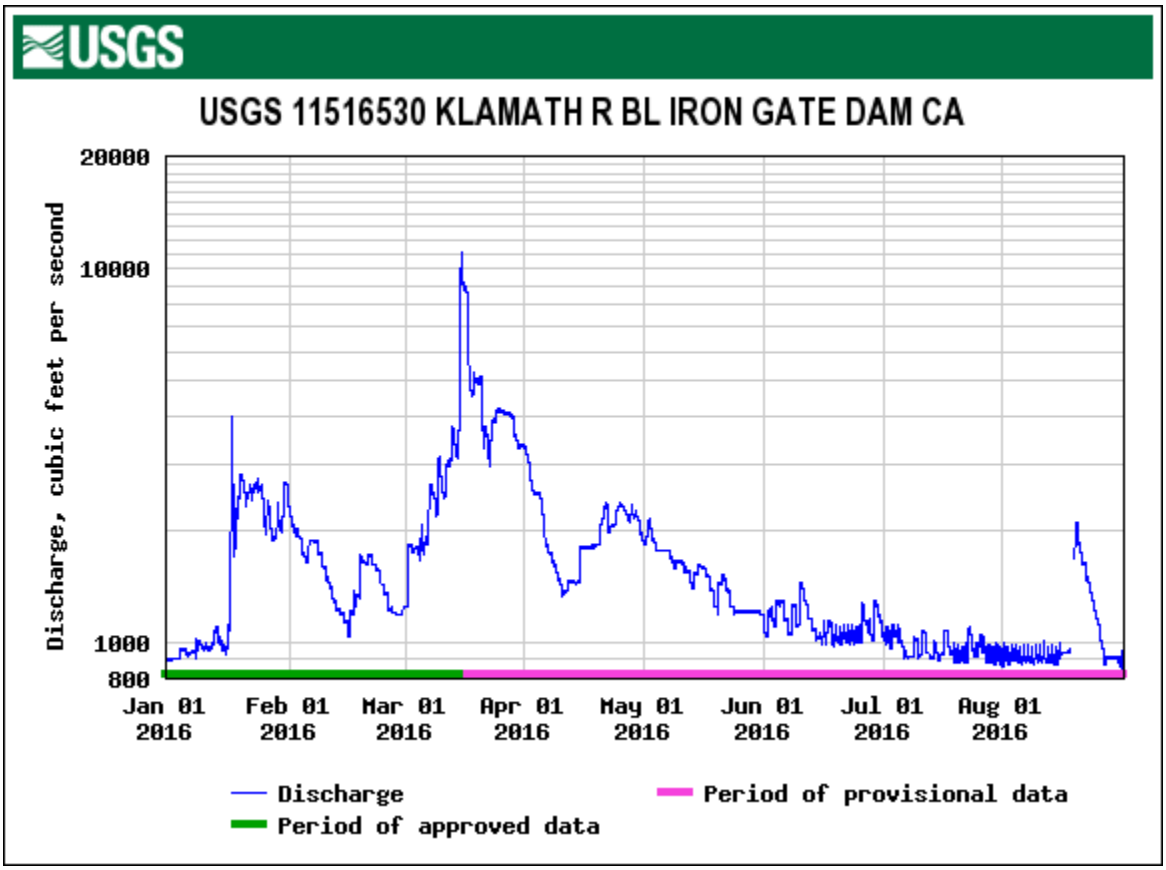


Figure 10. Daily discharge (cfs) below Iron Gate Dam from January 2016 through August 2016. Data collected after March 18, 2016 is provisional data. Data collected from USGS gaging station 11516530 at waterdata.usgs.gov.

In comparing river flows for previous years, the Palmer Hydrological Drought Index (NOAA 2016) for the 2016 water year in California was classified as a moderate drought year, an improvement from recent water years (2014 and 2015) that were ranked as extreme drought years.

Klamath River flows in 2016 were intermediate between low flow years (2009-2010) and high flow years (2011-2012, Figure 11). In 2009 and 2010, flows did not reach above 2000 cfs below IGD. In 2011, two peak spring flows exceeded 5000 cfs, the first of which was a manipulated pulse flow released from IGD in February. In 2012, spring flows were close to 4000 cfs. Flows in 2013, 2014, and 2015 were also intermediate, even with a pulse flow close to 1900 cfs in late May 2014.

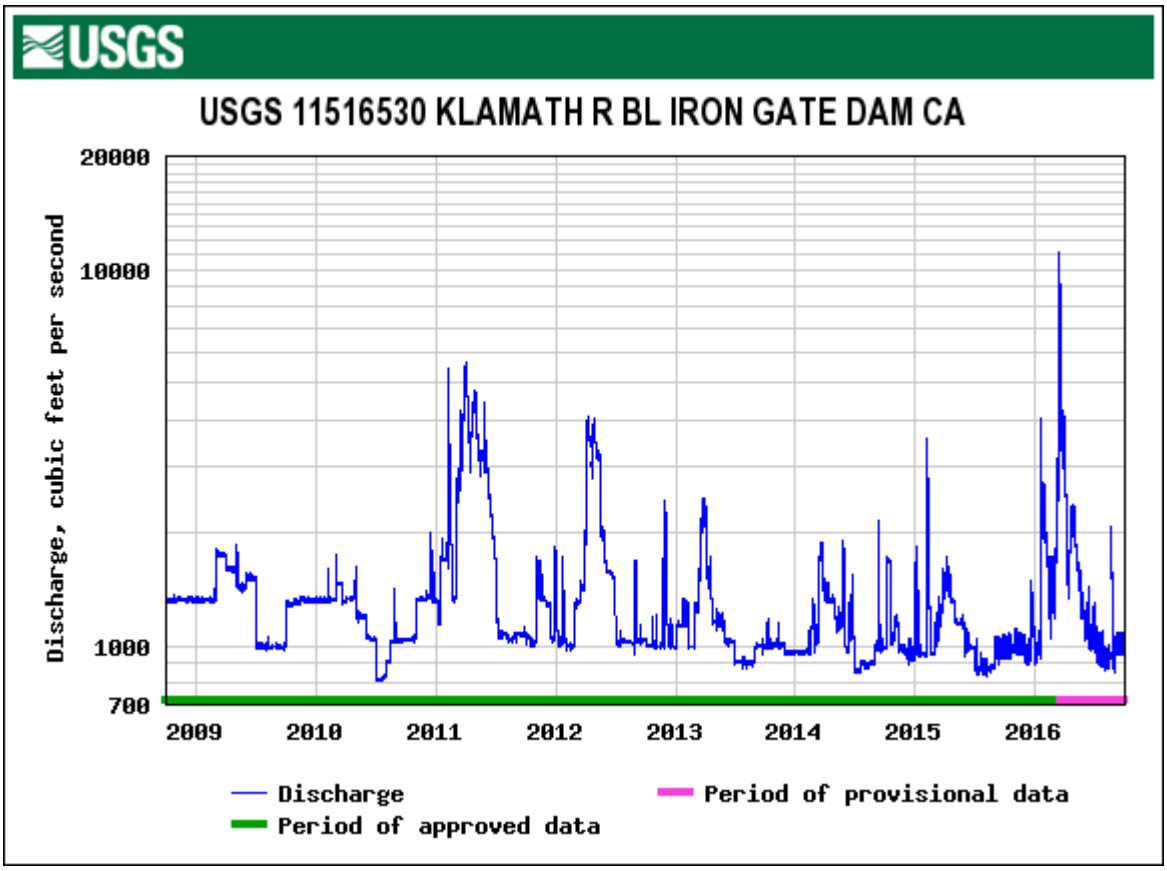


Figure 11. Daily discharge (cfs) below Iron Gate Dam from October 2009 through September 2016. Data collected after March 18, 2016 is provisional data. Data acquired from USGS waterdata.usgs.gov

Discussion

In the Klamath Basin, 2016 represented the first year of relatively normal river temperature and flow regimes, compared to the past two severe drought years (2014 and 2015). This change resulted in a significant decrease in infection levels and disease associated with the myxozoan parasite *C. shasta*. The *C. shasta* seasonal prevalence of infection by QPCR in Chinook salmon collected above the Trinity River confluence during the peak out-migration period (May-July) was 48%, considerably lower than 91% observed in 2015, and 81% in 2014. *Parvicapsula minibicornis* in Chinook salmon above the Trinity River confluence for the same period also decreased (89%) compared to 2015 (99%) and 2014 (92%). Clinical disease due to *C. shasta* was not observed by necropsy, QPCR or histology, and clinical disease associated with *P. minibicornis* was only observed in juvenile fish collected later in the season (June 26) and from the lower Salmon to Trinity (K2) river reach.

Natural Chinook Salmon

Ceratonova shasta prevalence by QPCR in natural fish was 27% in 2016, compared to 75% in 2015 and 76% in 2014. The first detection in 2016 (April 25) was approximately three weeks later than 2014 and 2015 (April 2-3) and occurred in the lower Scott to Salmon (K3) reach. The prevalence of infection at first detection was low (30%) and while water temperature was not available for this date and reach, the following week was reported to be 13.4℃ at Seiad Valley (Karuk temperature data). Natural fish likely benefited from an unusual water temperature profile below IGD that was markedly cooler than previous monitoring years. Three temperature decreases were also observed at Seiad Valley in May, June and July.

First detection in the upper Shasta to Scott (K4) reach occurred May 1 with 20% POI and rose significantly to 78% the following week. In the Scott to Shasta (K3) reach, POI rose to 90% by May 8 and remained high (83%) on the last sample for natural origin juvenile fish for this reach on May 15. Despite the elevated *C. shasta* POI for specific weeks and reaches, parasite DNA copy number did not increase to the 2-4 log threshold associated with clinical disease, indicating fish were infected but at low parasite numbers. *Parvicapsula minibicornis* in natural fish was also low in 2016 at 39%, compared to 92% in 2015.

The majority of juvenile Chinook tested by histology were natural origin sampled early in the season from the two upper reaches. Overall *C. shasta* prevalence of infection was low at 27% compared to 75% in 2015, and ranged from undetected to 30% over the three sample dates (April 17-May 15) compared to 70-80% in 2015. *Ceratonova shasta* pathology scores were low in 2016 indicating low levels of parasites and little intestinal necrosis (enteronecrosis). Natural fish had an overall *P. minibicornis* POI by histology of 39% compared to 92% in 2015.

Iron Gate Hatchery Coded-wire Tagged Chinook Salmon

Among coded-wire tagged (CWT) juvenile Chinook salmon released from Iron Gate Hatchery from May 17 – June 9, *C. shasta* was detected in 45% of fish screened by QPCR, compared to 90% in 2015. When comparing infection prevalence in IGH CWT by capture reach, *C.shasta*

POI in CWT Chinook was highest in the Scott to Salmon reach (K3) at 54%, followed closely by the Trinity to Estuary (K1) reach at 52%. In the Estuary, *C. shasta* POI was low at 30% indicating the majority of the fish sampled were not infected. The latest release group (June 9) likely benefited from the intermittent decreases in river temperatures (16-18°C) observed below IGD from approximately mid-June to the end of July.

In terms of CWT Chinook infection prevalence regarding exposure period, *Ceratonova shasta* prevalence of infection ranged from 51-72% in IGH CWT Chinook salmon residing 2-4 WAL. This compares to 92-93% in IGH CWT Chinook salmon in 2015 when POI was high across all exposure (WAL) groups. Mean DNA copy number in CWT Chinook was 8,500 in 2016, compared to 53,000 in 2015. The later infection peak of 2-4 WAL, as well as lower prevalence of infection in 2016, indicates marked hatchery Chinook experienced a lower exposure dose and delayed infection development compared to the previous two years. As noted in natural fish, parasite DNA levels were below the 2 log disease threshold for nearly all WAL groups. The exceptions were the 2WAL group (2.2 logs) and one fish residing 11WAL (3.0 logs). The vast majority of CWT Chinook had DNA levels at or below 1.5 logs.

Historical Comparison

For historical comparisons between monitoring years, data are restricted to all Chinook sampled during the peak migration period (May to end of July) in reaches above the Trinity confluence. The mean *C. shasta* prevalence of infection by QPCR for 2016 was 48%, compared to the mean for the entire monitoring program of 44% (2006-2016). The range of *C. shasta* POI been quite variable over the past eleven years (17-91% by QPCR and 2-62% by histology) and has correlated well with environmental conditions, primarily river temperature. The annual *C. shasta* POI in 2016 was a large decrease from the high POI (91% in 2015, and 81% in 2014) observed during two severe drought years.

For specific fish groups, river temperatures were advantageous for overall health of natural fish and no clinical disease was observed in fish sampled from the upper reaches in early to mid-May. Low prevalence by QPCR and histology suggests a positive prognosis for natural Chinook salmon as outmigration occurred prior to high spore counts in the main stem Klamath River. In Iron Gate CWT Chinook whose migration timing is later and often at higher river temperatures, infection exceeding the lower range (2 logs) of parasite DNA associated with clinical disease only occurred in fish residing 3 weeks post release. This indicates spore exposure below Iron Gate Hatchery was low, or not continuous, and hatchery fish also likely benefited from relatively cooler river temperatures profiles in the later (mid-June to mid-July) migration period. *Ceratonova shasta* POI was relatively low (30%) in the estuary, compared to recent years, indicating disease impacts for CWT Chinook were low in 2016.

In summary, 2016 was a moderately-low year for myxozoan disease impacts in natural and out-migrating juvenile Chinook salmon in the Klamath River. The environmental conditions in 2016 appear to correlate well with moderate disease levels observed in 2012-2013 (Table 3). The annual trends in *C. shasta* prevalence of infection in juvenile Chinook salmon demonstrate that while myxozoan exposure dose is key, river temperatures and flows are also important factors that influence disease development and severity in juvenile salmonids.

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Oregon State University

Photo contributions:
Cover Photo: Klamath River, USDA – Six Rivers National Forest.

Author Roles

The contributions of each author have been summarized below.

- Kimberly True – Project lead and coordination, data management and quality control, QPCR methodology and quality assurance, data analysis, and written report.
- Anne Voss – Data management and quality control, QPCR necropsy extraction and assays, pivot tables and environmental data figures, assistance with written report.
- Scott Foott – Project support, examination of histological specimens, diagnostic assessments, and report review.

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Appendix A – Samples Collected

Table 1. Number of fish collected for QPCR testing and histology (H) by Klamath River reach (reach code) and sampling week.

Week	Sample date	Shasta R. to Scott R. (K4)	Scott R. to Salmon R. (K3)	Salmon R. to Trinity R. (K2)	Trinity R. to Estuary (K1)	Klamath R. Estuary (K0)
1	27-Mar	20				
2	3-Apr	20				
3	10-Apr	19	21			
4	17-Apr	21 (H10)	20 (H10)			
5	24-Apr	20	20			
6	1-May	20 (H10)	20 (H10)			
7	8-May	18	20			
8	15-May	20 (H10)	18 (H11)			
9	22-May	18	21			
10	29-May	15 (H9)	20 (H10)			
11	5-Jun	21	3	14		
12	12-Jun	20	3	6		
13	19-Jun	17	25	22		17
14	26-Jun		21	21	18	16
15	3-Jul		18	24	9	15
16	10-Jul		8	31	4	7
17	17-Jul		5	20	19	12
18	24-Jul		13	22	7	12
19	31-Jul			22	1	15
20	7-Aug				9	11
21	14-Aug				2	

Table 2. *Ceratonova shasta* infection by QPCR in juvenile Chinook salmon sampled from 5 reaches within the Klamath River. The prevalence [% (number positive/number tested)] is presented for each sample reach by collection week and sample date.

Week	**Sample Date**	**Shasta R. to Scott R. (K4)**	**Scott R. to Salmon R. (K3)**	**Salmon R. to Trinity R. (K2)**	**Trinity R. to Estuary (K1)**	**Klamath R. Estuary (K0)**
1	27-Mar	0% (0/20)				
2	3-Apr	0% (0/20)				
3	10-Apr	0% (0/19)	0% (0/21)			
4	17-Apr	0% (0/21)	0% (0/20)			
5	24-Apr	0% (0/20)	30% (6/20)			
6	1-May	20% (4/20)	30% (6/20)			
7	8-May	78% (14/18)	90% (18/20)			
8	15-May	55% (11/20)	83% (15/18)			
9	22-May	78% (14/18)	10% (2/21)			
10	29-May	20% (3/15)	30% (6/20)			
11	5-Jun	19% (4/21)	33% (1/3)	64% (9/14)		
12	12-Jun	45% (9/20)	100% (3/3)	83% (5/6)		
13	19-Jun	41% (7/17)	68% (17/25)	64% (14/22)		47% (8/17)
14	26-Jun		95% (20/21)	67% (14/21)	100% (18/18)	63% (10/16)
15	3-Jul		39% (7/18)	50% (12/24)	78% (7/9)	27% (4/15)
16	10-Jul		13% (1/8)	19% (6/31)	25% (1/4)	14% (1/7)
17	17-Jul		0% (0/5)	35% (7/20)	16% (3/19)	17% (2/12)
18	24-Jul		31% (4/13)	45% (10/22)	43% (3/7)	25% (3/12)
19	31-Jul			73% (16/22)	0% (0/1)	13% (2/15)
20	7-Aug				33% (3/9)	9% (1/11)
21	14-Aug				50% (1/2)	
		K4 Total **27% (66/249)**	K3 Total **41% (106/256)**	K2 Total **51% (93/182)**	K1 Total **52% (36/69)**	K0 Total **30% (31/105)**

Table 3. *Parvicapsula minibicornis* infection by QPCR in juvenile Chinook salmon sampled from 5 reaches within the Klamath River. The prevalence [% (number positive/number tested)] is presented for each sample reach by collection week and sample date.

Week	**Sample Date**	**Shasta R. to Scott R. (K4)**	**Scott R. to Salmon R. (K3)**	**Salmon R. to Trinity R. (K2)**	**Trinity R. to Estuary (K1)**	**Klamath R. Estuary (K0)**
1	27-Mar	0% (0/20)				
2	3-Apr	0% (0/20)				
3	10-Apr	0% (0/19)	0% (0/21)			
4	17-Apr	0% (0/21)	0% (0/20)			
5	24-Apr	5% (1/20)	60% (12/20)			
6	1-May	65% (13/20)	55% (11/20)			
7	8-May	100% (18/18)	95% (19/20)			
8	15-May	95% (19/20)	89% (16/18)			
9	22-May	100% (18/18)	86% (18/21)			
10	29-May	73% (11/15)	85% (17/20)			
11	5-Jun	33% (7/21)	67% (2/3)	100% (14/14)		
12	12-Jun	80% (16/20)	100% (3/3)	100% (6/6)		
13	19-Jun	100% (17/17)	100% (25/25)	100% (22/22)		100% (17/17)
14	26-Jun		95% (20/21)	100% (21/21)	100% (18/18)	100% (16/16)
15	3-Jul		100% (18/18)	100% (24/24)	100% (9/9)	100% (15/15)
16	10-Jul		100% (8/8)	100% (31/31)	75% (3/4)	100% (7/7)
17	17-Jul		80% (4/5)	100% (20/20)	84% (16/19)	92% (11/12)
18	24-Jul		92% (12/13)	95% (21/22)	86% (6/7)	83% (10/12)
19	31-Jul			100% (22/22)	100% (1/1)	80% (12/15)
20	7-Aug				100% (9/9)	91% (10/11)
21	14-Aug				100% (2/2)	
		K4 Total **48% (120/249)**	K3 Total **72% (185/256)**	K2 Total **99% (181/182)**	K1 Total **93% (64/69)**	K0 Total **93% (98/105)**

Appendix B – Histological Summary

Table 1. 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.	*Ceratonova shasta* trophozoite stage
Chloromyxum sp	Chloromyxum species trophozoite stage
Pathology Score	**Mean kidney pathology score for sample group**
Intestine	
C. shasta troph.	*Ceratonova shasta* trophozoite stage
C. shasta myxosp.	*Ceratonova shasta* myxospore stage
Helminth	Trematode, nematode, or cestode
Pathology Score	**Mean intestine pathology score for sample group**
Other	
Adipose steatitis	Inflammation of visceral fat tissue
Adipose lipofuscin	Oxidized lipopigments within adipose cells
Gill	
Metacercaria	Immature trematode stage
Multif. Hyperplasia	Multifocal hyperplastic regions on lamellae

Table 2. Parasite prevalence of infection [number positive / number tested (%)], pathology score for kidney and intestine, and tissue abnormalities observed in histological sections of juvenile Klamath River Chinook salmon collected from the Shasta to Scott reach (K4). Collection dates are reported as Monday of given week.

Collection week	April 17	May 1	May 15	May 29	POI
Kidney					
Pm Troph.	5 /10 (50)	4 /10 (40)	8 /10 (80)	7 / 9 (78)	24 / 39 (62)
Pm Myxosp.	0 /10 (0)	0 /10 (0)	1 /10 (10)	0 /9 (0)	1 / 39 (3)
Metacercaria	0 /10 (0)	0 /10 (0)	0 / 10 (0)	0 /9 (0)	0 / 39 (0)
C.shasta troph.	0 /10 (0)	0 /10 (0)	1 /10 (10)	0 /9 (0)	1/ 39 (3)
Chloromyxum sp	0 /10 (0)	0 /10 (0)	0 /10 (0)	0 /9 (0)	0 / 39 (0)
Pathology Score	0.0	0.0	1.2	0.9	
Intestinal tract					
C.shasta troph.	1 /10 (0)	0 /10 (0)	3 /10 (30)	1 /9 (11)	5 / 39 (13)
C.shasta myxosp.	0 /10 (0)	0 /10 (0)	1 /10 (1)	0 /9 (9)	1 / 39 (3)
Helminth	0 /8 (0)	0 /10 (0)	0 /10 (0)	0 /9 (0)	0 / 37 (0)
Pathology Score	0.2	0.0	1.0	0.0	
Adipose steatitis	2 /10 (20)	3 /10 (30)	3 /9 (33)	3 /8 (38)	11 / 37 (30)
Adipose lipofuscin	0 /10 (0)	0 /10 (0)	0 /9 (0)	1 /8 (13)	1 / 37 (3)
Gill					
Metacercaria	7 /10 (70)	3 /9 (33)	7 /10 (70)	6 /9 (67)	23 /38 (61)
Multif. Hyperplasia	2 /10 (20)	0 /9 (0)	8 /10 (80)	2 /9 (22)	12 /38 (32)

Table 3. Parasite prevalence of infection [number positive / total (%)], pathology score for kidney and intestine, and tissue abnormalities observed in histological sections of juvenile Klamath River Chinook salmon collected from the Scott to Salmon River (K3). Collection dates are reported as Monday of given week.

Collection Week	April 17	May 1	May 15	May 29	POI
Kidney					
Pm Troph.	0 /9 (0)	0 /10 (0)	3 /10 (30)	9 /10 (90)	12 / 39 (31)
Pm Myxosp.	0 /9 (0)	0 /10 (0)	0 /10 (0)	0 /10 (0)	0 / 39 (0)
Metacercaria	0 /9 (0)	0 /10 (0)	0 /10 (0)	0 /10 (0)	0 / 39 (0)
C.shasta troph.	0 /9 (0)	0 /10 (0)	0 /10 (0)	0 /10 (0)	0 / 39 (0)
Chloromyxum sp	0 /9 (0)	0 /10 (0)	0 /10 (0)	0 /10 (0)	0 / 39 (0)
Pathology Score	0.0	0.0	0.0	0.6	
Intestinal tract					
C.shasta troph.	1 /10 (10)	1 /10 (10)	2 /11 (18)	1 /8 (13)	5 / 39 (13)
C.shasta myxosp.	0 /10 (0)	0 /10 (0)	0 /11 (0)	0 /8 (0)	0 / 39 (0)
Helminth	0 /10 (0)	1 /10 (10)	0 /11 (0)	0 /8 (0)	1 / 39 (3)
Pathology Score	0.0	0.3	0.09	0.0	
Adipose steatitis	3 /10 (30)	2 /10 (20)	1 /10 (10)	5 /6 (83)	11 / 36 (31)
Adipose lipofuscin	0 /10 (0)	0 /10 (0)	0 /10 (0)	0 /6 (0)	0 / 36 (0)
Gill					
Metacercaria	0 /10 (0)	1 /10 (10)	4 /11 (36)	7 /10 (70)	12 / 41 (29)
Multif. Hyperplasia	0 /10 (0)	1 /10 (10)	3 /11 (18)	5 /10 (50)	9 /41 (22)

Appendix C - Reviewer comments

Listed below are paraphrased comments provided by a reviewer of a draft of this report. The author's response is given below each comment.

Pg. 4 – Pre-Release Examination: Reviewer asked why only 32 fish were examined for pre-release examination at Iron Gate Hatchery.

Response: A target of 30 fish is based on Iron Gate Hatchery production numbers and a 95% confidence interval that at least one infected fish will be collected if the assumed pathogen prevalence level equals or exceeds 10%.

Pg. 6 – Sample Sites, Fish Groups, and Number Sampled: Reviewer asked how unknown fish are distinguished from natural and hatchery fish. Reviewer suggests clarifying the fish groups.

Response: Additional detail was added on page 6 to clarify fish groups. It is also described on page 13 that unknown fish are collected after hatchery release. This sentenced has been modified to explain that unknown fish are unmarked and therefore we do not know if this is a natural fish or an unmarked hatchery fish.

Pg. 7 – Parasite Infection Levels by Quantitative PCR Assays: Reviewer would find further detail about the quantitative PCR useful (Reaction volume used? Are samples tested in duplicate? What criteria determine if a sample is re-run?)

Response: The total reaction volume of the QPCR assay was stated on page 6 as 30µL. Samples are not tested in duplicate. Plate performance over the season is monitored and any plate that does not test within 3% of expected values for standards is re-extracted or re-tested (this methodology is described on pages 6 and 7).

Pg. 12 – Naturally produced Chinook salmon: In reference to the QPCR disease threshold analysis, the reviewer was interested to know if this will also be developed for *Parvicapsula minibicornis*?

Response: We would like to be able to assess *Parvicapsula minibicornis* in the same manner, however *C. shasta* is the predominant parasite, and *P. minibicornis* disease impacts and thresholds are more difficult to assess in dual infected juvenile Chinook.